Characterization of The Viable but Non-Culturable *Legionella pneumophila* in Water and the Role of 3-Hydroxybutyrate Dehydrogenase in Its Formation

by

Badii H. Q. Al-Bana

Submitted in partial fulfilment of the requirements
for the degree of Doctor of Philosophy

at

Dalhousie University
Halifax, Nova Scotia
September 2013

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Legionella pneumophila, the causative agent of Legionnaires' disease (LD), is an intracellular pathogen of freshwater protozoa that can also persist in the environment as a free-living bacterium. *L. pneumophila* has many morphological forms that fit within a developmental cycle. In water, *L. pneumophila* enters into a viable but non-culturable (VBNC) state that is largely uncharacterized. VBNC cells were produced from two developmental *L. pneumophila* forms, stationary phase forms (SPFs) and mature infectious forms (MIFs) by suspension in double deionized (dd) or tap-water at 45°C. Electron microscopy results showed that VBNC cells have a unique morphology and that in tap water they lose their poly 3-hydroxybutyrate inclusion bodies. Both SPFs and MIFs lost culturability faster in dd- than in tap water, and addition of salts to dd-water prolonged *L. pneumophila* culturability and enhanced viability. However, MIFs retained higher viability in dd- and tap water (85% and 51%, respectively) than SPFs (5% and 20%, respectively) as determined by the BacLight vital stain. Only ~1 VBNC cell out of 10^5 of those produced from SPFs in tap water regained culturability via infection of *Acanthamoeba*. All VBNC cells, except for those produced from SPFs in dd-water, resisted both digestion inside *Tetrahymena* spp. and detergent-mediated lysis. SDS-PAGE analysis and shotgun proteomics revealed a number of VBNC cell specific proteins; one of these was 3-hydroxybutyrate dehydrogenase (BdhA), which is involved in the metabolism of poly 3-hydroxybutyrate inclusion bodies. A *bdhA* mutant showed an early loss of culturability and a dramatic decrease in viability as compared to the parent strain, and complementing the mutant with a functional *bdhA* gene restored the parent's strain phenotypes. In conclusion, VBNC *L. pneumophila* has a distinct morphology and physiology that varies according to the developmental stage and the environmental conditions used to produce such VBNC cells. VBNC cells have a different protein profile and morphology than the culturable cells, suggesting that this state constitutes a distinct differentiated form within the developmental cycle of *L. pneumophila.* BdhA seems to influence *L. pneumophila* survival and hence VBNC cell formation. Collectively, the results from this study provide a better understanding of *L. pneumophila* VBNC form and the factors influencing its formation.

LIST OF ABBREVIATIONS USED

3HB, 3-hydroxybutyric acid

AHK, α-hydroxyketone

AHLs, N-acyl-L-homoserine lactones

Arf, ADP-ribosylation factor

bp, base pair

BSA, bovine serum albumin

c-di-GMP, bis-(3',5')-cyclic dimeric guanosine monophosphate

CFU, colony forming unit

COPI, coat protein complex I

CVC, central venous catheter

GTPase, GTP binding protein

LAI-1, *Legionella* autoinducer-1

LAMP, lysosome-associated membrane glycoprotein

LCV, *Legionella*-containing vacuole

LD, Legionnaires' disease

LD_{50}, lethal dose 50%

LPS, lipopolysaccharide

MIF, mature infectious form

Min, minutes

MOMP, major outer membrane protein

MRM, Multiple Reaction Monitoring

N, amino

ORF, open reading frame

PCR, polymerase chain reaction

PF, pontiac fever

PG, peptidoglycan

PHA, polyhydroxyalkanoates

PHB, poly β-hydroxybutrate

PM, plasma membrane

ppGpp, guanosine 3',5'- bispyrophsophate

RNAP, RNA polymerase

RF, replicative form

Rpf, resuscitation promoting factor

SDR, short-chain dehydrogenases/reductases

SDS, sodium dodecyl sulfate

Spp., species

SPF, stationary-phase form

T2SS, type II secretion system

T4SS, type IV secretion system

TA, toxin-antitoxin

TEM, transmission electron microscope

UTI, urinary tract infection

v-ATPase, vacuolar ATPase

VBNC, viable but non-culturable

ACKNOWLEDGEMENTS

My thanks are due to my supervisors, Dr. Garduno and Dr. Lee, for their guidance in the project and thorough reading and constructive editing of this thesis. I would like to express my gratitude and appreciation to Dr. Song Lee for accepting me as a lab member in the last year of my study; my project would not have been as successful without his input, guidance, and contributions. I would like to thank my committee members, Dr. John Rohde, Dr. Ross Davidson, and Dr. David Hoskin (who served earlier as a committee member), who have all provided valuable suggestions and guidance during the past four years. I would like also to thank Dr. Jean Marshall and Dr. Brent Johnston for their valuable help during my stay in the department. I would like to extend my gratitude to Dr. Trevor Charles for accepting the invititation to become my external examiner.

My thanks also to Dr. Celia Lima, David Allan, Lauren Davey, Maram Hulbah, Basil Dawod, Dr. Jeremy Benjamin, Julie Ryu, Mark Petten, Erin Brouwers, Madhu Esther, and Yahua Song for their help, friendship, and valuable technical assistance in the lab. I also thank members in the IWK for making the lab environment such a great place to work in.

I would like to acknowledge Dr. Gary Faulkner and Mary Ann Trevors for their help and expertise with the electron microscope and Dr. Alejandro Cohen from the Proteomic Core Facility at Dalhousie University for his valuable effort and help with shotgun proteomics. Also, My thanks to people in Dr. Yung-Hua Li lab for their valuable technical assistance.

My thanks also go to the Iraqi Ministry of High Education and Scientific Research represented by the Iraqi Cultural Office in Ottawa, which provided funds for my study.

Finally, I thank my beautiful wife Moreen for having confidence in me and for being always there for me to encourage and help; my lovely daughter Darielle, who gave me the enthusiasm to finish writing my thesis and to whom I am looking forward to giving a hug when I go back home. Special thanks also to my parents and my parents-in-law for their suppor

CHAPTER 1. INTRODUCTION

1.1. Biology of *L. pneumophila*

Legionella species (spp.) are Gram-negative bacteria that are ubiquitous in natural and artificial aquatic habitats and moist soil environments (Costa *et al*., 2005; Declerck *et al*., 2007a; Wallis and Robinson, 2005), where they parasitize and replicate intracellularly in various protozoa (Fields, 1996). *Legionella* can also survive as a free organism for a long period of time (Steinert *et al*., 1997). *L. pneumophila* was first identified as the causative agent of an atypical pneumonia affecting 182 people who were attending a Legionnaires' conference in Philadelphia in 1976; 29 of these cases were fatal (Fraser *et al*., 1977). Currently, there are 53 species and 70 serogroups in the genus *Legionella* (Guyard and Low, 2011) and, of these species, 25 are known to cause human disease (Gobin *et al*., 2009). The majority of disease outbrearks (91.5%) are caused by *Legionella pneumophila,* and serogroup 1 is the predominant causative agent (84.2%) (Yu *et al*., 2002). Other species (e.g., *L. longbeachae* and *L. micdadei)*, together with *L. pneumophila,* account for most human infections (Lam *et al*., 2011).

1.1.1. Microbial Ecology

1.1.1.1. Factors Influencing *L. pneumophila* Survival in Aquatic Environments

Various factors affect the presence of *L. pneumophila* in water. These include temperature, corrosion, scale, flow regimes (Lasheras *et al*., 2006; Liu *et al*., 2006), biofilms (Murga *et al*., 2001), inorganic and organic contents of the water, and the presence of host protozoa (Fliermans *et al*., 1981).

L. pneumophila survives as a free organism for a long period of time in low-nutrient environments under appropriate conditions (Paszko-Kolva 1992). In fact, studies have shown that *L. pneumophila* is capable of surviving for up to 14 months in tap water with only a modest loss of viability over the first few months (Schofield, 1985; Skaliy and McEachern, 1979). Temperature is one of the factors that affect *L. pneumophila* survival in water (Ohno *et al*., 2003). Generally, the optimal temperature for *L. pneumophila* growth is around 37°C (Yee and Wadowsky, 1982); the temperature for multiplication for some environmental

isolates has been found to range from 20 to 45°C (Kusnetsov *et al*., 1996; Wadowsky *et al*., 1985; Yee and Wadowsky, 1982). However, some *Legionella* spp. have been isolated from Antarctic freshwater lakes at 0°C (Carvalho *et al*., 2008). Moreover, the bacteria can persist in association with thermotolerant amoebae such as *Acanthamoeba*, *Naegleria*, *Hartmanella* and *Vahlkampfia* spp. at temperatures above 60°C (Taylor *et al*., 2009). This suggests that at least short-term survival is possible in waters of widely varying temperature (Fliermans *et al*., 1981b).

Survival of *L. pneumophila* at low temperatures relies on the Lsp Type II secretion system (T2SS) (Soderberg *et al*., 2008). *L. pneumophila* T2SS mutants are impaired for growth in medium, survival in tap water, and growth in *Acanthamoeba castellanii* or *Hartmanella vermiformis* at 25°C (but not at 37°C). RNA-modifying and catabolic enzymes, as well as a lipid A acyltransferase, are also implicated in survival at low temperatures (Albers *et al*., 2007; Soderberg and Cianciotto, 2010). *L. pneumophila* was able to survive in hot spring water at a temperature up to 45°C (Ohno *et al*., 2003). At 50°C, the survival rate of *L. pneumophila* strains was 25% after a 60 min incubation (Groothuis and Veenendaal 1983), but it was greatly reduced after 40 min of incubation above that temperature (Fliermans *et al*., 1981).

In addition to temperature, pH also influences *L. pneumophila* survival. The optimal pH value for *L. pneumophila* survival is between 6 and 8 (Ohno *et al*., 2003). In keeping with this, environmental isolates have been isolated from water with pH values ranging from 5 to 8.1, with the greatest frequency of isolation at pH 5-6.75 (Fliermans *et al*., 1981b). In hot spring water microcosms adjusted artificially to pH ranging from 2.0 to 10.0, the lowest pH killed *L. pneumophila* within 1 day of incubation at 42°C; however, *L. pneumophila* survival was maintained at pH 5.0 (Ohno *et al*., 2003).

L. pneumophila is growth-defective at low salt concentrations (100 mM NaCl), but can survive high salt concentrations (500 mM NaCl) at temperatures below 20°C (Heller *et al*., 1998). Moreover, *L. pneumophila* was able to survive for a long time in tap water as long as certain minerals involved in starvation survival (e.g. iron, zinc, and potassium) were present (States *et al*., 1985). Similarly, the addition of trace elements such as Fe, Mn, Mg, Ca, Zn, and Cu, as well as phosphate, stimulates growth of *Legionella* spp. in laboratory culture (Reeves *et al*., 1981).

1.1.1.2. Association of *L. pneumophila* with Biofilms

Biofilms are aggregations of microorganisms that secrete extracellular polymeric substances such as polysaccharides to form a protective matrix that adheres to surfaces (Donlan, 2002). The microbiological makeup of these films differs depending on system conditions such as water flow rate, temperature, exposure to light, and nutrient content. This mix of factors results in a wide range of diverse microbial ecosystems containing bacteria, fungi, protozoa, and algae (Costerton and Wilson, 2004). *Legionella* spp., in their natural environments, present as planktonic cells, and also as monospecies or multispecies biofilms (Declerck *et al*., 2009; Declerck, 2010; Murga *et al*., 2001), which can be adherent at solid-water interfaces and floating at water-air interfaces. Biofilms might provide the amino acids and organic carbon that are essential for *L. pneumophila* growth (Declerck, 2010), and they might also constitute a protective niche against stressful environmental conditions and biocides (Borella *et al*., 2005). Although it remains unexplored for *Legionella*, the production and development of biofilms *in vivo* promote persistence, colonization of the airways, and increase resistance to antibiotics among other pathogenic bacteria (Hall-Stoodley and Stoodley, 2009).

Some authors argue that other microbial species are indispensable in order for *L. pneumophila* to multiply in a biofilm under such conditions, and that *Legionella* is capable of growing on the excreted nutrients from other microorganisms (Declerck *et al*., 2007b; Kuiper *et al*., 2004). Research by Temmerman *et al.* (2006) suggested that *L. pneumophila,* within biofilms, was able to feed (via necrotrophy) on dead bacteria and possibly other organic constituents, since heat-killed Gram-negative bacteria supported the growth of *L. pneumophila* in filter-sterilized tap water. Moreover, in oligotrophic conditions, *L. pneumophila* was able to form microcolonies within laboratory-derived multi-organism biofilms devoid of amoebic life (Rogers and Keevil, 1992) and on growth media deficient in L-cysteine and ferric pyro-phosphate when in association with *Flavobacterium breve* (Wadowsky and Yee, 1983). In addition, *L. pneumophila* was able to replicate in association with photosynthetic cyanobacteria in algal mats, implying the use of algal products as nutrients. *Legionella*'s capacity to grow in association with cyanobacteria might be expected to be found in heated water systems that are exposed to light, since both organisms multiply over an elevated temperature range (Taylor *et al*., 2009).

Within a biofilm, *Legionella* can become filamentous and multinucleated to facilitate rapid division into planktonic form when advantageous (e.g., when parasitic hosts are present) (Taylor *et al*., 2009). The morphology of *L. pneumophila* biofilms depends on the temperature: at 25°C, rod-shaped bacteria produce a loose biofilm, whereas at 37- 42°C filamentous bacteria form a thick and dense mycelial biofilm mat (Piao *et al*., 2006). A comparison of the transcriptomes of sessile and planktonic *L. pneumophila* indicates the upregulation of genes implicated in the protection from oxidative stress and the acquisition of iron in the biofilm (Hindre *et al*., 2008). Accordingly, iron regulates biofilm formation of *L. pneumophila* as well as the expression of siderophore biosynthesis genes (Hilbi *et al*., 2011).

Unlike other bacterial species, *L. pneumophila* does not produce common classes of autoinducers, such as N-acyl-L-homoserine lactones (AHLs) or the furanosyl borate diester AI-2. However, *L. pneumophila* possesses the *lqs* gene cluster containing homologues of the *cqs* (*Vibrio cholerae* quorum sensing) genes (Tiaden *et al*., 2007). The *lqs* gene cluster encodes the autoinducer synthase LqsA, the putative cognate sensor kinase LqsS, a protein of unknown function (HdeD), and the response regulator LqsR. The Lqs components produce and likely respond to the signalling molecule LAI-1 (*Legionella* autoinducer-1), which was identified by mass spectrometry as the α-hydroxyketone (AHK) compound 3-hydroxypentadecan-4-one (Spirig *et al*., 2008) and is therefore distinct from the *V. cholerae* AHK CAI-1 (Cholera autoinducer-1; 3-hydroxytridecan-4-one) (Higgins *et al*., 2007).

Interestingly, the *Pseudomonas aeruginosa* homoserine lactone autoinducer (3OC12-HSL) was recently shown to exert bacteriostatic activity and decrease biofilm formation of *L. pneumophila* (Kimura *et al*., 2009). These effects are correlated to the transcriptional down-regulation of *lqsR* gene in *L. pneumophila*. This finding suggests a connection between 3OC12-HSL and the uniquely identified quorum sensing system in *L. pneumophila* (Kimura *et al*., 2009). Recently, a glycosaminoglycan (GAG)-binding adhesin of *L. pneumophila*, Lcl, was found to be involved in the early steps of surface attachment and intercellular interactions. Furthermore, Lcl was differentially regulated during growth phases and biofilm formation, and its transcriptional regulation was mediated by 3OC12-HSL (Mallegol *et al*., 2012). Therefore, other bacterial species that could be found together with *Legionella* within a biofilm might be able to control *L. pneumophila* growth and biofilm formation through quorum sensing.

1.1.1.3. Association of *L. pneumophila* with Protozoa

Amoebae are recognized as being both reservoirs and vehicles of pathogenic microorganisms in the environment. Free-living amoebae generally have 2 stages of development: the trophozoite and the cyst. The trophozoite is the active metabolic stage, feeding on bacteria and multiplying by binary fission; encystement can occur as a consequence of hostile pH conditions, osmotic pressure, temperature, or even unfulfilled nutritional needs of the amoeba (reviewed by Greub and Raoult, 2004).

Legionella spp. replicate in various protozoa, including amoebae (*Acanthamoeba*, *Hartmanella*, *Dictyostelium*, *Naegleria*, *Echinamoeba* or *Vahlkampfia* spp.) and ciliates (*Tetrahymena* spp.) (Fields, 1996; Solomon and Isberg 2000; Steinert and Heuner, 2005). However, intracellular growth of *L. pneumophila* in *Tetrahymena* spp. is inconsistent. Unlike *Tetrahymena pyriformis*, *T. tropicalis* (Berk *et al*., 2008) and *T. thermophila* do not support *Legionella* replication inside their food vacuoles (Steinert *et al*., 2002). Survival and transmission of *L. pneumophila* to humans is strongly linked to the presence of amoebae in water (Greub and Raoult, 2004). *Acanthamoeba*, *Hartmannella*, and *Naegleria* are most commonly isolated from *Legionella*-contaminated plumbing systems (Steinert *et al*., 2002). Recently, 48% of amoeba cultures, isolated from sediments from estuarine systems in the USA, were positive for the presence of *Legionella* spp., and four percent of these cultures contained *L. pneumophila* (Gast *et al*., 2011).

Domestic water supply is a relatively nutrient-free environment, and parasitization of protozoa provides *Legionella* with the nutrients necessary for its growth (Wadowsky *et al*., 1988). Protozoa show a greater resistance than bacteria to treatment with various thiocarbamates, isothiazolones, and quaternary ammonium compounds (Sutherland and Berk, 1996). Similarly, disinfection with chlorine, chlorine dioxide, ozone, monochloramine, and copper-silver ionization is less effective on protozoa than bacteria (Thomas *et al*., 2004). In addition, once parasitized protozoa become encysted, cysts provide a protective niche for *Legionella* cells against environmental stresses, high temperature, desiccation, radiation, detergents, antibiotics, osmotic stress and biocidal chemicals (Storey *et al*., 2004; Taylor *et al*., 2009).

For example, encysted amoeba-bound *Legionella* survives exposure to 100 ppm free chlorine and up to 80°C for 10 min, conditions which would normally kill most if not all

Legionella within a system (Storey *et al*., 2004). Similarly, in the absence of *A. polyphaga*, *L. pneumophila* became non-culturable after exposure to 256 ppm of sodium hypochlorite (NaOCl), wheras intracellular *L. pneumophila* within *A. polyphaga* was resistant to 1024 ppm of NaOCl (Garcia *et al*., 2007). *L. pneumophila* in the planktonic phase that were exposed to Ag^{2+} and Cu^{2+} were completely inactivated (more than 7 log reduction) within 30 min, while intracellular bacteria showed much higher resistance against the exposure to the same concentrations of Ag^{2+} and Cu^{2+} (Hwang *et al*., 2006).

Rowbotham (1983) found that at a certain stage after the ingestion of bacteria, amoebae lyse and release numerous free bacteria or a few vesicles (a single vesicle can contain up to 10^4 bacteria). On the other hand, protozoa may empty their digestive vacuoles, which contain bacterial cells, often prior to encystment (Berk *et al*., 1998). The expelled bacteria are in the form of bacterial clusters surrounded by bacterial debris and are called pellets (Berk *et al*., 2008). When *A. polyphaga* and *A. castellanii* expelled pellets containing viable *L. pneumophila* cells, it was found that more than 90% of these pellets were small enough to be inhaled (i.e., less than 5 mm in diameter) (Berk *et al*., 1998), supporting the hypothesis that humans infected with *Legionella* would possibly have inhaled a pellet that was filled with the bacteria rather than having inhaled free bacteria (Greub and Raoult, 2004).

In addition, these pellets contained viable bacteria despite a 24 h exposure to biocides used in cooling towers. Moreover, the bacteria aggregated together within these pellets and did not disperse despite freezing and thawing treatments (-70 ^{o}C and 35 ^{o}C) and ultrasound (Berk *et al*., 1998). Considering that amoebae, in the trophozoite or cyst stages, usually adhere strongly to a physical substrate, this observation suggests that these pellets would further facilitate the bacterial dissemination as aerosols rather than the amoebae themselves (Berk *et al*., 1998).

1.1.2. Legionellosis

Legionella infections take two distinct forms: Pontiac fever (PF) and Legionnaires' disease (LD) (Fraser *et al*., 1977; Glick *et al*., 1978). PF is a mild self-limiting illness that mimics influenza with a short incubation period (typically 30-90 h) and a high infection rate that can exceed 90% of exposed people (Remen *et al*., 2011). However, it has been estimated

that water containing >10^3 cfu *Legionella* per litre was associated with an increased risk of PF (Hautemaniere *et al*., 2011). Because *Legionella* is not isolated from PF patients (in spite of these patients being seropositive for *Legionella*) (reviewed in Steinert *et al*., 2002), it has been speculated that PF is caused by viable but non-culturable (VBNC) forms of *Legionella*.

LD, on the other hand, is a severe pneumonic disease that develops within 2 to 10 days after the initial exposure. It has an attack rate in the general population of 0.1-5% (Remen *et al*., 2011) and accounts for 2-9% of cases of community-acquired pneumonia (von Baum *et al*., 2008). Its symptoms begin with a mild cough, malaise, muscle aches, low fever and gastrointestinal symptoms, and later manifestations include high fever, alveolitis and bronchiolitis. Considerable lung damage, with patchy infiltrated regions, can be observed by X-ray radiography (Winn, 1988). Histological reports describe intra- and extracellular bacteria in phagocytes, fibroblasts and alveolar epithelial cells (Fields, 1996).

Host-related risk factors for LD include increasing age, smoking, male gender, chronic lung disease, diabetes, lung cancer, and immunosuppressive treatment (Marston, 1994). On the other hand, polymorphisms of the Toll-like receptors 4 and 5 were shown to be associated with resistance to LD (Hawn *et al*., 2003; Hawn *et al*., 2005). Environmental factors such as high humidity and increased rainfall also increase the risk for legionellosis (Fisman *et al*., 2005). Disease occurrence was found to display a late summer to early autumn seasonality, presumably due to enhanced proliferation of the bacteria in warmer aquatic environments (Fisman *et al*., 2005; Marston *et al*., 1994).

Legionellosis is a major health concern for public health professionals. It has been estimated that between 10,000 to more than 100,000 cases of LD occur per year in the United States, resulting in 8000-18,000 hospitalizions each year with a case-fatality rate of approximately 8% (Hicks *et al*., 2007; Shelton *et al*, 2000). In Europe, the most recent report estimated the LD rate in 2008 to be 11.8 cases per million (Joseph and Ricketts, 2010). The largest outbreak of the disease (449 confirmed cases) was attributed to cooling towers in a city hospital in Murcia, Spain, in July 2001 (Garcia-Fulgueiras *et al*., 2003).

LD emerged in the second half of the twentieth century partly as a result of the development of artificial water systems, which have allowed *Legionella* to gain direct contact with susceptible human populations (Lam *et al*., 2011). Indeed, with the exception of natural hot springs where temperature ranges from 35^{o}C to 40^{o}C, the sources of legionellosis are

exclusively man-made water systems (Guyard and Low, 2011). Several outbreaks have been linked to a variety of aerosol-producing devices, such as cooling towers (Garcia-Fulgueiras *et al*., 2003), whirlpool spas (Den Boer, 2002), decorative fountains (O'Loughlin, 2007), industrial air scrubbers (Nygard *et al*., 2008), showers, hospital ventilation systems (Fields *et al*., 2002), and dental-unit water lines (Atlas *et al*., 1995).

Contaminated aerosols inhaled by patients reach the alveoli of the lung, where bacteria are engulfed by macrophages. Instead of being killed by macrophages, *L. pneumophila* survives and replicates in a protected niche within these phagocytes, leading to the tissue damage and inflammation that produce the symptoms of LD. Since person-to-person transmission of *L. pneumophila* infection has not yet been observed, the protozoan hosts are believed to have provided the primary evolutionary pressure from which the bacterium acquired and maintained its ability to overcome the defense mechanisms of phagocytes (reviewed in Fields *et al*., 2002).

Legionellosis can be diagnosed by isolation of *L. pneumophila* on specialized media, serologic diagnosis by antigen-specific immune response, or nucleic acid amplification technique that is not routinely performed on individuals suffering from pneumonia. Erythromycin was the most effective treatment against LD during the first outbreak in 1976 and remained the drug of choice until it was replaced by azithromycin and fluoroquinolones (reviewed in Fields *et al*., 2002).

1.1.3. The Developmental Cycle and Morphological Features of *L. pneumophila* Forms

To tolerate environmental fluctuations, many bacteria alter their cellular physiology and morphology, a process known as differentiation. The alternation between differentiated forms in bacteria within a cycle is called a developmental cycle. By employing cellular differentiation, bacterial pathogens can evade host defense mechanisms and promote self-preservation. For example, the sexually transmitted bacterium *Chlamydia trachomatis* has a dimorphic developmental cycle, alternating between an extracellular, metabolically inert elementary body (required for transmission) and an intracellular, metabolically active reticulate body that undergoes repeated cycles of cell division (Abdelrahman and Belland, 2005). Likewise, the aetiologic agent of human Q fever, *Coxiella burnetii*, differentiates

between a replicative large cell variant and a resilient small cell variant (Heinzen *et al*., 1999).

Similarly, *L. pneumophila* possesses a developmental cycle in which the bacteria alternate between differentiated forms (Fig.1.1) (Byrne and Swanson, 1998; Faulkner and Garduno, 2002; Garduno *et al*., 2002a). In pioneering studies performed by Rowbotham (1986), the bacteria were found to alternate between two morphologically distinct forms in amoebae, changing motility, shape, surface and stores of energy-rich polymers.

In broth cultures, *L. pneumophila* alternates between a replicative form (RF) and a stationary-phase form (SPF). SPF is sodium-sensitive, cytotoxic, osmotically resistant, competent to evade macrophage lysosomes, infectious, and motile (Byrne and Swanson, 1998). Ultrastructural analysis using a HeLa cell model (Faulkner and Garduno, 2002; Garduno *et al*., 2002a; Garduno *et al*. 1998a) revealed that *L. pneumophila* alternates intracellulary between a vegetative, metabolically active RF and a metabolically dormant, highly infectious cyst-like form termed the mature infectious form (MIF) (Garduno *et al*., 2002a), with various intermediate (RF-to-MIF or MIF-to-RF) morphological forms also reported (Faulkner and Garduno, 2002). MIFs give rise to morphologically distinct intermediates in nutrient-rich laboratory media as well, which in turn give rise to RFs (Garduno *et al*., 2002b).

While in transit through *T. tropicalis*, SPFs can rapidly and directly (in the absence of bacterial replication) differentiate into MIFs, indicating that SPFs and MIFs constitute a differentiation continuum (Faulkner *et al*., 2008). MIFs are formed in natural amoebic hosts and, to a lesser extent, in macrophages, but they do not develop *in vitro* (Faulkner and Garduno, 2002; Garduno *et al*., 2002a; Greub and Raoult, 2003).

Compared with SPF bacteria, MIFs produced from infection of HeLa cells were 10-fold more infectious (by plaque assay), displayed increased resistance to rifampin (3- to 5-fold) and gentamicin (10- to 1,000-fold), resisted detergent-mediated lysis, tolerated high pH, and had a very low respiration rate that is consistent with decreased metabolic activity (Garduno *et al*., 2002a). MIFs released from *T. tropicalis* were also more resistant to various stresses than SPFs. MIFs were more resistant to gentamicin, survived better in a nutrient-poor environment, and were more infectious to human pneumocyte cells compared with SPFs (Koubar *et al*., 2011).

In a murine lung infection model, co-infection with *L. pneumophila* and *H. vermiformis* potentiated the replication of *L. pneumophila,* resulting in a more severe pneumonia and a higher mortality rate compared to infection with *L. pneumophila* alone (Brieland *et al*., 1996). Moreover, *Legionella* cells that have replicated within amoebic hosts tend to infect other macrophages at a rate ten times higher than extracellularly replicating *Legionella* (Cirillo *et al*., 1994). These traits, displayed by MIFs and SPFs, promote the dissemination of *L. pneumophila* from one host to another and are referred to as 'transmissive' traits (Byrne and Swanson, 1998; Molofsky and Swanson, 2004). Ultrastructurally, both SPF and RF bacteria appear as slender rods and display wavy outer membranes, well-defined inner membranes and electronlucent periplasmic spaces. Both SPF and RF cytoplasmic regions are uniform in electron density. However, SPFs display cytoplasmic inclusions (Faulkner and Garduno, 2002; Garduno *et al*. 2002) (Fig.1.1A and C). In contrast, MIFs appear as stubby rods with a pleomorphic envelope that is irregular in shape and a dense cytoplasm containing cytoplasmic inclusions of poly-β-hydroxybutyrate (Garduno *et al*., 2002), which can be utilized by *L. pneumophila* as an energy source during starvation (James *et al*, 1999). Moreover, MIFs display a thickening of the inner leaflet of the outer membrane and long invaginations of the inner membrane that run along the cytoplasmic inclusions (Fig.1.1B) (Faulkner and Garduno, 2002; Garduno *et al*., 2002).

Giménez stain has been used to discriminate among the differentiated forms of *L. pneumophila* (Garduno *et al*., 2002). RFs typically display a green staining while SPF forms display a homogeneous dull red staining. On the other hand, MIFs exhibit a bright shade of red that is often bipolar (Fig.1.2) (Garduno *et al*., 2002).

A less studied morphotype of *L. pneumophila* is its filamentous form. *L. pneumophila* filaments are multinucleate and can easily reach lengths of more than 50 μm (Piao *et al*., 2006). Filamentous growth occurs in some bacterial species to increase fitness against adverse environmental conditions and, in the case of pathogens, against host immune responses (Justice *et al*., 2008). Consequently, bacterial filamentation could be an important contributor to virulence (Justice *et al*., 2008; Declerck, 2010). Filamentous *L. pneumophila* occurs in the environment and has been found in lung tissue from autopsies, sputum and bronchoalveolar lavage from infected patients and inside cultured epithelial cells, macrophages and amoeba (cited in Prashar *et al*., 2012).

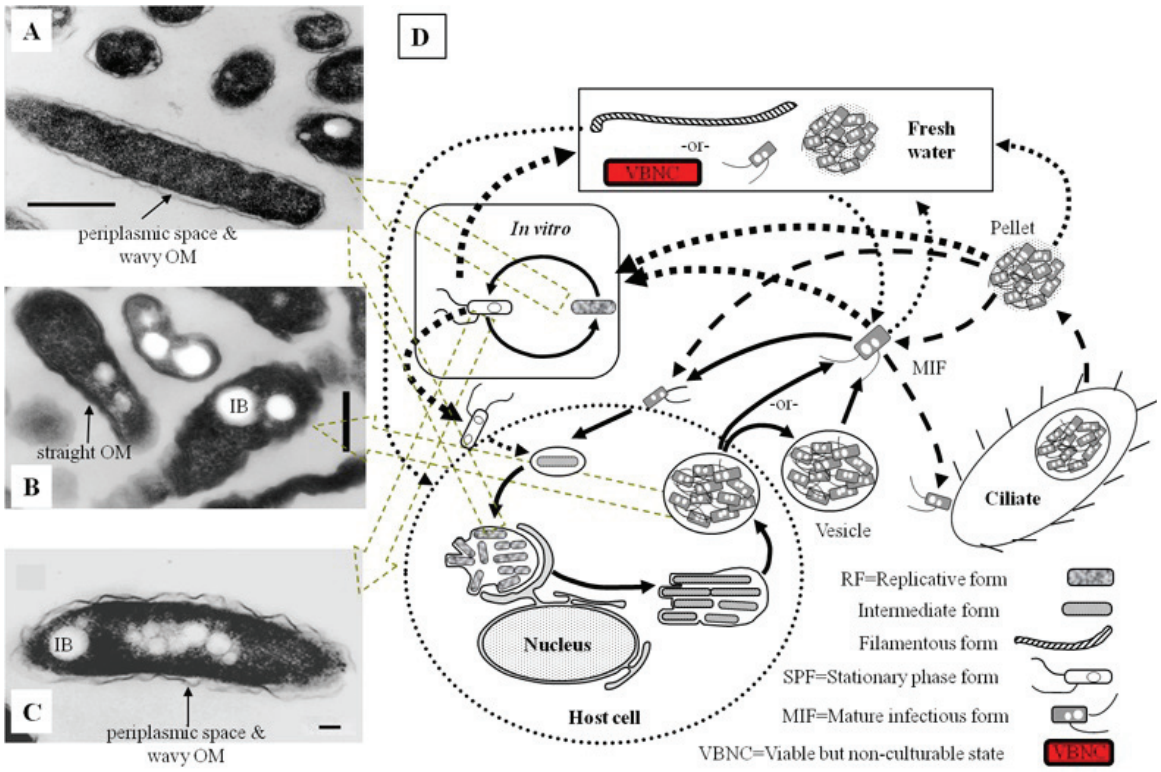


Fig.1.1. Schematic representation of the developmental cycle of *L. pneumophila*. The mature infectious forms (MIFs) or the stationary phase forms (SPFs) may attach to and enter a host cell (protozoan, macrophage, or cell line). After entry, they inhibit phagosome-lysosome fusion and recruit vesicles from the endoplasmic reticulum to form the replicative compartment, which is called the Legionella containing vacuole (LCV). Expression of the Dot/Icm proteins and other factors is required to abolish phagosome-lysosome fusion and provide further conditioning of the phagosome. Active replication takes place inside the LCVs. Replicative forms (RFs) appear as slender rods and display wavy outer membranes, well-defined inner membranes and electronlucent periplasmic spaces (panel A) (Bar 0.5 μm). Intermediate forms with a distinct morphology arise, while replication continues. Termination of intracellular replication leads to full differentiation of MIFs, which acquire a full-virulence phenotype. MIFs appear as stubby rods with a pleomorphic envelope that is irregular in shape and have a dense cytoplasm containing cytoplasmic inclusions. MIFs also display a thickening of the inner leaflet of the outer membrane and long invaginations of the inner membrane (panel B) (Bar 0.1 μm). Flagellum expression and cytotoxicity are upregulated. MIFs are released to the extracellular medium, either free, inside vesicles, or within pellets. MIFs may initiate another intracellular cycle. The loop formed from infection of the host cell by MIFs to the release of MIFs from the host cell and begining of new round of host cell infection is indicated with solid lines arrows and represent the "host cell" loop (panel D). Free MIFs released into water may become part of a biofilm, thus, entering the "water or biofilm" loop of the differentiation network (indicated by the circular-dot line arrows), or get ingested by ciliates, which act as packagers of *L. pneumophila* into pellets (ciliate loop indicated by the broken line arrows). Ciliate- produced pellets are infectious and able to initiate intracellular infections, as well as grow *in vitro* (D panel). When placed in a

suitable artificial growth medium, free or pelleted MIFs enter the "*in vitro*" loop (indicated by the squared-dot line arrows) of the differentiation network, where *L. pneumophila* alternates between the RF and the SPF (D panel). SPFs display a morphology similar to RFs in addition to the presence of cytoplasmic inclusions (panel C) (Bar 0.1 μm). SPFs may undergo filamentation or initiate biofilms. *L. pneumophila* may differentiate into the filamentous form, or enter the VBNC state, which may in turn, effeciently persist in the environment or initiate intracellular infection in amoeba (re-enetring the host cell loop) (panel D).Entry into the transmissive phase (SPFs and MIFs) is linked to enhanced virulence and improved resistance to environmental challenges, through a complex regulatory network (see text for details) (panel A, B , and C are reproduced from Garduno *et al*., 2002a; Faulkner and Garduno, 2002) (Apendix IV); (panel D is adapted with modifications from Garduno *et al.*, 2008)

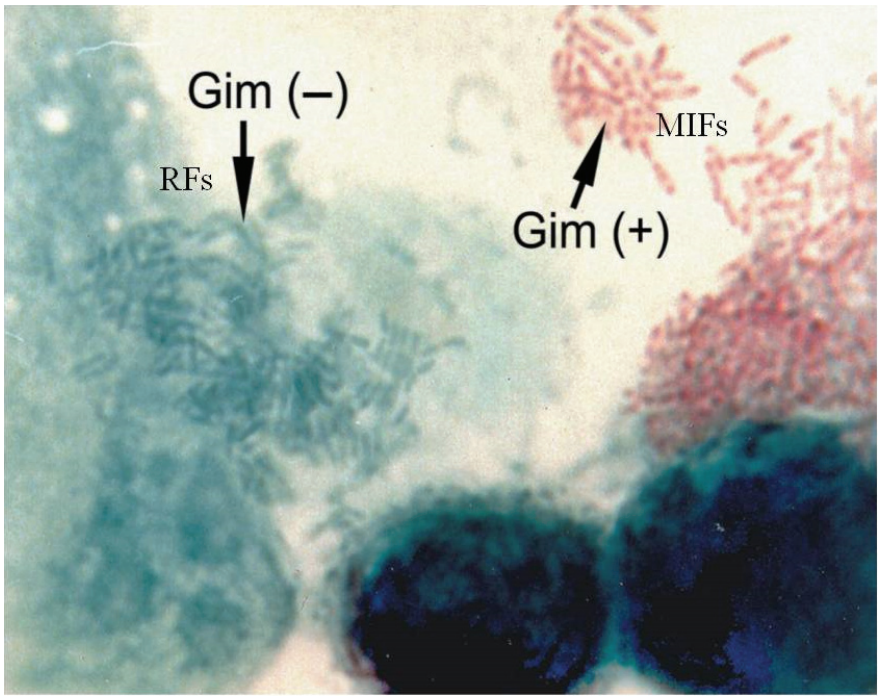


Fig.1.2. Giménez phenotypes observed in HeLa cell monolayers infected with *L. pneumophila*. MIFs display a bright shade of red [Gim(+) reaction] whereas the RFs display a green stain [Gim(-) reaction] (reproduced with modification from Garduno *et al*., 2002a) (Apendix IV).

Recently, filamentous *L. pneumophila* was demonstrated to invade lung epithelial cells by a mechanism not shared by non-filamentous *L. pneumophila* that involves the engagement of host cell β1 integrin and E-cadherin receptors. The internalized filamentous *L. pneumophila* occupy an intracellular compartment decorated with endoplasmic reticulum markers, where they differentiate into short rods and replicate to produce infective progeny. However, the molecular mechanisms that control the differentiation of *Legionella* from filaments to short rods is unknown; one possibility is that this phenomenon is triggered by the availability of intracellular nutrients (Prashar *et al*., 2012).

1.1.3.1. Evidence Supporting The Developmental Cycle of *L. pneumophila*

Further evidence supporting the developmental cycle of *L. pneumophila* was provided by Bruggemann *et al*. (2006), who used a DNA microarray chip comprised of all genes found in the *L. pneumophila* genome to monitor the *L. pneumophila* transcriptome during infection of *A. castellanii*. Expression of several hundred *L. pneumophila* genes was up- or down-regulated following the intracellular life cycle in protozoa (Bruggemann *et al*., 2006). Genes associated with the replicative phase included those encoding factors that promote replication (e.g., putative amino acid transporters), enzymes of the tricarboxylic acid (TCA) cycle, components of the electron transport chain, and some virulence-associated factors like CsrA (Bruggemann *et al*., 2006). Eventually, as nutrients and oxygen became limited, genes involved in the replicative phase were down-regulated and those involved in the transmissive phase were up-regulated, including genes encoding Dot/Icm-secreted effector proteins, EnhABC, LetE, FliA, integration host factor (IHF), several uncharacterized transcriptional regulators, and two-component systems (Bruggemann *et al*., 2006).

A family of regulators possessing a GGDEF/EAL motif was also up-regulated exclusively during the transmissive phase *in vivo* (Bruggemann *et al*., 2006). Members of this family are known to regulate the transition between motile and sessile bacteria found in biofilms when nutrients are scarce. This process is regulated through the diguanylate cyclase and phosphodiesterase activities of GGDEF/EAL proteins, which control intracellular concentrations of bis-(3',5')-cyclic dimeric guanosine monophosphate (c-di-GMP) (Romling and Amikam, 2006).

The ability of the c-di-GMP signaling network to control different levels of gene expression and function (transcriptional activity, post-transcriptional activity, enzymatic activity, and protein-protein interactions) is partially explained by the diversity of c-di-GMP binding modules. For example, c-di-GMP controls the production of *P.aeruginosa* extracellular polysaccharide (EPS) by altering DNA binding of the FleQ transcriptional regulator (Hickman and Harwood, 2008). Cyclic di-GMP also regulates gene expression post-transcriptionally by binding to riboswitches and affecting mRNA translation (Sudarsan *et al*., 2008).

In addition to its role in transcriptional and post-transcriptional gene regulation, c-di-GMP allosterically controls enzyme activity: binding of c-di-GMP to the PilZ domain of cellulose synthase, BcsA, is required for optimal activity of this enzyme (Benach *et al*., 2007). In *E. coli*, binding of c-di-GMP to the YcgR receptor protein results in altered flagellar motor output (Boehm *et al*., 2010). In *S. typhimurium*, mutation in an EAL-domain protein, influencing c-di-GMP levels, has been linked to the ability of the organism to kill macrophages and resist peroxides (Hisert *et al*., 2005). Recently, the *L. pneumophila* genome was found to encode 22 predicted proteins containing domains related to cyclic diguanylate synthesis, hydrolysis, and recognition; over-expression of several of these proteins strongly decreased the ability of *L. pneumophila* to grow inside host cells (Levi *et al*., 2011).

By comparing the two-dimensional patterns of protein spots obtained from MIFs and SPF bacteria, as well as using pulse-chase radiolabeling experiments, numerous proteins with an altered level of expression were identified. In particular, a dramatic increase in the levels of a 20-kDa protein associated with MIFs purified from HeLa cells was noted (Garduno *et al*., 2002a; Garduno *et al*., 2002b). MIFs were enriched for a 20-kDa protein named MagA (for MIF-associated gene); while MagA has no known function, its deletion resulted in MIFs exhibiting differences in Giménez staining, as well as an apparent increase in cytopathology to HeLa cells (Hiltz *et al*., 2004).

Proteomic analysis revealed a difference in protein expression between RF and SPF grown *in vitro* as well (Hayashi *et al*, 2010). Sixty-eight proteins differed significantly in their expression, with 64 of them upregulated in SPF. The up-regulated proteins included enzymes related to glycolysis, ketone body biogenesis and PHB biogenesis, suggesting that *L. pneumophila* may utilize sugars and lipids as energy sources when amino acids become

scarce. Proteins related to motility (flagella components and twitching motility associated proteins) were also up-regulated, suggesting that they enhance infectivity of the bacteria in host cells under certain conditions. Furthermore, nine up-regulated proteins of unknown function were found. Two of them were identified as novel bacterial factors associated with hemolysis of sheep red blood cells. Another two proteins were translocated into macrophages via the Icm/Dot type IV secretion apparatus as effector candidates in a reporter assay with *Bordetella pertussis* adenylate cyclase (Hayashi *et al*, 2010).

1.1.4. Regulation of *L. pneumophila* Differentiation

Byrne and Swanson (1998) proposed a model in which *L. pneumophila* expresses transmission traits when nutrients are limiting and replication traits when growth conditions are favourable. When nutrients are limiting within the host, *L. pneumophila* expresses factors to lyse the spent host, to survive and disperse into the environment, and to re-establish infection by avoiding lysosome degradation (Byrne and Swanson, 1998). Sauer *et al*. (2005a) linked nutrient acquisition to *L. pneumophila* differentiation by demonstrating that acquisition of an essential amino acid (threonine) by phagosomal transporter A (PhtA) is required for transmissive forms of *L. pneumophila* to differentiate to replicative forms in broth and in murine macrophages. Thus, *L. pneumophila* utilizes its Pht transporter to gauge amino acid availability before entering the replicative phase (Sauer *et al*., 2005).

The global change in gene expression of *Legionella* is mediated by the alarmone ppGpp (guanosine 3', 5'- bispyrophsophate). When nutrients are abundant, transmissive bacteria hydrolyze ppGpp, resulting in initiation of cell division and repression of transmission factors. As the replicating bacteria consume nutrients, vacuolar conditions presumably deteriorate and stimulate ppGpp production, prompting the progeny to re-enter the transmissive state (Sauer *et al*., 2005; Molofsky and Swanson, 2003). Cellular pools of ppGpp are generated by the ppGpp synthase RelA and by the bifunctional synthase/hydrolase SpoT. RelA associates with bacterial ribosomes and responds to amino acid starvation by sensing uncharged tRNA (Potrykus and Cashel, 2008). Then, by using ATP as a phosphate donor, RelA phosphorylates GDP and GTP, to synthesize ppGpp and its precursor, pppGpp (guanosine 3'-diphosphate, 5'-triphosphate), respectively (Srivatsan and Wang, 2008).

In a similar fashion, SpoT, the bifunctional ppGpp synthase/hydrolase, responds to fatty acid starvation by associating with acyl-carrier protein, which is critical for fatty acid biosynthesis (Battesti and Bouveret, 2006). SpoT can also generate ppGpp in response to a variety of stresses, including carbon source deprivation, phosphate starvation, and iron starvation (Srivatsan and Wang, 2008). The ppGpp effector molecule then binds directly to the β and β' subunits of the RNA polymerase (RNAP) core enzyme. As a result, the transcription of transmissive phase genes in *L. pneumophila* such as flagellar genes, Dot/Icm Type IV secretion system components, and regulatory RNAs is up-regulated, while the expression of replication-related genes, such as ribosomal genes and others involved in ATP synthesis, is inhibited (Fig.1.3) (Dalebroux *et al*., 2010; Magnusson *et al*., 2005; Srivatsan and Wang, 2008).

In *L. pneumophila*, interaction of ppGpp with RNAP is cooperative with the protein DksA, which binds to the secondary channel of RNAP (Haugen *et al*., 2008). By binding to RNAP, DksA modifies kinetic properties of specific promoters by sensitizing them to changes in ppGpp concentration (Paul *et al*., 2004). DksA and ppGpp can cooperate to up or down-regulate a gene based on its promoter sequence. In broth, DksA promotes differentiation when ppGpp levels increase as judged by the flagellin subunit, *flaA*, expression and evasion of degradation by macrophages (Dalebroux *et al*., 2010). DksA and ppGpp can also perform certain functions in the complete absence of the other. For macrophage transmission, ppGpp is essential, whereas DksA is dispensable, indicating that ppGpp can act autonomously (Dalebroux *et al*., 2010). Over-expressed *dksA* in *L. pneumophila* partially rescued motility and cytotoxicity of $ppGpp^0$ mutant (*relA* and *spoT* double mutant). However, unlike $ppGpp^0$ mutant *L. pneumophila*, which is degraded following the primary replication period in macrophages, *dksA* mutants survive in macrophages and replicate to wild-type levels (Dalebroux *et al*., 2010).

DksA and ppGpp can also regulate gene expression indirectly through their interactions with RNAP. In the vegetative state of *E. coli* growth, the housekeeping sigma factor, σ^{70}, is associated with RNAP and is frequently bound to ribosomal RNA promoters (Bremer and Dennis, 1996). However, upon activation of the stringent response, ppGpp and DksA bind RNAP and dissociate it from σ^{70}, freeing the core polymerase to access alternate sigma factors such as σ^{54} (Bernardo *et al*., 2006). Subsequently, RNAP is directed to stress

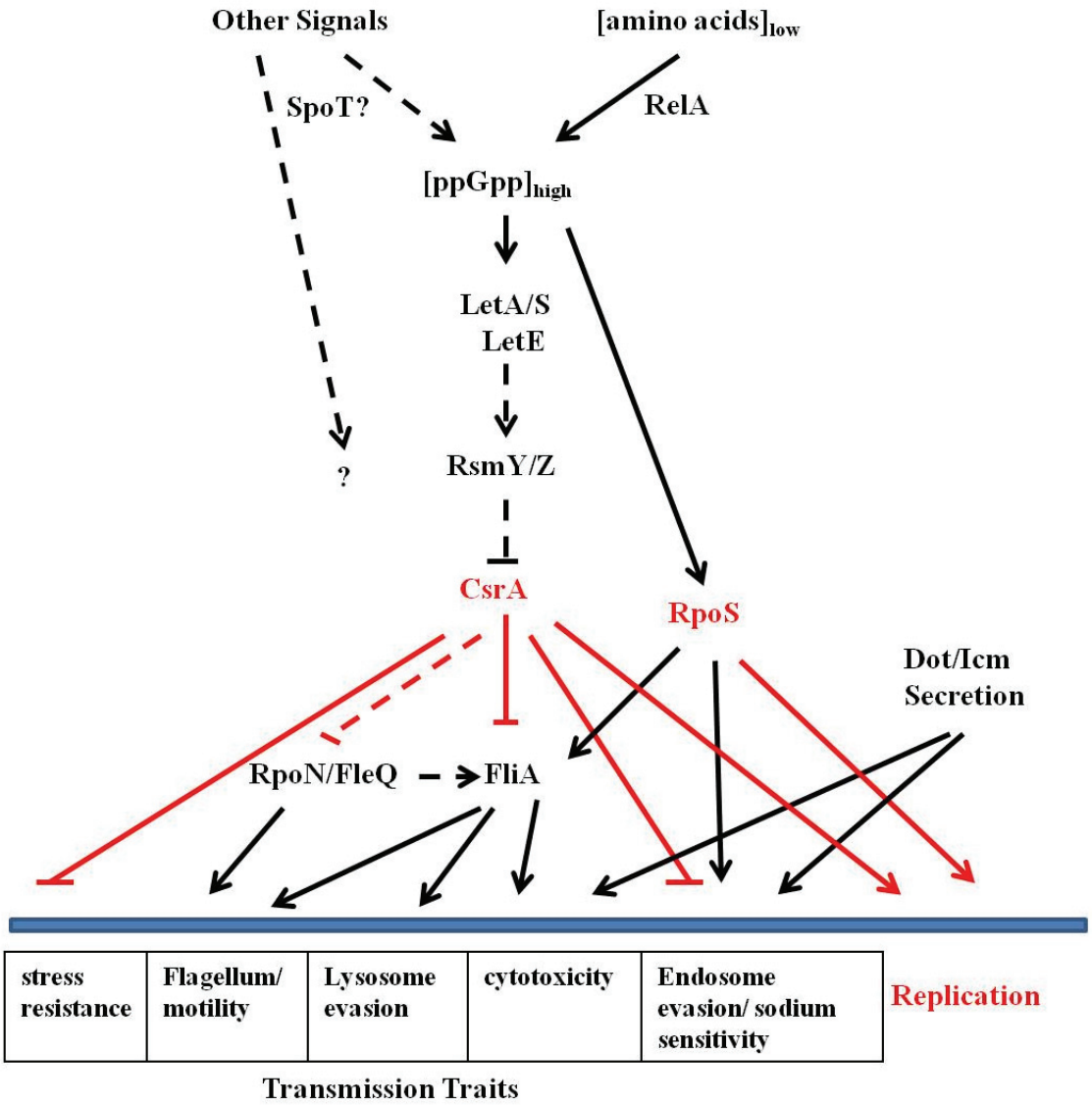


Fig.1.3. A model for the regulation of *L. pneumophila* differentiation. Arrows indicate activation and bars indicate inhibition. Replicative phase regulatory interactions are represented by solid red lines, while transmission phase regulatory pathways are indicated by solid black lines. Speculative interactions are designated by dotted lines (reproduced with permission from Molofsky and Swanson, 2004) (Apendix IV).

response-related genes by these alternative sigma factors. Thus, many genes under the control of an alternate sigma factor may be regulated by ppGpp and /or DksA by an indirect mechanism (Szalewska-Palasz *et al*., 2007).

Since *L. pneumophila* persists in a variety of aquatic and soil environments, it is conceivable that metabolic cues other than amino acids and fatty acids also induce its differentiation (Edwards *et al*., 2013). One common way that microbes respond to external stimuli is via two-component signal transduction systems. For many two-component systems, the cues that initiate autophosphorylation and the subsequent phosphorelay are unknown. However, it is believed that a variety of environmental stimuli or conditions can activate these systems (reviewed in Calva and Oropeza, 2006).

At the core of *L. pneumophila*'s differentiation circuitry is the *Legionella* transmission activator and sensor (LetA/LetS), a two-component system that regulates all known transmission traits and is required by *L. pneumophila* in order to respond to elevated ppGpp (Hammer *et al*., 2002; Lynch *et al*., 2003; Shi *et al*., 2006). A *letA* mutation led to dramatically decreased transcriptional levels of *dotA* (encoding for a component of the type IVB secretion system) and *ralF* (encoding for a type IVB secretion system substrate). In addition, the *letA* mutation dramatically decreased the secretion of macrophage infectivity protein (Mip) (Shi *et al*., 2006).

LetA/LetS belongs to a family of signal-transducing proteins that employ a four-step His-Asp-His-Asp phosphorelay to regulate gene expression. However, rather than acting as on/off switches, the LetA/S system exhibits rheostat activity, which likely confers versatility as microbes adapt to fluctuating environments. This was found when a threonine substitution at position 311 of *letS* resulted in a mutant resembling the wild type for some traits, and *letS* null mutants for others, while displaying intermediate levels of infectivity, cytotoxicity, and lysosome evasion (Edwards *et al*., 2010).

The mechanism by which ppGpp activates the LetA/S system remains unknown. Nicotinic acid, a precursor of the central metabolite nictotinamide adenine dinucleotide (NAD+), was postulated to modulate the LetA/S system (Edwards *et al*., 2013) since nicotinic acid modulates the activity of microbial two-component systems such as BvgA/BvgS (Miller *et al*., 1989) and EvgA/EvgS (Utsumi *et al*., 1994). Indeed, *L. pneumophila* differentiation was triggered by nicotinic acid (Edwards, *et al*., 2013).

The LetA transcriptional activator positively regulates the transcription of two non-coding regulatory RNAs, RsmY and RsmZ (Rasis and Segal, 2009). These sRNAs have repeated GGA motifs in their sequences (Valverde *et al*., 2003) that are essential for their binding to the RNA binding protein, CsrA (Heeb *et al*, 2006). CsrA acts as a translational repressor by binding to mRNAs that contain a CsrA binding site at or near the ribosomal binding site (Wang *et al*., 2005; Baker *et al*., 2002).

In *L. pneumophila*, CsrA is expressed during the exponential growth phase and was shown to repress a panel of transmission traits such as stress resistance, flagellation, contact-dependent cytotoxicity, coccoid morphology, lysosome evasion, intracellular growth, and expression of *rpoS*, *letE*, and *fliA* (Fettes *et al*., 2001; Forsbach-Birk *et al*., 2004; Hammer *et al*., 2002; Heuner *et al*., 2002; Molofsky and Swanson, 2003). Interaction of RsmY and RsmZ with CsrA is thought to relieve its ability to bind transcripts critical for the transmissive phenotype (Molofsky and Swanson, 2003; Rasis and Segal, 2009).

During intracellular replication, CsrA negatively regulates specific flagellar gene regulators. Conditional *csrA* null mutant *L. pneumophila* is unable to replicate in host cells and exhibits motility when nutrients are replete (Molofsky and Swanson, 2003). In accordance with these findings, reduced expression of *csrA* leads to accumulation of *flaA* and *fliA* transcripts, the latter encodes the alternative sigma factor FliA, which in turn regulates expression of flagellar genes and other transmissive traits (see below) (Forsbach *et al*., 2004). Conversely, over-expression of *csrA* in stationary phase bacteria represses motility and leads to reduced levels of *fliA* and *flaA* transcripts (Molofsky and Swanson, 2003; Fettes *et al*., 2001).

The small activator protein, LetE, enhances the expression of various LetA/S-dependent stationary growth phase traits and promotes contact-dependent cytotoxicity and macrophage infectivity (Hammer *et al*., 2002; Bachman and Swanson, 2004). LetE induces the characteristic shape, pigment, and heat resistance of stationary phase *L. pneumophila* (Bachman and Swanson, 2004b).

Another factor purportedly downstream of ppGpp in the *L. pneumophila* virulence cascade is the stationary phase sigma factor RpoS, which is essential for intracellular replication of *L. pneumophila* in *A. castellani* and is partially required for growth in macrophages (Hovel-Miner *et al*., 2009; Bachman and Swanson, 2001). Although RpoS

translation efficiency and protein levels have not been monitored in *L. pneumophila*, the *rpoS* transcript is more abundant in exponential than in stationary phase (Bachman and Swanson, 2004a). Mutant RFs lacking either *rpoS* or the response regulator gene *letA* were unable to produce normal SPFs *in vitro* and did not fully differentiate into MIFs *in vivo*, further supporting the existence of a common mechanism of differentiation shared by SPFs and MIFs (Faulkner *et al*., 2008).

As with *E. coli*, *rpoS* expression by *L. pneumophila* is sensitive to ppGpp pools, since transcript levels increase following artificial induction of ppGpp (Brown *et al*., 2002). Thus, it appears that ppGpp controls RpoS expression to affect transmission and replication in *L. pneumophila*; however, details regarding the interplay within this complex regulatory mechanism remain to be clarified. RpoS is also integrated into the *L. pneumophila* CsrA regulatory system. In particular, this sigma factor is required for maximal *rsmY* and *rsmZ* gene expression (Rasis and Segal, 2009; Hovel-Miner *et al*., 2009). Therefore LetA/S and RpoS cooperate, by mechanisms yet to be defined, to relieve CsrA-mediated repression of transmissive transcripts.

Consistent with this model, LetA/S and RpoS are required for robust accumulation of *fliA* mRNA in stationary phase *L. pneumophila* (Bachman and Swanson, 2004b). However, the basal level of *fliA* transcripts in *rpoS* mutant *L. pneumophila* is still sufficient to induce activation of *flaA*, leading to a modest accumulation of *flaA* mRNA and partial motility of mutant bacteria (Bachman and Swanson, 2004b; Bachman and Swanson, 2001). Contrary to this, basal levels of *fliA* are not sufficient for *flaA* expression in *letA/S* mutant bacteria, suggesting that LetA/S affects FliA activity independent of RpoS. This incomplete overlap between LetA/S and RpoS implies that dual pathways likely contribute to optimal flagellar expression of *L. pneumophila*. Thus, regulatory interactions between ppGpp, LetA/S, RpoS and CsrA are critical for flagellar assembly and enable *L. pneumophila* to rapidly transition between replicative and transmissive virulence programs in host cells (Bachman and Swanson, 2004b).

In addition to the Csr regulatory system, prokaryotes can employ other noncoding RNAs to modulate gene expression. These sRNAs require the Hfq chaperone protein and, through complementary base pairing with mRNAs, can modify either the translation or stability of their mRNA targets (reviewed in Gottesman, 2004). A gene in *L. pneumophila*

that has significant homology to published *hfq* genes demonstrated regulation by RpoS and the transcriptional regulator LetA (McNealy *et al*., 2005). Hfq appears to play a major role in exponential phase regulatory cascades of *L. pneumophila*. Mutants lacking *hfq* demonstrate defects in growth and slight defects in virulence in both amoeba and macrophage infection models (McNealy *et al*., 2005).

Data indicates that Hfq contributes to virulence in a number of bacterial pathogens, presumably by altering the interaction of sRNAs with *rpoS*, thereby affecting *rpoS* translation. In the replicative phase, RpoS induces the expression of *hfq;* exponential phase transcripts, such as *csrA* and *fur* (ferric uptake regulator), are then stabilized by Hfq and most likely by sRNAs (McNealy *et al*., 2005). Upon entering stationary phase, ppGpp accumulates and the LetA/S two-component system is induced (Hammer *et al*., 2002; Hammer and Swanson, 1999). As a result, LetA activates transmissive phase traits, while directly or indirectly repressing *hfq* transcription (McNealy *et al*., 2005).

The small DNA binding regulatory proteins integration host factor (IHF), which acts as an accessory factor that influences regulatory processes such as replication and transcription by bending the DNA (Goosen and van de Putte, 1995), and HU, a heat-stable nucleoid protein paralogue of IHF (Dame and Goosen, 2002), are reciprocally expressed over the developmental cycle: HU is expressed during exponential phase and IHF is expressed postexponentially. IHF mutants failed to grow in *A. castellanii* but were infectious for HeLa cells, though electron microscopic examination revealed defects in late-stage differentiation (thickened cell wall, intracytoplasmic membranes, and inclusions of PHB), and were depressed for the developmental marker MagA (Morash *et al*., 2009).

L. pneumophila flagellar gene transcription occurs in a hierarchy of four classes (Albert-Weissenberger *et al*., 2010; Bruggemann *et al*., 2006). The master regulator FleQ and the alternative sigma factors RpoN (σ^{54}) and FliA (σ^{28}) control expression of particular gene classes. Albert-Weissenberger *et al*. (2010) found that the Class I factors FleQ and RpoN control transcription of 14 out of 31 Class II genes encoding elements of the flagellar basal body, rod, hook, and regulatory proteins.

FleQ also contributes to activation of some Class III genes, including FliA (Albert-Weissenberger *et al*., 2010), which is critical for final assembly of the organelle. It targets additional Class III elements, including motor components, and all Class IV elements,

including the filament FlaA and the capping protein FliD (Heuner *et al*., 2002; Bruggemann *et al*., 2006; Albert-Weissenberger *et al*., 2010). The role of FliA is not limited to flagellar regulation; it is critical for *L. pneumophila* to resist degradation in macrophage lysosomes (Molofsky *et al*., 2005; Bruggemann *et al*., 2006). Moreover, FliA is essential for intracellular replication in particular protozoan hosts. Thus, FliA provides an additional link between the flagellar cascade and virulence of *L. pneumophila* (Heuner *et al*., 2002).

1.1.5. Pathogenesis of *L. pneumophila*

Phagocytes eliminate engulfed microorganisms by delivering them into the lysosomal system. Bacteria not adapted for an intracellular life cycle are effectively digested within the phagolysosome, an acidic environment that contains various activated hydrolytic enzymes (reviewed in Scott *et al*., 2003). *L. pneumophila* possesses many of the traditional bacterial determinants that are important for pathogenicity in other bacteria, including lipopolysaccharide (LPS), flagella, pili, a type II secretion system (T2SS), and outer membrane proteins. However, the ability to manipulate host cell processes from within an intracellular vacuole requires a unique arsenal. In the case of *L. pneumophila*, this arsenal includes a type IV secretion system (T4SS) that translocates effector proteins, including many proteins with eukaryotic similarity, into the host cell, where they act on diverse host cell pathways (reviewed in Newton *et al*., 2010).

The intracellular infection of *L. pneumophila* in the human macrophage model is remarkably similar to the protozoan model. In fact, most of the genes required for intracellular replication in the human host are also required for replication in the protozoan host (Gao *et al*., 1997). Indeed, the current perspective is that the ability of *L. pneumophila* to infect and replicate in mammalian cells is a consequence of its evolutionary capacity to infect and replicate in protozoan hosts (Abu Kwaik *et al*., 1998). Within minutes of internalization, the bacterial phagosome, also called the *Legionella*-containing vacuole (LCV), is covered by small smooth vesicles that likely originate from the endoplasmic reticulum (ER). The phagosomal membranes come to resemble those of the ER in thickness and protein composition, and later become decorated with ribosomes. Other host organelles, such as mitochondria, are also recruited to the area of the LCV (Tilney *et al*., 2001). This change in membrane structure occurs concomitantly with the successful evasion of the endocytic

pathway (Horwitz, 1983). The LCV compartment not only protects *L. pneumophila* from being recognized by the cellular immune system but also provides the bacterium with nutrients for its replication (Price *et al*., 2011).

1.1.5.1. Attachment and Entry/Uptake of *L. pneumophila* by Host Cells

Attachment to and invasion of the protozoa *H. vermiformis* and *A. polyphaga* by *L. pneumophila* is mediated by a protozoan 170-kDa lectin that is inhibited by galactose/N-acetylgalactosamine (Harb *et al*., 1998; Venkataraman *et al*., 1997). Upon attachment, very fast dephosphorylation of tyrosine-phosphorylated proteins, including the 170-kDa receptor itself and cytoskeletal-associated proteins, occurs (Venkataraman *et al*., 1998). It is interesting that *Entamoeba histolytica* also encodes a 170-kDa lectin that mediates its attachment to mammalian epithelial cells (Ravdin *et al*., 1986). Inhibition studies demonstrated the functional similarity of these lectins. *L. pneumophila* attachment to and invasion of *H. vermiformis* was decreased in a dose-dependent manner by soluble galactose, N-acetyl-D-galactosamine, or two monoclonal antibodies specific to the 170-kDa protein of *E. histolytica* (Venkataraman *et al*., 1997). The bacterial ligand(s) responsible for lectin binding have yet to be identified.

The recognition and initial events in *Acanthamoeba* spp. phagocytosis are similar to those in mammalian macrophages, notably the presence of a D(+)-mannose inhibitable receptor (Allen and Dawidowicz, 1990a; Allen and Dawidowicz, 1990b; Lock *et al*., 1987). Interestingly, the importance of this receptor in *Legionella* uptake may not be specific for the entire *Acanthamoeba* genus. The addition of the same concentration of D-mannose to the culture medium blocks *A. castellanii* uptake of *L. pneumophila*, but does not block uptake of *A. polyphaga*. Furthermore, addition of cyclohexamide, an inhibitor of protein synthesis, and cytochalasin D, a microfilament disrupter, will inhibit *A. castellanii* but not *A. polyphaga* uptake of *L. pneumophila* (Harb *et al*., 1998; Declerck *et al*., 2007c). On the other hand, uptake of *L. pneumophila* by *H. vermiformis* is reduced by methylamine, an inhibitor of adsorptive pinocytosis, and by cyclohexamide, but not by cytochalasin D. These findings suggest considerable heterogeneity in uptake mechanisms by different protozoan hosts (King *et al*., 1991).

Recently, E-cadherin and β1 integrin receptors have been found to mediate

attachment of the filamentous forms of *L. pneumophila* to lung epithelial cells. The activation of these receptors induced the formation of actin-enriched membrane surface structures that were designated hooks and membrane wraps. While hooks are filopodia-like protrusions that trap bacteria at the host cell surface, membrane wraps are rearrangements of opposing lamellar protuberances that engulf filamentous bacteria. *L. pneumophila* entrapped by these structures are gradually internalized through a zipper mechanism of phagocytosis (Prashar *et al*., 2012).

Some of the bacterial factors implicated in *L. pneumophila* host cell invasion include EnhC, LpnE, RtxA, LvhB2, and HtpB (reviewed in Newton *et al*., 2010), although few of these have been shown definitively to play a direct role in bacterial uptake. For example, EnhC is a periplasmic protein that is required for the maintenance of cell wall integrity, so its contribution to bacterial invasion is likely to be indirect (Liu *et al*., 2008). The best-characterized of the entry proteins is the surface-located chaperonin HtpB or Hsp60 (the 60-kDa heat shock protein), which interacts with undetermined host cell receptor(s), triggering cell signaling and secretion of IL-1 (Retzlaff *et al*., 1996).

HtpB is upregulated in the presence of eukaryotic cells and, *in vitro,* in response to H_2O_2, heat, and osmotic shock (reviewed in Swanson and Hammer, 2000). HtpB-specific antibody inhibits entry of *L. pneumophila* and purified HtpB protein stimulates uptake of latex beads by HeLa cells (Garduno *et al*., 1998b). HtpB accumulates in the LCV after bacterial uptake, and it has been suggested that HtpB contributes to the recruitment of mitochondria to the nascent LCV, as inert beads coated with HtpB are associated with mitochondria following invasion (Chong *et al*., 2009). Thus, HtpB may play a dual role in bacterial entry and the early development of the LCV, suggesting that these two events are closely linked. LadC, is another *L. pneumophila* protein that may be involved in initial interactions with host cells: mutations in LadC caused a defect in host cell adherence. This protein localizes to the bacterial inner membrane, and its role in adherence is not yet known (Newton *et al*., 2008).

It has been found that the Dot/Icm system enhances the efficiency of *L. pneumophila* uptake into phagocytic host cells. A role for translocated Dot/Icm effectors during uptake is supported by reduced internalization by macrophage-like HL-60 cells of nonfunctional Dot/Icm mutants compared with the wild type (Hilbi *et al*., 2001). Only a few described

effectors have been suggested to function during uptake. For example, LaiA/SdeA may play a role in adherence and uptake (Chang *et al*., 2005). *L. pneumophila laiA/sdeA* mutants have defects in adhesion and invasion in epithelial cells as well as reduced mortality in permissive A/J mice. However, the mechanism by which this protein may influence adherence and uptake remains to be determined (Chang *et al*., 2005).

Type IV pili, which mediate host cell attachment by pathogenic *Neisseria* spp., *P. aeruginosa*, and other bacterial spp., may also function as *L. pneumophila* adhesins (reviewed in Swanson and Hammer, 2000). An insertional mutation in the putative *L. pneumophila* pilin structural gene, *pilEL*, reduced the bacterial adherence to *A. polyphaga* and the mammalian monocytic U937 and epithelial HeLa cell lines by ~50%, although intracellular replication was not affected (Stone and Abu Kwaik, 1998). Thus *L. pneumophila* pili may contribute to binding to mammalian macrophages and alveolar epithelial cells, and may serve as the ligand for the protozoan lectin. However, the role of the type IV pilus in Legionnaires' disease remains to be confirmed (Swanson and Hammer, 2000).

1.1.5.2. The Dot/Icm Secretion System

A region of the *L. pneumophila* genome, termed *icm* (intracellular multiplication), was identified based on the ability of this region to restore normal trafficking and replication in the avirulent strain 25D (Marra *et al*., 1992). Additionally, genetic screens aimed at isolating *L. pneumophila* mutants that were defective for inhibition of phagosome/lysosome fusion and association of host cell organelles led to the identification of a second genetic locus that was termed *dot* (defect in organelle trafficking) (Berger and Isberg, 1993). Computational analysis of the proteins predicted to be encoded by the *dot* and *icm* genes revealed that these products would likely function as a type IV secretion system (T4SS) (reviewed in Hubber and Roy, 2010).

T4SSs are often found in bacteria that interact with eukaryotic hosts. Many of these systems are now recognized as transport machines that have a dedicated role in the transport of virulence proteins, known as effectors, across the outer membrane of the bacterial cell and usually directly into eukaryotic host cells. Because mutations disabling the Dot/Icm system resulted in bacteria unable to replicate intracellularly and avoid fusion with lysosomes, this system was proposed to play a critical role in infection by translocating effectors that alter the

dynamics of membrane transport in the host cell (reviewed in Hubber and Roy, 2010). To date, there are at least 275 experimentally confirmed Dot/Icm substrates (Zhu *et al*., 2011).

Many Dot/icm effectors have eukaryotic-like functions. Phylogenetic analyses demonstrated that lateral gene transfer from eukaryotic hosts, as well as bacterial genes that became eukaryotic-like by gradual adaptation to the intracellular milieu or gene fragment acquisition, contributed to the existing repertoire of eukaryotic-like proteins (ELPs), which comprise over 3% of the putative proteome of *L. pneumophila* strains (Lurie-Weinberger *et al*., 2010).

1.1.5.3. Biogenesis of *Legionella* Containing Vacuole and Acquisition of Nutrients

The unusual biogenesis of the LCV arises from the simultaneous delay of endosome fusion and the recruitment of vesicles and membrane from the host cell secretory pathway (reviewed in Newton *et al*., 2010). Professional phagocytes such as fresh water amoebae and human macrophages destroy invading microorganisms or foreign particles by engulfing them into phagosomes. Phagosomes traditionally mature into digestive vacuoles via the endocytic pathway, which involves a progressive interaction with the endosomal network that leads to acidification of the vacuole and degradation of most of the microbes (Garin *et al*., 2001).

The majority of LCVs containing wild type bacteria do not acquire early endosome markers such as Rab5 or the lysosome marker LAMP-1 during the early phase of infection (< 1 h). However, phagosomes containing Dot/Icm-deficient mutants do ultimately fuse with lysosomes (Berger and Isberg, 1993), indicating that interactions with the endocytic pathway are blocked immediately by effectors introduced by the bacterium. Bacterial factors beyond Dot/Icm effectors have been implicated in the evasion of the endocytic pathway, including the LPS-rich outer membrane vesicles released by *L. pneumophila* (Fernandez-Moreira *et al*., 2006), the multifunctional chaperone HtpB exposed on the bacterial surface (Garduno *et al*., 1998b), and *L. pneumophila* proteins containing Sel-1 repeats, such as LpnE, EnhC and LidL (Newton *et al*., 2007). Because the mechanisms of action of these proteins remain enigmatic, it is unclear whether they directly contribute to the arrest of LCV maturation.

L. pneumophila hijacks the host secretory pathway, which transports proteins synthesized in the ER to the Golgi complex and finally to downstream cellular destinations or

the extracellular environment (Mellman and Warren, 2000). Interception of vesicles originating from the ER facilitates the conversion of the plasma membrane of the LCV into membranes with ER characteristics (Kagan and Roy, 2002).

Members of the Arf (ADP-ribosylation factor), Rab and Sar families of small GTPases are primary regulators of vesicle trafficking. Arf1 regulates COPI-coated retrograde trafficking from the cis-Golgi compartment to the ER (reviewed in D'Souza-Schorey and Chvrier, 2006). This small GTPase is recruited and enriched on the LCV membrane in a Dot/Icm-dependent manner. A Sec7 domain is conserved in all guanine nucleotide exchange factors (GEFs), which activate membrane-associated GTPases by inducing the exchange of GDP with GTP, of Arf small GTPases. *L. pneumophila* codes for a protein with Sec7 domain called RalF. RalF is required for the recruitment of Arf1 by the LCV and is a GEF for Arf1 (Nagai *et al*., 2002).

Rab1 GTPase also plays important roles in vesicle trafficking between the ER and the Golgi apparatus (Stenmark, 2009). Like Arf1, Rab1 is recruited to the LCV in a process that requires the Dot/Icm transporter (Kagan *et al*., 2004). SidM/DrrA is a GEF factor for Rab1 and is required for the recruitment of this small GTPase by the LCV. The SidM/DrrA-mediated Rab1 recruitment is enhanced by LidA, another Dot/Icm substrate, which interacts with Rab1, irrespective of its nucleotide binding status (Machner and Isberg, 2006). The recruitment of Rab1 by SidM/DrrA directly contributes to the fusion of ER-derived vesicles to the LCVs (Arasaki *et al*., 2012).

In addition to the endocytic and secretory pathways, *L. pneumophila* also exploits the host ubiquitination pathway. Ubiquitination is a post-translational modification that regulates the activity, half-life, and cellular localization of a wide variety of proteins. Ubiquitination involves an enzymatic cascade resulting in the formation of an isopeptide bond between ubiquitin and internal lysine (K) residues of a substrate protein. This process involves a ubiquitin-activating enzyme (E1), which forms a thioester bond between a catalytic cysteine and the carboxy terminal glycine (G) residue of ubiquitin. The ubiquitin is then transferred to a ubiquitin-conjugating enzyme (E2). Finally, a ubiquitin ligase (E3) promotes the covalent conjugation between G of ubiquitin to one or more K residues in a specific substrate. Polyubiquitination occurs when additional ubiquitin molecules are attached to a lysine residue of the previous ubiquitin and form a ubiquitin chain. (reviewed in Xu and Luo, 2013).

The importance of the ubiquitination machinery in *L. pneumophila* infection is clearly demonstrated by the enrichment of ubiquitinated proteins on its phagosome, as well as the fact that pharmaceutical inhibition of proteasomal degradation causes arrest in intracellular bacterial growth (Dorer *et al*., 2006). At least 6 Dot/Icm substrates bear features associated with typical eukaryotic E3 ubiquitin ligases (reviewed in Xu and Luo, 2013). Among these proteins is AnkB, which is composed of eukaryotic domains and motifs that include an F-box domain, two ankyrin repeats (ANKs) and a C-terminal CAAX farnesylation motif, where C represents a cysteine residue, A denotes an aliphatic amino acid, and X any amino acid (Al-Quadan and Abu-Kwaik, 2011).

The F-box domain of AnkB interacts with the host Skp1 component of the SCF (Skp1-Cullin-F-box) ubiquitin ligase complex and functions as a platform for docking of polyubiquitinated proteins to the LCV membrane within evolutionarily distant hosts, macrophages and amoeba (Price *et al*., 2009).

The two ANK protein-protein interaction domains of AnkB are also essential for the biological functions of AnkB: deletion of these domains in U937 macrophage-like cells and *A. polyphaga* resulted in defects in intracellular proliferation and decoration of the LCV with polyubiquitinated proteins. Price *et al.* (2010) suggested that the two ANK domains might be involved in binding the substrates and linking them to the SCF complex bound to the F-box domain to trigger their polyubiquitination.

Interestingly, a recent report suggests that AnkB is involved in the production of amino acids to provide nutrients for *L. pneumophila* inside the LCV (Price *et al*., 2011). AnkB promotes proteasome-mediated generation of free amino acids that are essential energy and carbon sources. Polyubiquitinated proteins assembled by AnkB on the LCV are preferentially enriched for Lys48-linked polyubiquitinated proteins (a hallmark for proteasomal degradation) that generate 2-24 amino acid peptides. Substitution of Lys48 to Arg abolishes the decoration of the LCV with polyubiquitinated proteins and blocks intracellular proliferation (Price *et al*., 2011). However, this inhibition is bypassed by excess amino acid supplementation to the infected amoeba or macrophage cells.

Cells infected by the wild type strain have high levels of free amino acids compared to the *ankB* mutant-infected cells, which are similar to uninfected cells (Price *et al*., 2011). Cysteine or serine supplementation rescues the *ankB* mutant as efficiently as the mixture of

amino acids. Cysteine and serine are converted by *Legionella* into pyruvate that feeds the TCA cycle, and *L. pneumophila* relies on amino acids as the major sources of carbon and energy production through the TCA cycle.

Interestingly, supplementation of amoeba or macrophages with pyruvate or citrate rescues the *ankB* mutant for intracellular proliferation within amoeba and macrophages and for its starvation response, similar to the supplementation by the mixture of amino acids. Remarkably, injection of mice with the mixture of amino acids or with cysteine specifically rescues the *ankB* mutant for intrapulmonary proliferation, similar to genetic complementation (Price *et al*., 2011). These findings indicate that the ultimate goal of exploitation of multiple evolutionarily conserved eukaryotic processes by AnkB is to generate amino acids required as sources of carbon and energy for intracellular growth of *Legionella*.

This indicates a tremendous patho-adaptation of *Legionella* to the intracellular life within human cells and amoeba. Remarkably, the amino acids essential for *Legionella*, due to deficiency of de novo synthesis, are also essential for the two evolutionarily distant hosts (with the exception of cysteine, which is scarce in human cells) (reviewed in Al-Quadan *et al*., 2012).

1.2. The Viable But Non-Culturable State in Bacteria

The environmental conditions in which prokaryotes live generally undergo great seasonal and circadian changes, such as temperature variations and changes in levels of solar radiation and in the availability of nutrients, the latter alternating between situations of feast and famine. These changes are particularly pronounced in aquatic environments. When environmental conditions become adverse, bacteria must be able to withstand stress and to adopt strategies that enable survival until suitable conditions for growth and development are re-established. Some bacterial genera deal with adverse conditions by developing resistant structures (e.g., endospores, myxospores, cysts, chlamidospores and achinetes) from vegetative cells by transformation processes. However, some other bacteria enter into a viable but non-culturable (VBNC) state (reviewed in Barcina and Arana, 2009).

VBNC bacteria are defined as bacteria that are living but unable to grow or divide when inoculated into routinely used bacteriological culture media (Xu *et al*.,1982). However, they maintain a number of cellular functions such as respiration (Oliver, 1995), transcription

(Lleo *et al*., 2000), and protein synthesis (Rahman *et al*., 1994). Some VBNC cells can resuscitate (i.e. regain culturability) when suitable conditions becomes available. If it is possible to pass from the VBNC state to the culturable state, then the VBNC phenotype could be considered as part of the life-cycle of non-differentiating bacteria (Barcina and Arana, 2009).

A regular decline in colony forming units occurs following exposure to one or more environmental stresses, although total cell counts generally remain fairly constant during this period of decline. The key test that determines whether such cells are dead or alive but in a VBNC state is the viability count (Oliver, 2005). Viability can be monitored using a variety of criteria, including cellular membrane integrity, uptake and incorporation of radiolabeled amino acids, protection of genomic DNA from DNase I digestion (Pawlowski *et al*., 2011), production of ATP (Lindback *et al*., 2010), phage susceptibility (Ben Said *et al*., 2010), and detection of mRNA, which has a very short half-life) (Coutard *et al*., 2005). For example, Rahman *et al*. (1994), studying *S. dysenteriae* cells that had been in the VBNC state for 4-8 weeks, found that such cells were capable of active uptake of methionine and its incorporation into proteins (Rahman *et al*., 1994).

Xu *et al*. (1982) were the first to find experimental evidence of the existence of the VBNC state in pathogenic bacteria. They showed that *E. coli* and *Vibrio cholerae* cells that were suspended in artificial seawater quickly lost their ability to grow on the culture media normally used for their detection. Subsequently, many investigators have reported on VBNC bacteria, with more than 60 species of bacteria shown to demonstrate the phenomenon (Table 1.1). The list includes Gram positive and Gram negative bacteria, non-pathogens and a large number of human pathogens, including *Campylobacter* spp., *Klebsiella* spp., *L. pneumophila*, *Micrococcus* spp., *Mycobacterium tuberculosis*, *Pseudomonas* spp., *Salmonella* spp., *Vibrio* spp., *Escherichia coli* and *Enterococcus faecalis* (reviewed in Oliver, 2010 and Oliver, 2005).

The time required for cells to enter the VBNC state varies markedly based on type of bacteria, initial inoculum, phase of growth, and the inducing conditions. Reports of the process taking months are not uncommon, while others have reported days for the same bacteria (reviewed in Oliver, 2000). For example, at 4^{o}C the time taken to enter the VBNC state was 5 months for the *V. cholerae* non-O1/non-O139, 6 months for Enterohemorrhagic

Table 1.1. Pathogens known to enter the VBNC state

Aeromonas hydrophila	*Helicobacter pylori*	*Serratia marcescens*
A. salmonicida	*Klebsiella aerogenes*	*Shigella dysenteriae*
Agrobacterium tumefaciens	*K. pneumoniae*	*S. flexneri*
Burkholderia cepacia	*K. planticola*	*S. sonnei*
B. pseudomallei	*Legionella pneumophila*	*Streptococcus faecalis*
Campylobacter coli	*Listeria monocytogenes*	*Vibrio alginolyticus*
C. jejuni	*Mycobacterium tuberculosis*	*V. anguillarum*
C. lari	*M. smegmatis*	*V. campbellii*
Cytophaga allerginae	*Pasteurella piscicida*	*V. cholerae*
Enterobacter aerogenes	*Pseudomonas aeruginosa*	*V. harveyi*
E. cloacae	*P. syringae*	*V. mimicus*
Enterococcus faecalis	*Ralstonia solanacearum*	*V. parahaemolyticus*
E. hirae	*Rhizobium leguminosarum*	*V. shiloi*
E. faecium	*R. meliloti*	*V. vulnificus (types 1 & 2)*
Erwinia amylovora	*Salmonella enterica*	*Xanthomonas campestris*
Escherichia coli	*S. typhi*	*X. axonopodis pv. citri*
Francisella tularensis	*S. typhimurium*	

E. coli (EHEC), 7 months for Enterotoxigenic *E. coli* (ETEC) , 11 months for Enteropathogenic *E. coli* (EPEC), 12 months for *S. flexneri*, and 18 months for *S. enterica* (Senoh *et al*., 2012).

In another experiment, *Yersinia pestis* entered the VBNC state within 21 days in tap water microcosm at 4^{o}C, while *Y. pestis* incubated in artificial sea water or sterilized river water at 4^{o}C, exhibited a lesser extent of decline in culturability after a 28 day period (Pawlowski *et al*., 2011). Moreover, when water was held at 25°C, *Brucella suis* and *B. melitensis* were culturable only for 2 days, while *Y. pestis* strains were culturable for 21 days. When the water was held at 5°C, *B. suis* and *B. melitensis* were culturable up to 2 weeks, yet *Y. pestis* were culturable for only 1 or 2 days (Gilbert and Rose, 2012).

The age of the cells has been shown to have a dramatic effect on the time required for lab-grown cells to become nonculturable. For example, whereas *V. vulnificus* cells from the logarithmic phase of growth generally require less than ten days to become completely nonculturable at 5^{o}C, those taken from the stationary phase require over a month (Oliver *et al*., 1991).

The percentage of the viable cells at the time of losing culturability also varies with the inducing conditions. For example 90% of *E. coli* O157:H7 cells entered into a VBNC state in chloraminated tap water within 15 min, while 14% of them entered into a VBNC state in river water within 14 weeks (Liu *et al*., 2009).

Three different models have been proposed to explain the VBNC phenomenon. The first model suggests that starving cells become nonculturable due to cellular stochastic deterioration and that they show some signs of senescence, as with any other aging organisms. Proponents of the stochastic deterioration model argue that loss of culturability is intimately linked to oxidative damage. The accumulated data suggest that there is an increased demand for oxidation management in cells subjected to nutrient starvation, and a significant number of the genes and regulons induced by stasis are indeed part of such an induced defence machinery. However, this machinery obviously fails to fully combat starvation-induced oxidation since oxidative modifications of proteins, such as carbonylation and illegitimate disulfide bond formation, increase during stasis in wild-type *E. coli* cells (reviewed in Nystrom, 2003).

Non-culturability state may be reversible depending on the magnitude of the cellular

damage. Nevertheless, if starvation and oxidative damage are allowed to proceed for an extended period of time, the non-culturable cells are destined to irreversibly lose their life-supporting activities (Ericsson *et al*., 2000). In *Salmonella*, both RpoS and RpoE are required for protection against oxidative damage in stationary phase: nearly all cells of a *Salmonella* population lacking both RpoE and RpoS became non-culturable after 24 hours in stationary phase, but the plating efficiency of these mutants was completely preserved under anaerobic stationary phase conditions (Testerman *et al*., 2002).

The second model suggests that non-culturability is the culmination of an adaptive pathway generating dormant survival forms, similar to spore formation in differentiating bacteria (reviewed in Nystrom, 2003). In a study conducted by Ravel *et al* (1994), a *Vibrio cholerae* transposon mutant with an accelerated loss of culturability was isolated during a screen involving prolonged incubation in stationary phase. These mutant cells remained intact and were metabolically active. However, no information is available on where the mutation mapped on the chromosome. Nevertheless, this mutant is the first bacterial isolate exhibiting an altered VBNC response, and suggests that entry into the VBNC state is under genetic control. Perhaps an equally informative approach would be to isolate mutants with a retarded rather than accelerated loss of culturability in stationary phase. If VBNC formation is a physiological adaptation similar to spore formation, then such mutants could be selected (Nystrom, 2003).

The third model states that starved cells lose viability due to activation of genetic modules mediating programmed cell death (reviewed in Nystrom, 2003). Bacteria harbor toxin-antitoxin (TA) loci on the chromosome, and it has been suggested that these loci mediate programmed cell death upon nutritional stress and growth arrest (Aizenman *et al*., 1996). The origin of this proposition stems from work on the TA locus consisting of *mazE* (antitoxin) and *mazF* (toxin) genes in *E. coli*, which form an operon with the upstream gene, *relA*, of the stringent response. Ectopic overproduction of MazF effectively reduced the viable counts of the population, suggesting that MazF is a *bona fide* toxin.

Moreover, it was demonstrated that artificial elevation of ppGpp levels reduced transcription of the *mazEF* operon. Based on these results, it was argued that programmed cell death is triggered whenever conditions, such as nutrient starvation and growth arrest, elicit ppGpp accumulation, which in turn will block further production of MazEF and allow

the more stable toxin, MazF, to express its killing function. Aizenman *et al*. (1996) proposed that the *mazEF* operon provides the cells with a system for altruistic cell death during starvation conditions in that programmed deterioration of part of the population may enable the rest to survive or even grow on constituents leaking out of dead siblings.

Pedersen *et al*. (2002) demonstrated that the MazF and the RelE toxins do not, in fact, kill the cell. Overproduction of the toxins rather elicits a bacteriostatic condition that can be fully reversed by ectopic production of the cognate antitoxins. In other words, elevated levels of the toxins lock the cells in a growth-arrested, G0-like state, which is incompatible with colony formation on nutrient agar plates unless the cognate antitoxin is similarly elevated to counteract this state. The RelE toxin was shown to primarily inhibit translation, and it was suggested that the function of this toxin is to modulate the global rates of protein synthesis during nutritional stress conditions. As such, the TA modules may serve as a back-up system to the stringent response and check superfluous macromolecular synthesis in a ppGpp-independent fashion during stasis (reviewed in Nystrom, 2003).

Several authors have proposed that in the case of serious damage by stressful factors, some cells may excrete organic molecules that provide for other members of the population, thereby assuring species survival until better times come (Arana *et al*., 2004; Aertsen and Michiels, 2004; Cuny *et al*., 2005). For example, Arana *et al*. (2004) simultaneously studied the changes in an *E. coli* population in adverse conditions as well as the chemical variations in the surrounding medium. They detected that throughout the survival process, which included entrance into the VBNC state, *E. coli* released into its surroundings organic molecules (amino acids, proteins and carbohydrates). The variations in the concentrations of amino acids and carbohydrates in the medium throughout the survival process indicated the coexistence of a double process of excretion and uptake (Arana *et al*., 2004).

Accordingly, Barcina and Arana (2009) proposed that the VBNC phenotype may represent an intermediate stage in an altruistic death process, which forms part of a survival strategy of species. As part of the response to environmental stress, a percentage of the population releases organic molecules into the surrounding medium. The excretion of these molecules may, among other things, cause a loss of culturability and the consequent appearance of a VBNC subpopulation. At the same time, the organic material excreted may be used by other members of the population to repair cell damage and alleviate stress

(Barcina and Arana, 2009).

1.2.1. Factors Influencing Differentiation into The VBNC State

A variety of factors, both chemical and environmental, have been reported to induce the VBNC state. These typically are stressors that might be lethal without entry into this dormant state. These factors include nutrient starvation (Cook and Bolster, 2007), incubation outside the normal temperature range of growth (Wong and Wang, 2004), elevated or lowered osmotic concentrations (Asakura *et al*., 2008; Wong and Liu, 2008), oxygen concentrations (Kana *et al*., 2008), commonly used food preservatives (Cunningham *et al.*, 2009), heavy metals (Ghezzi and Steck, 1999), exposure to white light (Gourmelon *et al*., 1994), pasteurization (Gunasekera *et al*., 2002) and chlorination (Oliver *et al*., 2005).

Moreover, association with eukaryotic host cells has also been linked to VBNC state formation. For example, *A. castellanii* was found to induce the VBNC state in *Aeromonas hydrophila* (Rahman *et al*., 2008). In a different system, Sussman *et al.* (2003) reported the association of VBNC cells of the coral pathogen, *Vibrio shiloi*, with a marine fireworm. Induction of the VBNC state under these stressful environments has important implications for public health. For example, induction of the VBNC state in *E. coli*, often associated with foodborne illness, was already reported under low pH (Zhao and Mathews, 2000), high salinity (Roth *et al*., 1988), UV radiation in combination with high salinity (Pommepuy *et al*., 1996), starvation conditions (Smith *et al*., 1994), low water availability (Artz *et al*., 2006), and high copper concentrations (Grey and Steck, 2001a).

Simultaneous exposure to starvation and a temperature outside the normal range for bacterial growth is commonly encountered by bacteria in the environment. *E. coli* O157:H7 entered the VBNC state in response to exposure to starvation and low temperature (at 4 ^{o}C). However, starvation alone did not induce entry into the VBNC state within 1.5 years (Liu *et al*., 2009). *Vibrio* spp. also entered the VBNC state by starvation in artificial sea water at 5^{o}C (Baffone *et al*., 2003). Steinert *et al* (1997) found that 1% of the *L. pneumophila* cells placed in tap water at 20^{o}C entered the VBNC state after 125 days of incubation, whereas the remaining 99% of the cells died.

In another study, Ohno *et al.* (2003) induced the VBNC state in *L. pneumophila* in hot spring and tap water at high temperature. The decreased ratio of cultivable cells and viable

cells in microcosms of hot spring water depended on the rise in temperature. Viability was maintained at temperture up to 45°C but not at 50°C (Ohno *et al*., 2003). Heat shock treatment at 70°C for 30 min caused 10 to 25% of cells to enter the VBNC state (Allegra *et al*., 2008). At 4 ^{o}C, the decimal rate of decline of colony forming cells was approximately 29 days; at 37^{o}C, it was 13 days (Hussong *et al*., 1987).

After short term starvation at 30^{o}C, *L. pneumophila* entered the VBNC state on day 35 while keeping viability up to 10%. During extended starvation, only 1% of the *L. pneumophila* cells still showed activity on day 200 (Yamamoto *et al*., 1996). Within 30 days of incubation in synthetic drinking water at 25 °C, culturability of *L. pneumophila* was rapidly reduced to below the detection limit, while the viability varied in only 0.1 log reduction during the same period, and these levels were sustained constantly for 190 days (Hwang *et al*., 2006).

In addition to the effect of temperature and starvation, Ohno *et al.* (2003) found that *L. pneumophila* survival in a microcosm at 42°C was also influenced by pH. At pH 2.0, bacteria were rapidly killed. Culturability at pH 5.0 and 10.0 were not detected at days 14 and 21 after incubation, respectively; however, viability at pH 5.0 was maintained over the entire study period of 61 days.

Water salt contents and concentrations affect *L. pneumophila* culturability as well. Dilution of hot spring water, and thus reduction of its salt contents, by distilled water was reported to hasten the loss of *L. pneumophila* culturability, with a decline in metabolic activity—particularly at high dilution—while bacteria maintained in a microcosm of distilled water lost both culturability and metabolic activity within 5 days of incubation at 42°C (Ohno *et al*., 2003). Moreover, *L. pneumophila* was able to maintain its viability for a long time in tap water by reducing its metabolic activities and losing its culturability as long as certain minerals that are involved in starvation survival (e.g., iron, zinc, and potassium) were present (States *et al*., 1985). The effect of salt on the survival of *L. pneumophila* was studied by Heller *et al*. (1998), who found that *L. pneumophila* could survive in salt solutions up to 3% NaCl at low but not high temperatures.

Bacterial growth phase also has an effect on induction of the VBNC state. In water microcosms with an inoculum size of 10^6 cfu/mL of RF cells, no colony was detected by 63 days after incubation at 42°C. However, in SPF cells, the decrease in culturable cells was

more rapid. In contrast, with an inoculum size of 10^8 cfu/mL, the SPF cells culturability lasted longer than the RF cells (Ohno *et al*., 2003). In another study, Hambleton *et al*. (1983) reported that *L. pneumophila* cell populations in the exponential phase survived poorly in aerosols with a humidity of 65% compared to post-exponential phase cells.

Another factor influencing survival of *L. pneumophila* was subculturing. Compared to freshly isolated strains, a strain subcultured 20 times was found to be significantly more susceptible to 2 ppm monochloramine after 24 h and not capable of entering to VBNC state (Turetgen, 2008).

1.2.2. Factors Inducing Resuscitation from The VBNC State

Entry of bacteria into the VBNC state can be of significance only if the VBNC cells are capable of regaining culturability. Indeed an important characteristic of the VBNC cells that distinguishes them from dead cells is the ability to resuscitate in natural environments as a consequence of environmental changes (Whitesides and Oliver, 1997). In fact, many factors have been found to induce recovery from the VBNC state.

Removal of environmental stress (e.g., temperature up-shift in cold-stressed cells) resulted in resuscitation of diverse species of *Vibrio* (Whiteside and Oliver, 1997; Wong *et al*., 2004a), *Helicobacter* (Kurokawa *et al*., 1999), *Salmonella* (Gupte *et al*., 2003), *Enterococcus* (Lleo *et al*., 2001), and *Aeromonas* (Maalej *et al*, 2004). In addition, nutrient supplementation to VBNC *Pasteurella pisticida* (Magariños *et al*., 1997), *Enterococcus* spp. (Lleo *et al*., 2001), *V. harveyi*, *V. fischeri* (Ramaiah *et al*., 2002), and *Micrococcus* produced by starvation (Mukamolova *et al*., 1998), and removal of salinity stress in the case of VBNC *E. coli* (Ohtomo and Saito, 2001) resulted in their resuscitation. Moreover, VBNC of *E. coli* O104:H4 (Aurass *et al*., 2011) and the plant-pathogenic bacteria *Erwinia amylovora*, produced under toxic concentrations of copper ions, was resuscitated by copper-ion chelation (Ordax *et al*., 2006).

Mukamolova *et al*. (2003) suggested that VBNC results form an imbalance between anabolism and catabolism, leading to the intracellular production of free radicals, and that cells can be recovered in presence of H_2O_2 degrading agents. Supplementation of the media with catalase or sodium pyruvate resulted in resuscitation of *V. parahaemolyticus, Aeromonas hydrophila* (Wai *et al*., 2000; Mizunoe *et al*., 2000) and *E. coli* O157:H7

(Asakura *et al*., 2005); similarly, the antioxidant oxyrase was used to resuscitate *E. coli* (Reissbrodt *et al*., 2002). The use of diluted culture medium has been recommended to stimulate resuscitation (Whitesides and Oliver, 1997), thereby avoiding an increase in cellular stress derived from the oxidative metabolism (Wong *et al*., 2004a).

The importance of removing free radicals in resuscitation of VBNC cells was further studied in *Staphylococcus aureus*. VBNC *S. aureus* produced in natural seawater at 4^{o}C showed a significant decrease in viability after 22 days of entry into the VBNC state. Masmoudi *et al*. (2010) showed that mutational inactivation of catalase (*katA*) or superoxide dismutase (*sodA*) rendered the bacteria hypersensitive to seawater stress; thus, part of the seawater lethality on *S. aureus* at low temperature was mediated by reactive oxygen species during microcosm-survival process. Shifting the temperature of VBNC wild type cells from 4 to 22^{o}C induced a partial recovery of the population. However, deficiencies in *katA* or *sodA* prevented this resuscitation ability.

Association with eukaryotic cells has been reported to induce resuscitaion in a number of VBNC bacteria. VBNC *V. cholerae* non-O1/non-O139, *V. parahaemolyticus*, enterohemorrhagic *E. coli*, enterotoxigenic *E. coli*, enteropathogenic *E. coli*, *Shigella flexneri*, and *Salmonella enterica* were converted to the culturable state by co-culture with selected eukaryotic cells (e.g., HT-29, Caco-2, T84, HeLa, Intestine 407, and CHO cells) (Senoh *et al*., 2012). Moreover, animal models of infection have also been reported to induce resuscitaion of VBNC cells in many bacterial spp, as with the recovery of *C. jejuni* in fertilized eggs via amniotic and yolk sac injection (Chaveerach *et al*., 2003), as well as after feeding suckling mice (Baffone *et al*., 2006; Jones *et al*., 1991) or inoculating 1-day-old chicks (Cappelier *et al*., 1999a).

Culturable cells *V. parahaemolyticus* were recovered on selective agar medium from the VBNC cultures injected into suckling mice, probably as a result of *in vivo* resuscitation (Wong *et al*., 2004b). Resuscitation has also been approached by the inoculation of natural hosts. VBNC *Ralstonia solanacearum*, the tropical agent that causes bacterial wilt disease (brown rot) in potato (van Elsas *et al*., 2000), could be resuscitated to culturable state following injection into tomato plants (Grey and Steck, 2001b).

Resuscitation promoting factors (Rpfs), a family of secreted proteins widely distributed among actinobacteria, are known to be involved in resuscitation of dormant cells.

M. luteus Rpf protein consists of an N-terminal domain conserved between the Rpf proteins as well as a C-terminal LysM domain thath is dispensable for resuscitation activity but is thought to cause binding to peptidoglycan, thus localizing Rpf to the cell wall (Mukamolova *et al*., 2002a). In *M. luteus*, there appears to be only one *rpf*, which is absolutely required for growth, as its deletion could only be achieved in the presence of a plasmid copy of this gene (Mukamolova *et al*., 2002a).

However, in *M. tuberculosis* there are five genes containing an *rpf* domain (Mukamolova *et al*., 2002b), which are collectively dispensable for *in vitro* growth (Kana *et al*., 2008; Mukamolova *et al*. 2002b). Mutations in any single one of these genes did not prevent resuscitation or growth, although such mutations did influence colony morphology (Downing *et al*., 2004). Mutating RpfA/C/D or RpfB/D/E simultaneously led to cells unable to resuscitate and with reduced virulence in a mouse infection model. Therefore, there is some but not complete redundancy between the *M. tuberculosis rpfs* (Downing *et al*., 2005).

A crystal structure of the domain conserved among the Rpf family revealed a c-type lysozyme-like fold (Ruggiero *et al*., 2009), and enzymatic activity has been predicted to cleave the glycosidic bond between N-acetylglucosamine and N-glycolyl-muramic acid in the cell wall peptidoglycan (Cohen-Gonsaud *et al*., 2005). *Micrococcus luteus* Rpf has been found to be active against mycobacteria (Mukamolova *et al*., 1999) and recombinant mycobacterial Rpf proteins were active against *M. luteus* as well as mycobacteria (Mukamolova *et al*., 2002b). Recently, a recombinant truncated form of *M. luteus* Rpf hydrolyzed isolated peptidoglycan of *M. smegmatis* and *M. tuberculosis,* liberating peptidoglycan fragments of different size, which showed a stimulatory activity toward dormant mycobacterial cells that is similar to the activity of recombinant Rpf.

Large peptidoglycan fragments obtained either by Rpf digestion or by peptidoglycan ultrasonication also revealed resuscitation activities, suggesting that peptidoglycan fragments could either directly activate the resuscitation pathway or serve as a substrate for endogenous Rpf, resulting in low molecular weight products with resuscitation activity. While both suggestions are plausible, the observation that peptidoglycan-dependent resuscitation activity is suppressed by a specific Rpf inhibitor (4-benzoyl-2-nitrophenylthiocyanate) provides additional support for the second of these possibilities (Nikitushkin *et al*., 2013).

An evidence of a resuscitation factor in *Salmonella*, YeaZ has been described. Unlike

the Gram-positive Rpfs, YeaZ does not have cross-species activity (Panutdaporn *et al*., 2006). YeaZ is a paralogue of Gcp, a protein found in all genomes sequenced so far, including eukaryotes (Nichols *et al*., 2006). The best-characterised Gcp is from *Mannheima haemolytica*, where it has been shown to be a secreted sialoglycoprotease cleaving glycosylated host cell surface proteins (Sutherland *et al*., 1992). The amino acid sequence encoded by the *Salmonella* Typhimurium *rpf* gene shares 24.2% homology with *M. luteus* Rpf. Recombinant Rpf proteins of *Salmonella* Typhimurium after expression in *E. coli* not only promoted proliferation but also induced resuscitation of VBNC *S. oranienburg* cells to the culturable state in a dose-dependent manner (Panutdaporn *et al*., 2006).

Another class of resuscitation factors was reported by Reissbrodt *et al*. (2002). This novel factor was described as a heat-stable autoinducer of growth, which was secreted by a variety of Gram-positive and Gram-negative bacterial species when incubated in media containing the human catecholamine hormone norepinephrine (Freestone *et al*., 1999). Norepinephrine is produced in large amounts in humans following severe tissue injury and is considered to be a stress-related hormone. The bacterial growth stimulation observed in the presence of this hormone appeared to be due to non-nutritional factors (Lyte *et al*.,1996). These showed a high degree of cross-species activity, and appeared to be a family of signaling molecules (Freestone *et al*., 1999).

Subsequently, Sperandio *et al*. (2003) identified the factors to represent a novel quorum-sensing system, which they termed AI-3. Because both epinephrine and norepinephrine could substitute for AI-3 in activating enterohemorrhagic *E. coli* virulence gene expression—and becausethe effects of AI-3 and epinephrine/ norepinephrine could be blocked by adrenergic receptor antagonists—they suggested that these compounds have a similar structure.

Reissbrodt *et al*. (2002) found that the autoinducers present in the spent media of *Y. ruckeri* resulted in resuscitation from the VBNC state of several strains of *Salmonella enterica* serovar Typhimurium and of two *E. coli* O157:H7 strains. These findings would appear to have major implications for the resuscitation of enteropathogens from the VBNC state, especially those occurring in the human intestinal tract, at a time (e.g. tissue damage) when the host may be under significant physiological stress (Oliver, 2010).

The number of resuscitated VBNC cells from different bacteria (*V. cholerae* non-

O1/non-O139, V. *parahaemolyticus*, enterohemorrhagic *E. coli*, enterotoxigenic *E. coli*, enteropathogenic *E. coli*, *S. flexneri*, and *S. enterica*) was determined by serially diluting an aliquot of each microcosm two-fold and inoculating 0.1 mL of each dilution onto a confluent culture of different eukaryotic cells. The turbidity of media in each well was observed visually and the highest dilution showing turbidity was recorded. The number of resuscitated bacteria differed with the bacterial species and cell line used (Senoh *et al*., 2012). However, the real number of VBNC cells that are able to resuscitate is very few. For example, in *E. faecalis*, *E. hirae* (Lleo *et al*., 2001), and *S. meliloti*, the resuscitation ratio was 1 recovered VBNC cell in every 10^5 viable cells (Basaglia *et al*., 2007).

The time spent in the VBNC state seems to influence the ability of bacteria to resuscitate. Resuscitation of VBNC *V. cholerae* O139, *V. cholerae* non-O1/non-O139, *V. parahaemolyticus*, EHEC, and ETEC occurred for about 2 weeks after entry into the VBNC state compared to 3 weeks in EPEC and 4 weeks in *S. flexneri* and *S. enteritica*. VBNC bacteria that required more time to become VBNC required a long time to resuscitate (Senoh *et al*., 2012). *C. jejuni* VBNC cells regain their culturability depending on how long they have been in the VBNC state, which affects the number of respiring bacteria (Baffone *et al*., 2006). VBNC *E. faecalis* and *E. hirae* were resuscitable as soon as the cells entered the VBNC state and decreased gradually to undetectable levels over the following 3 months. However, *E. faecium* were resuscitable over a 2 week interval (Lleo *et al*., 2001).

Colwell *et al*. (1996) studies showed that human volunteers develop clinical symptoms of cholera after ingestion of VBNC *V. cholerae*. However, after repeating the experiments with microcosm-containing cells that were non-culturable for 1 month, no *V. cholerae* could be isolated from the stools of volunteers, suggesting that the ability to recover from the intestinal tract may depend on the age of the VBNC cells.

It has been shown that resuscitaion is the result of true recovery of VBNC cells rather than multiplication of few culturable cells in the sample. Exposure of *E. coli* cells to high salinity stress decreased the number of CFUs significantly. When the culture was relieved from the stressful condition, the number of CFUs returned within 2 hr to the same level as before the stress. This recovery in the number of CFUs seemed to be independent of DNA synthesis and cell division, as the same phenomenon was also observed in the presence of nalidixic acid. Moreover, the total number of cells was the same before and after relief from

saline stress (Ohtomo and Saito, 2001).

In addition, a study by Whitesides and Oliver (1997), which employed extensively diluted populations of VBNC *V. vulnificus* cells, showed that it was extremely unlikely for any culturable cells to be present. Resuscitation of these populations occurred at such a rapid rate that, if it were due to regrowth of culturable cells, they would have had a doubling time of approximately six minutes. This is clearly an impossible generation time for cells incubated at a suboptimal temperature without nutrient or aeration.

Continuous gene expression was found to be required for resuscitation of VBNC bacteria. For example, chloramphenicol inhibition experiments of VBNC *V. parahaemolyticus* revealed that protein synthesis in the first 24 h of temperature upshift was critical in determining the success of the three-day resuscitation period (Wong *et al*., 2004a).

Resuscitation of the *L. pneumophila* serogroup 1 VBNC was first reported when heat treatment (10-30 mins at 45°C) had led to culture of *L. pneumophila* from underground water collected post-chlorination (Colbourne and Dennis, 1989). This is in contrast to James *et al* (1999) that reported *L. pneumophila* could not be resuscitated by heat shock. Resuscitation of VBNC *L. pneumophila* produced by starvation in environmental water at 4^{o}C and 37^{o}C was also documented to occur following infection of chick embryos resulting in chick embryo mortality (Hussong *et al*., 1987).

Steinert *et al* (1997) were the first to demonstrate that VBNC *L. pneumophila* could be resuscitated in the presence of *A. castellani*, and this was subsequently confirmed by other studies using VBNC cells obtained after starvation, heat, chlorine and other biocide treatments (Ohno *et al*., 2003; Dusserre *et al*., 2008). Other species of amoeba, such as *A. polyphaga*, have also been reported to induce resuscitaion of VBNC *L. pneumophila* (Dusserre *et al*., 2008; Garcia *et al*., 2007). However, VBNC *L. pneumophila* did not regain culturability after co-culturing with the ciliate *T. pyriformis* (Yamamoto *et al*., 1996). Such observations suggest that VBNC *L. pneumophila* can resuscitate only in amoebae. Moreover, these bacteria infect amoeba and macrophages in a similar way, so it could be speculated that VBNC cells can recover their culturability and pathogenicity in macrophages, thus posing a public health concern (Alleron *et al*., 2008). However, no resuscitation of *L. pneumophila* has been observed in mammalian cells.

1.2.3. Virulence, Gene Expression and Environmental Fitness of Bacteria in The VBNC State

1.2.3.1. Bacterial Virulence in The VBNC State

VBNC microorganisms could pose a public health threat, as many assays that test for food and drinking water safety are culture-based. VBNC cells in water and food could be transported to humans where, in the case of VBNC pathogens, they may cause infections (reviewed in Oliver, 2010). Bacterial populations in the VBNC state have importance regardless of whether or not they have the capacity to resuscitate, as pathogenic bacteria in this state are still able to produce toxins, thus having a negative effect on their hosts. Moreover, despite the fact that bacteria in this state drastically reduce their activity, the global result of their activity if they are present in high density can be of quantitative importance (reviewed in Barcina and Arana, 2009).

The medical implications of the existence of pathogenic bacteria in the VBNC state are numerous. For example, it appears that the 'latent' or the 'dormant' phase of *M. tuberculosis* infections (Young *et al*., 2009) represents the VBNC state in this pathogen, and that the recurrence of tuberculosis years after a person was thought to be tuberculosis-free is due to resuscitation of this pathogen from the VBNC state (Pai *et al*., 2000). The presence of VBNC bacteria has also been utilized to explain a number of chronic bacterial infections in humans, such as gastritis or urinary tract infections (UTIs). In the case of gastritis, *H. pylori* has been detected in the gastric mucosa in a VBNC state (Catrenich and Makin, 1991).

With regard to chronic UTIs, round non-culturable *E. coli* have been detected in the wall of the bladder and have been implicated in the maintenance of the infection focus during recurrences in humans and in mouse models (Anderson *et al*., 2004; Rivers and Steck, 2001). Moreover, VBNC *S. epidermidis* and *S. aureus* have been detected within biofilms from central venous catheters (CVCs). Viable cells were associated with CVCs from febrile patients, some of whom showed *S. epidermidis*-positive blood cultures, suggesting that CVC-associated biofilms can be reservoirs for staphylococci in the VBNC state (Zandri *et al*., 2012).

Studies performed *in vitro* and in animal models also confirmed the pathogenicity of VBNC and resuscitated VBNC bacteria. Colwell *et al*. (1985) inoculated VBNC cells of *V. cholerae* into ligated rabbit ileal loops and observed enteropathogenicity in all samples.

Intestinal fluid was also reported to contain culturable *V. cholerae* cells. In subsequent studies, human volunteers developed clinical symptoms of cholera and had culturable *V. cholerae* cells in their stool after ingesting VBNC *V. cholerae* cells (Colwell *et al*., 1996).

Oliver and Bockian (1995) found that mice inoculated with *V. vulnificus* VBNC cells died, and culturable cells were recovered from both blood and the peritoneal cavity. VBNC *V. vulnificus* retain the capsule, an acidic polysaccharide capsule with antiphagocytic activity that is essential for the virulence of this bacterium (Simpson *et al*., 1987; Linder and Oliver, 1989). This supports the observation that VBNC *V. vulnificus* can cause mortality in mice. Moreover, RT-PCR showed that VBNC *V. vulnificus* continued to express the cytolysin/hemolysin gene (*vvhA*) (Fischer-Le Saux *et al*., 2002).

In another study, resuscitated VBNC *V. harveyi* cells injected intraperitoneally into zebra fish resulted in their death with a lethal dose 50% (LD_{50}) similar to that for groups inoculated with culturable cells (Sun *et al*., 2008). Moreover, a continued expression of virulence determinants such as *ctxA* (the cholera toxin gene) and *tcpA*, encoding the structural subunit of the toxin-coregulated pilus, was demonstrated in VBNC cells belonging to different *Vibrio* species (*V. cholerae*, *V. parahaemolyticus*, *V. vulnificus* and *V. mimicus*) (Vora *et al*., 2005; Mishra *et al*., 2012).

VBNC *C. jejuni* resuscitated in embryonated eggs regained their adhesion properties to HeLa cells (Cappelier *et al*., 1999) and, in another study, caused death in suckling mice (Jones *et al*., 1991). The ability of *E. faecalis* cells in the VBNC state to adhere to cultured heart and urinary tract epithelial cells was shown by Pruzzo *et al*. (2002). While they observed a 50-70% decrease in the adherence ability of VBNC cells compared to actively growing cells, adherence values were similar to those of growing cells following resuscitation. In addition, Rahman *et al*. (1994) found that VBNC *S. dysenteriae* retained cytopathogenicity for cultured HeLa cells, as well as the ability to produce active shiga toxin and to adhere to intestinal epithelial cells (Rahman *et al*., 1996).

Copper-induced VBNC *E. amylovora* cells were virulent for the first 5 days, and resuscitated cells regained their pathogenicity on immature fruits over 9 months (Ordax *et al*., 2006). Resuscitated *E. coli* O104:H4 VBNC cells, produced by copper ion stress, preserved their fitness, major virulence gene markers (*stx2*, *aggR*, *aggA* genes), and specific phenotypes (ESBL resistance, autoaggregation typical for EAEC strains) (Aurass *et al*., 2011). VBNC *E.*

coli O157:H7 strains produced at 8°C on lettuce plants (Dinu and Bach, 2011), in river water, buffer, deionized water, or chloraminated treated water retained expression of the *stx1* and *stx2* (Shiga-like toxins) genes (reviewed in Trevors, 2011). In VBNC ETEC cells, genes encoding ETEC toxins (STh and LT), which are colonization factors (CS7 and CS17), were expressed during 3 months in VBNC state (Lothigius *et al*., 2010).

Peptidoglycan synthesis and expression of the virulence factors CagA, VacA and UreA was also observed by *H. pylori* for at least 32 h while in VBNC state (reviewed in Oliver, 2010). Resuscitated *A. hydrophila* cells also regained their ability to lyse human erythrocytes and to attach to and kill McCoy cells (Maalej *et al*., 2004). Finally, resuscitated *L. pneumophila* JR32 in *A. castellanii* regained the capacity to survive inside human monocytes and to intraperitoneally infect guinea pigs, which is considered a parameter for virulence (Steinert *et al*., 1997).

As was noted by del Mar Lleo *et al*. (2007), antibiotics, which are highly active on growing cells, do not necessarily act on VBNC cells. It seems likely that, because VBNC cells demonstrate such low metabolic activity, they effectively become resistant to antibiotics, and yet are able to resuscitate and reinitiate infections. Ehrlich *et al*. (2002) reported that antibiotic resistant VBNC cells of *Haemophilus influenzae* present in biofilms are able to initiate chronic ear infections. In some reports, antibiotic resistance is exceptional. Vancomycin was reported to be effective against VBNC cells of *E. faecalis* only when at 500 times the MIC (Lleo *et al*., 2007). Anuchin *et al*. (2009) observed drastic increases in resistance to hydromycin and doxycyclin in dormant cells of *M. smegmatis* compared with 48-h cultures.

Subsequently, VBNC *H. pylori* cells were reported to exhibit antibiotic resistance, which likely accounts for the frequent reinfections suffered by persons who undergo remission despite antibiotic treatment (reviewed in Oliver 2010). Similarly, uropathogenic *E. coli* cells, typically not detected by standard methods, were not eliminated by antibiotic treatment (Rivers and Steck, 2001). Therefore, recurrent urinary tract infections are most likely a result of cells in this temporarily dormant state.

1.2.3.2. Gene Expression in The VBNC Bacteria

Despite their dormant state, VBNC cell populations still express some genes. For

example, in 10-month-old *E. coli* O157:H7 VBNC cells induced by river water, mRNA of the lipopolysaccharide gene (*rfbE*) and the flagellin gene (*fliC*) were still detected (Liu *et al*., 2009). In VBNC *V. cholerae* O1, RT-PCR revealed that the DNA pol II (27.43-fold), *fliG* (12.44-fold), ABC transporter (27.11-fold), *relA* (60.76-fold) and *flaC* (15.29-fold) were significantly up-regulated in VBNC cells. The expression of DNA pol II, *fliG*, ABC transporter, *relA* and *flaC* was 3.3, 1.1, 5.9, 5.8 and 1.2-fold, respectively, for resuscitated cells (Mishra *et al*., 2012). Both the *relA* and *rpoS* stress response genes had a significantly higher expression in the case of *V. cholerae* VBNC cells (4^{o}C) compared to starving cells (15^{o}C) (Gonzalez-Escalona *et al*., 2006).

Regardless of whether the cells remained culturable or entered the VBNC state, continued expression of the major stress factor, RpoS, was observed for as long as 14 days (cited in Oliver, 2010). This is consistent with the findings of Boaretti *et al*. (2003), who reported RpoS to be involved in the persistence of *E. coli* in the VBNC state. Moreover, in *V. cholerae* strain El Tor 3083 VBNC cells, *relA* mRNA was selectively increased 3.2-folds (Gonzalez-Escalona *et al*., 2006). The relatively high expression of *relA* in the VBNC state compared with the starved cells suggests that the VBNC bacteria may be capable of adapting to environmental signals via such stringent responses (Asakura *et al*., 2007).

In the research by Del Mar Lleo *et al*. (2000), non-culturable *E. faecalis* cells were capable of expressing the *pbp5* gene for at least 3 months in the water microcosm. Moreover, VBNC *H. pylori* cells in a freshwater stream maintained the expression of *murG*, a glycosyltransferase (reviewed in Oliver, 2010). This enzyme has been shown by Signoretto *et al*. (2002) to be required during the late stages of peptidoglycan assembly in *E. faecalis* cells entering the VBNC state, which may explain continued production of this enzyme in non-culturable cells.

In another study, the global transcription pattern of the VBNC *V. cholerae* O1 cells was compared with that of stationary-phase cells grown in rich medium. A total of 100 genes exhibited greater than five fold expression in the VBNC state; and these genes were mostly those responsible for cellular processes—49% encoded proteins for transport and binding, cellular processes, DNA metabolism, cell envelope, energy metabolism and regulatory functions. Furthermore, qRT-PCR analysis verified the changes in the expression levels, showing that the iron (III) ABC transporter, *polB*, *fliG* and *flaC* mRNAs were increased in

the non-culturable state. Upregulation of genes responsible for the transport of iron, magnesium, potassium and cobalamin, may account for the protective function of the dormant pathogen (Asakura *et al*., 2007).

Proteomics has revealed that new proteins are synthesized when the environmental conditions become inadequate for growth. Some proteins are expressed in response to factors such as thermal stress, oxidative stress, starvation and pH changes. Some of these proteins are specific to the stressor in question, while others provide cross protection against various types of stress (Barcina and Arana, 2009). The protein expression patterns between culturable and VBNC populations have been compared by Kong *et al*. (2004), who demonstrated that low temperature incubation of *V. vulnificus* prevents both catalase (KatG) activity and its *de novo* synthesis, making the cells hydrogen peroxide-sensitive and paralleling the entry into the VBNC state. This appears, to date, to be the only bacterium for which a molecular basis of the VBNC state has been described.

In another study, deletion of *ompW* in the oxidative stress-sensitive enterohemorrhagic *E. coli* O157:H7 strain increased recovery from dormancy, while overexpression of OmpW in the stress-resistant strain decreased recovery when exposed to oxidative stress, suggesting that high levels of OmpW sensitize the bacteria to stress (Asakura *et al*., 2008).

Protein profiles of *V. parahaemolyticus* VBNC and starved cells were determined by two-dimensional polyacrylamide gel electrophoresis, with the proteins enhanced in the VBNC state or strongly down-regulated in the starved cells identified by mass spectrophotometry (Lai *et al*., 2009). The 13 up-regulated proteins are known to be associated with transcription (two homologues of alpha subunit RNA polymerase), translation (ribosomal protein S1, two homologues of elongation factor TU, elongation factor EF-G), ATP synthase (F1 alpha subunit), gluconeogenesis-related metabolism (dihydrolipoamide acetyltransferase, glyceraldehyde 3-phosphate dehydrogenase), and antioxidants (2 homologues of peroxiredoxins, AhpC/Tsa family); in addition, there was a conserved hypothetical protein with unknown function. Expressions of the genes encoding four of these proteins (EF-TU, GAPD, AhpC and conserved hypothetical protein) were at high levels in the second week of VBNC induction. These proteins may play important roles in the induction or maintenance of VBNC *V. parahaemolyticus* (Lai *et al*., 2009). Also using

proteomics, Muela *et al*. (2008) studied modifications in the outer membrane of *E. coli* cells during entry into the VBNC state. They observed a drastic rearrangement of the outer membrane subproteome with the appearance of over 106 new spots including outer membrane proteins: TolC and two isoforms of OmpT, OmpA and NlpA (lipoprotein 28).

The genetic pathways underlying the VBNC response in *E. faecalis* are in part the same as those leading to the starvation response, as indicated by the presence of similar expression profiles for certain proteins (e.g., enoyl-ACP reductase). However, major differences in the expression patterns of other proteins (e.g., EF-Ts, fructose-bisphosphate aldolase, and mannose-specific PTS system) were observed. This suggests that the underlying pathways in the starvation and VBNC states are not overlapping but rather share some crossing points (Heim *et al*., 2002).

1.2.3.3. Environmental Fitness of Bacteria in The VBNC State

Regarding environmental fitness, VBNC *V. parahaemolyticus* were highly resistant to thermal (42^{o}C, 27^{o}C), low-salinity (0% NaCl), or acid (pH 4.0) inactivation (Wong and Wang, 2004), and H_2O_2 (Su *et al*., 2013). Moreover, VBNC *V. parahaemolyticus* resistance to lethal heat challenge (50°C for 12 min) and lethal low salinity (0.2% NaCl) was found to increase significantly with incubation time under VBNC induction conditions (Su *et al*., 2013). Weichart & Kjelleberg (1996) reported that VBNC *V. vulnificus* cells exhibited initial sonication sensitivity similar to that of growing cells but that resistance increased with increased cold incubation, with final resistance equaling that of starved cells.

In addition, Rowe *et al*. (1998) reported that VBNC cells of *C. jejuni* were more resistant to chlorine than were culturable cells, and Anuchin *et al*. (2009) reported that dormant forms of *M. smegmatis* show elevated resistance to heat (up to 80^{o}C) compared to the culturable bacteria. Finally, VBNC *L. pneumophila* produced by starvation in sterile ultrapure water at 37^{o}C for 33-40 days became more resistant to the disinfection effects of both heating and chlorination than the culturable cells (Chang *et al*., 2007).

1.2.4. Morphological and Structural Changes in The VBNC State

Bacterial cell morphology is not static: under certain conditions, such as nutrient deficiency, high or low temperature, and other stress factors, the cell shape may undergo

dramatic changes. During physiological nutrient starvation in bacteria, carbohydrate molecules are depleted first, followed by proteins and some RNA. The chromosomal DNA can be compressed and surrounded by dense cytoplasm.

Recovery from starvation occurs when the stress conditions are removed and conditions become more optimal for growth. RNA synthesis occurs first, followed by protein synthesis, increased cell size, DNA replication and finally cell division (reviewed in Trevors *et al*., 2012). In the VBNC state, bacterial cells become smaller and change from a rod to a spherical shape (Clements and Foster, 1998), the nuclear region in the cytoplasm becomes dense, and the total cellular lipid, carbohydrate and PHB (poly β-hydroxybutrate) decreases, suggesting these molecules are being consumed to prolong the survival. In addition, RNA, DNA and protein content each decreases. The decline in DNA content is a puzzle, as the genetic instructions are necessary and required for subsequent cell growth and division. It is plausible that once the DNA content passes a declining threshold amount, recovery of the cells is not possible and death is the outcome (reviewed in Trevors *et al*., 2012).

Alterations to the cell walls of both Gram-positive and Gram-negative bacteria have been described in connection with the VBNC state, likely with the function of rendering the cells more resistant to stress (Signoretto *et al*., 2000; Signoretto *et al*., 2002; Costa *et al*., 1999). Cells entering the VBNC state typically exhibit significant dwarfing; for example, cells of *V. vulnificus* are 2 μm long in the log phase but only 0.6 μm in diameter in the VBNC state (reviewed in Oliver, 2010).

In *E. coli* O104:H4, a statistically significant decrease in length and diameter of the VBNC cells (length ~ 1.2 mm, diameter ~ 0.8) was observed compared with both the parental cells and resuscitated cells (length ~ 1.4 mm, diameter ~ 0.9) (Aurass *et al*., 2011). The periplasmic space was greatly increased in the nonculturable *Y. pestis* cells compared to the laboratory grown bacteria. Additionally, the cytoplasm of the nonculturable cells had condensed into a small rounded cytosol with a volume of approximately half that of the laboratory grown cells. The most apparent morphological change in the non-culturable cells was a shift from rod-shaped to a more cocci-like cell shape (Pawlowski *et al*., 2011).

VBNC *Edwardsiella tarda* cells gradually changed in shape from short rods to coccoid and decreased in size, but the resuscitative cells did not show any obvious differences from the normal cells (Du *et al*., 2007). VBNC *Salmonella pullorum* transformed

to the spherical-rods or spherical shape and aggregated (Li *et al*., 2012). The rod-shaped *V. parahaemolyticus* cells in the exponential phase became coccoid cells in the VBNC state, with aberrantly shaped cells formed in the initial stage. The rod-shaped exponential phase cells had a uniformly distributed and densely stained cytoplasm with some densely stained granules. In contrast, the coccoid VBNC state had a loosely bound cell envelope, and the cytoplasm of most of these cells was lightly stained. The cells that were almost empty of cytoplasm contained densely stained granules that were probably bound to the cytoplasmic membrane region. In the aberrantly shaped cells, the cell wall was loosened and flexible, allowing the cells to bulge; also, and the formation of a new and thin cell wall—or the expansion of the existing cell wall—was also discerned, primarily at the polar position and enclosing an empty cellular space. Moreover, cell buds with empty cytoplasm were also noticed. The thickness of the cell wall increased with the VBNC induction time (Su *et al*., 2013).

In addition to the cell rounding observed in the VBNC state, changes in colony morphology were also observed in cells while diffrentiating to the VBNC state. For example, colonies of *V. cholerae* O1 cells inoculated in freshwater microcosm and incubated at 4^{o}C progressively decreased in size, lost their sheen, and became rugose from day 7 onwards on tryptic soy agar (Mishra *et al*., 2012).

The composition of membrane fatty acids changes in the VBNC cells (Day and Oliver, 2004). The role of changes in the membrane fatty acid (FA) composition of *V. vulnificus* as a contributing factor to the ability of this organism to enter into and survive in the VBNC state was tested by analyzing the cells' FAs during the initial hours of temperature and nutrient downshift. Prior to downshift, the predominant FAs were 16:0, 16:1 and 18:0. During the first four hours of downshift, statistically significant changes occurred in the 15:0, 16:1, 16:0, 17:0, and 18:0 FAs. These results indicate that changes in FA composition occur prior to entry into the VBNC state, suggesting that the ability to maintain membrane fluidity may be a factor in this physiological response. Moreover, cells in which fatty acid synthesis was inhibited did not survive, indicating that active fatty acid metabolism is essential for entry of cells into the VBNC state (Day and Oliver, 2004). *V. parahaemolyticus* also underwent major changes in fatty acid composition when entering the VBNC state, with a rapid increase in 15:0 fatty acid and saturated/unsaturated ratio coupled with a rapid decrease

in 16:1 (Wong *et al*., 2004b).

Signoretto *et al*. (2002), in studying the cell wall peptidoglycan of *E. coli* entering the VBNC state, reported a threefold increase in unusual DAP-DAP cross-linking, an increase in mucopeptides bearing a covalently bound lipoprotein, and a shortening of the average length of glycan strands in comparison with exponentially growing cells. Del Mar Lleo *et al*. (2007) examined the effects of several antibiotics acting on peptidoglycan or protein synthesis in *E. faecalis* and found several β-lactams that block resuscitation of VBNC cells. Surprisingly, vancomycin, even when used at 100 times its minimum inhibitory concentration (MIC), was totally ineffective in this regard. The authors suggested that this insensitivity is due to the lack of synthesis of D-ala-D-ala, the specific target of this antibiotic, by the metabolically relatively inactive VBNC cells (Lleo *et al*., 2007). In that peptidoglycan, rearrangements have been observed in both Gram-positive and -negative cells as they enter the VBNC state; such events may be hallmarks of the VBNC state (Oliver, 2010).

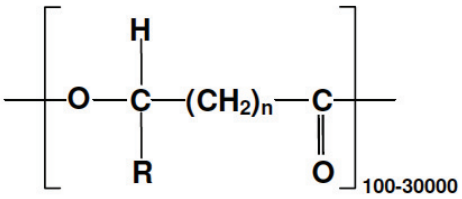
1.3. Poly 3-Hydroxybutyrate

L. pneumophila cells accumulate poly 3-hydroxybutyrate (PHB) in their cytoplasm in the form of inclusions (Mauchline *et al*., 1992; James *et al*., 1999), and PHB has been found to be important for *L. pneumophila* survival in starvation (James *et al*., 1999). In the current study, the role of PHB in *L. pneumophila* entry into the VBNC state was evaluated.

1.3.1. Metabolism and Regulation of Poly 3-Hydroxybutyrate in Bacteria

Polyhydroxyalkanoates (PHAs) form a class of important industrial biopolymers. A wide variety of microorganisms, including numerous Gram-positive and Gram-negative bacteria, accumulate PHAs in their cytoplasm as hydrophobic granules that function as intracellular carbon/energy storage compounds or reducing power for coping with changing and often oligotrophic environments (reviewed in Khanna and Srivastava, 2005). More than 100 different monomer units have been identified as constituents of PHA. One of the most abundant PHAs is PHB, a homopolymer of 3-hydroxybutyrate (3HB) (Fig.1.4) (reviewed in Reddy *et al*., 2003).

PHB is often studied as an archetype of natural biodegradable plastic with similar physical and chemical properties as that of petrochemical-based plastics (Anderson and



n=1	R=hydrogen	Poly(3-hydroxypropionate)
	R=methyl	Poly(3-hydroxybutyrate)
	R=ethyl	Poly(3-hydroxyvalerate)
	R=propyl	Poly(3-hydroxyhexanoate)
	R=pentyl	Poly(3-hydroxyoctanoate)
	R=nonyl	Poly(3-hydroxydodecanoate)
n=2	R=hydrogen	Poly(4-hydroxybutyrate)
n=3	R=hydrogen	Poly(5-hydroxyvalerate)

Fig. 1.4. General chemical structure of polyhydroxyalkanoates (Reproduced with permission from Lee, 1996) (Apendix IV).

Dawes, 1990). The biosynthesis and degradation of PHAs is a cyclic mechanism that has already been described in many bacteria, including *Ralstonia eutropha* and *Azotobacter beijerinckii* (Slater *et al*., 1988; Senior and Dawes, 1973; Oeding and Schlegel, 1973). By now, more than 100 bacterial species have been found to synthesize PHA as reserve polymers in response to nutritional imbalance caused by a deficiency of nitrogen, phosphorus, potassium, magnesium and oxygen sources, as well as an excess of carbon sources, in the medium (reviewed in Trotsenko and Belova, 2000). While facing different stresses, such as low nutrient availability and detrimental physical, chemical, or biological factors, these bacteria begin to degrade PHAs in order to overcome the unfavorable conditions (Wang *et al*., 2009).

The PHB biosynthesis pathway requires the successive action of three enzymes, as shown in Fig.1.5. β-ketoacyl-CoA thiolase (PhbA) promotes the condensation of two molecules of acetyl CoA in a reversible manner to form acetoacetyl-CoA. This is followed by reduction of acetoacetyl-CoA to 3-hydroxybutyryl-CoA by an NADPH- or NADH-dependent acetoacetyl-CoA reductase (PhbB) and then polymerization of the 3-hydroxybutyryl-CoA by PHB polymerase or synthase (PhbC) in order to form PHB, with concomitant release of coenzyme A. The formation of PHB requires large quantities of acetyl-CoA and reducing equivalents, either as NADH or NADPH (Anderson and Dawes, 1990).
PHB degradation is divided into intracellular and extracellular degradation. Intracellular PHB degradation is initiated by the intracellular PHB depolymerase (PhaZ), which catalyzes the breakdown of PHB granules into 3HB (Uchino *et al*., 2008). In addition to PHB depolymerase, 3HB-oligomer hydrolase is also involved in this reaction. The enzyme hydrolyses 3HB oligomers to monomers (Kobayashi *et al*., 2005). In general, researchers believe that the product of PHB degradation is 3HB (Kobayashi *et al*., 2005; York *et al*., 2003). However, Uchino and coworkers (2007) suggested that instead of the free acid, PHB degradation released 3-hydroxybutyryl-CoA as primary products, which could be converted back to PHB when necessary without wasting any energy.

3HB can either be oxidized to acetoacetate by 3-hydroxybutyric acid dehydrogenase (EC 1.1.1.30; BdhA) in a process requiring nicotinamide adenine dinucleotide (NAD+) (Aneja, *et al*., 2005) or activated into a CoA derivative by enzymes such as acyl-CoA synthetases (Ruth *et al*., 2008). Subsequently, acetoacetate is converted to acetoacetyl-

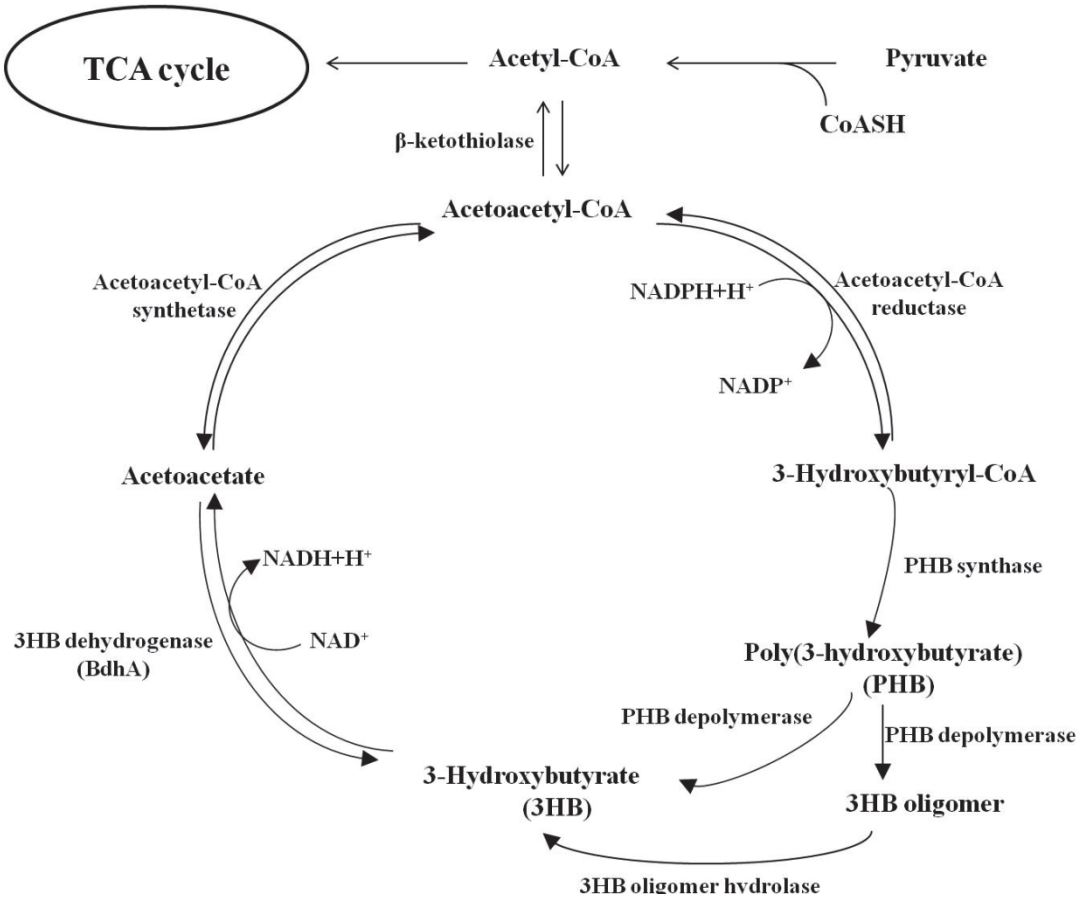


Fig.1.5. Metabolic pathway of PHB in bacteria (Reproduced with permission from Shiraki *et al*., 2006) (Apendix IV).

CoA with the presence of acetoacetyl-CoA synthase, ATP and CoA (Cai *et al*., 2000). A ketothiolase cleaves the acetoacetyl-CoA to yield two molecules of acetyl-CoA, which are metabolized via the glyoxylate shunt and tricarboxylic acid cycle (Senior and Dawes, 1973) in order to synthesize ATP, which would enhance the survival and resistance of bacteria in stressful environments (Ruiz *et al*., 2001).

Extracellular degradation, on the other hand, is the utilization of PHA granules that are released from other bacteria after cell lysis. Several bacteria express and secrete extracellular depolymerases in different environments (Tanio *et al*., 1982; Jendrossek *et al*., 1996). The product of extracellular digestion, 3HB or 3HB-dimers, are further hydrolyzed by a constitutive intracellular esterase to yield a monomer, which is taken into the cells and oxidized to acetoacetic acid by a constitutive BdhA (cited in Delafield *et al*., 1965).

PHAs are stored in the bacterial cytoplasm as discrete, water-insoluble granules that can contribute up to 75% (w/w) of the bacterial dry cell weight. They represent ideal storage materials because these polymers exert negligible osmotic pressure in the bacterial cells (reviewed in Anderson and Dawes, 1990). The occurrence of PHB as intracellular inclusions was first reported for *Bacillus megaterium* by Maurice Lemoigne in 1925, who reported the presence of 3HB in the autolysate of *B. megaterium* (cited in Potter and Steinbüchel, 2005). The physical properties of PHB granules are very different, and two physical states—intracellular native PHB granules and partially crystalline PHB granules—can be distinguished. Intracellular native PHB granules are in an amorphous rubbery state, and the surface is surrounded with a layer consisting of phospholipids and granule-associated proteins (Steinbüchel *et al*., 1995; Mayer *et al*., 1996). The granule-associated proteins play a major role in the synthesis and degradation of PHAs and in the formation of PHA granules. However, during extraction of PHB granules from the cell, the phospholipid and protein layer is damaged or lost (Griebel and Merrick, 1971).

Under phase-contrast microscope and transmission electron microscope, the granules appear transparent with round/oval shape (Loo and Sudesh, 2007) and a size that varies from 100 to 800 nm (Loo and Sudesh, 2007; Shively, 1974). Electron microscopic studies revealed a dense membrane of a thickness of 15-20 nm at the surface of PHB granules (Lundgren *et al*., 1964). Chemical analyses have shown that inclusion bodies contain approximately 97.5% PHA, 2% protein, and 0.5% lipid (Griebel *et al*., 1968), although some estimates of the lipid

contents are considerably higher (Steinbuchel *et al*., 1995).

Four types of granule-associated proteins are found within bacterial genera producing PHAs: (i) PHA synthase; (ii) PHA depolymerases and 3HB-oligomer hydroxylase; (iii) phasins (PhaPs), which are thought to be the major structural proteins of the membrane surrounding the inclusion; and (iv) the transcriptional repressor of phasin expresssion, PhaR (reviewed in Pötter and Steinbüchel, 2005). Phasins represent a class of noncatalytic proteins consisting of a hydrophobic domain, which associates with the surface of the PHB granules, and of a predominantly hydrophilic/amphiphilic domain exposed to the cytoplasm of the cell. This layer of phasins stabilizes PHA granules and prevents coalescence of separated granules (Steinbuchel *et al*., 1995; Mayer *et al*., 1996).

Based on data from studies on *R. eutropha*, the regulation of PHB biosynthesis by PhaR was proposed to proceed as follows. If the cells are cultured under conditions not permissive for PHB biosynthesis, PhaR (which has no PHB granules to bind to) will exist free in the cytoplasm. Therefore, the cytoplasmic concentration of PhaR becomes sufficiently high to repress transcription of PhaP. If conditions permissive for PHB biosynthesis start, the constitutively expressed PHA synthase (PhaC) starts to synthesize PHB molecules that remain covalently linked to the enzyme.

Small micelles are formed and become larger constituting the nascent PHB granules. PhaC no longer covers the PHB granule surface entirely, and proteins with a binding capacity to the hydrophobic surface, such as PhaR, bind to the granules. This lowers the cytoplasmic concentration of free PhaR so that it can no longer repress transcription of PhaP. PhaP is then synthesized and subsequently binds to the PHB granules. When the PHB granules reach the maximum possible size according to the physiological conditions, almost the entire surface will be covered by PhaP protein, which is displacing PhaR protein from the PHB granules. Consequently, the cytoplasmic concentration of PhaR increases, ultimately exceeding the threshold concentration required to repress transcription of PhaP. As a result, PhaP protein is no longer synthesized. This mode of regulation ensures that PhaP is not produced in higher amounts than required to cover the surface of PHB granules. In addition, the binding capacity of PhaR to the promoter region of its own gene prevents overexpression of this repressor protein, which is under autocontrol (Potter and Steinbuchel, 2005).

In addition to PhaR, PhaF is another regulator of PHB biosynthesis. Prieto *et al*.

(1999) have proposed a model describing PhaF-mediated regulation of protein expression in *P. oleovorans* that is similar to the *R. eutropha* PhaR/PhaP system. According to the model, PhaF binds DNA, responds to PHA or factors present during PHA synthesis, and regulates the expression of a granule-associated protein. However, PhaF and PhaR are not homologs and, unlike PhaR, PhaF also regulates expression of PHA synthase.

In *Azotobacter vinelandii*, PhbR, a transcriptional activator that is a member of the AraC family of activators, and RpoS have been found to upregulate the *phbBAC* operon through pB1 and pB2 promoters, respectively (Peralta-Gil *et al*., 2002). Moreover, CydR (an Fnr-like regulatory protein) and GacA/GacS two-component regulatory system regulate the PHB synthesis in negative and positive manners, respectively (Peralta-Gil *et al*., 2002; Castaneda *et al*., 2000; Wu *et al*., 2001).

PHB synthesis is not only controlled by protein regulators that affect the levels of PHB operon enzymes, but it is also influenced by the availability of substrate, acetyl-CoA (Peralta-Gil *et al*., 2002). Acetyl-CoA serves as an allosteric activator for β-ketothiolase and is also a precursor for PHB polymer. The PHB biosynthetic pathway competes for acetyl-CoA with other metabolic pathways such as the TCA cycle, the acetate formation pathway, and the fatty acid biosynthesis pathway that consume acetyl-CoA, thereby leading to the reduced availability of acetyl-CoA for PHB biosynthesis.

A diminished carbon flux through the TCA cycle or other pathways would result in higher production of PHB polymers by increasing the level of acetyl-CoA (Segura and Espin, 2004; Aldor and Keasling, 2003). PHB can also be synthesized through de novo fatty acid synthesis and β-oxidation pathways (Aldor and Keasling, 2003). Another regulator for PHB biosynthesis identified in *Azotobacter vinelandii* is *arrF*, which encodes an iron-responsive small RNA that is under negative control by the ferric uptake regulator protein. Δ*arrF* mutant was found to overproduce PHB. Protein expression changes in the mutant included upregulation of 6-phosphogluconolactonase and the E1 component of pyruvate dehydrogenase complex, leading to the production of NADPH and acetyl-CoA, and downregulation of proteins in the TCA cycle that consume acetyl-CoA. Among genes upregulated in the mutant is *phbF*, which encodes the PHB synthesis regulator PhbF. The deletion of *arrF*, therefore, would increase PhbF levels, favoring PHB synthesis in the mutant (Pyla *et al*., 2010).

L. pneumophila is also one of the microorganisms that accumulate PHB, and, in *L. pneumophila* serogroup 1, PHB yields of up to 16% of cell dry weight could be extracted from culture samples. The PHB was located in electron-dense intracellular inclusions, which fluoresce bright yellow when stained with the lipophilic dye Nile red. PHB accumulation increased three fold during iron-limited culture and was inversely related to the concentration of iron metabolized (James *et al*., 1999).

This is consistent with the physiology of PHB formation, which is promoted during unbalanced growth when an essential nutrient other than the carbon and energy source is limiting (Steinbuchel and Schlegel, 1991; Dawes and Senior, 1973). Moreover, several studies have noted the formation of inclusions resembling PHB granules by *L. pneumophila* during intracellular growth in aquatic amoebae (Vandenesch *et al*., 1990; Fields *et al*., 1986). Restricted availability of essential nutrients, such as iron, is likely to promote PHB formation by *L. pneumophila* inside the amoebal host. Therefore, besides providing protection and supporting proliferation in hostile environments, amoeba may contribute to the environmental persistence of *Legionella* by inducing a PHB-rich phenotype that is physiologically more prepared for extracellular survival in low-nutrient environments (James *et al*., 1999).

1.3.2. Role of PHB in Bacterial Survival

In PHA-producing bacteria, PHA is a major determinant for overcoming periods of carbon and energy starvation. The accumulation and degradation of PHA can improve the establishment, proliferation, and survival of PHA-producing bacteria under environmental stress conditions imposed in water and soil (reviewed in Kadouri *et al*., 2005). Several studies, in which different PHA-producing bacteria (e.g., *P. oleovorans* (Ruiz *et al*., 2001; Ruiz *et al*., 1999), *C. necator* (*Ralstonia eutropha*) (López *et al*., 1995), *Rhizobium tropici* (Povolo and Casella, 2004), *Rhizobium leguminosarum* (Lodwig *et al*., 2005), and *A. brasilense* (Tal and Okon, 1985)) were incubated either under starvation conditions in natural oligotrophic environments or under conditions mimicking natural settings showed that wild-type strains containing PHAs survived starvation better than either PHA-polymerase or PHA depolymerase mutants (López *et al*., 1995; Ruiz *et al*., 1999).

Even when the PHB biosynthesis and degradation genes were expressed in the non-

PHB producing *E. coli*, a starvation experiment demonstrated that the complete PHB mobilization system in *E. coli* served as an intracellular energy and carbon storage system, which increased the survival rate of the host when carbon resources were limited. Moreover, a stress tolerance experiment indicated that *E. coli* strains with a PHB production and mobilization system exhibited an enhanced stress resistance capability (Wang *et al*., 2009).

Studies on *Spirillum* spp. indicated that starvation survival had a direct relationship with PHB content. An increase in the polymer content from 10 to 18 wt% resulted in as much as a 50% increase in starvation survival (Matin *et al*., 1979). Furthermore, Anderson and Dawes (1990) observed that the presence of PHB in a cell delayed the degradation of cellular components such as RNA and proteins during nutrient starvation. In addition, accumulated PHA in *A. brasilense* (Kadouri *et al*., 2002) and *C. necator* (Handrick *et al*., 2000) supported cell multiplication in the absence of an exogenous carbon source.

PHA compounds appear to endow cells that produce it with increased resistance to other types of environmental stresses, as was shown when *P. oleovorans* and a PHA depolymerization-minus mutant strain were used to assess the effect of PHA on survival and resistance to various stress agents. When exposed to 20% ethanol, the wild-type strain survived better than the PHA depolymerase-minus strain, with survival rates of 23% and 9%, respectively. Also, a 47^{o}C heat shock was less damaging to the wild type than to the mutant strain (Ruiz *et al*., 2001).

Similar observations were made for psychrotrophic strains of *Rhizobium* under cold stress (Sardesai and Babu, 2001). After exposure of *A. brasilense* to stress and adverse conditions such as UV irradiation, desiccation, and osmotic pressure, the survival of PHA-rich bacteria was higher than that of PHA-poor ones (Tal and Okon, 1985). Similar results were obtained working with *phaC* (Kadouri *et al*., 2002) and *phaZ* mutants of *A. brasilense* (Kadouri *et al*., 2003a; Kadouri *et al*., 2003b). The ability of these mutants to tolerate such stresses and to grow in the presence of hydrogen peroxide was significantly affected. As the two PHA mutants are similarly altered, with one blocked in the anabolic route (*phaC* mutant) and the other in the catabolic route (*phaZ* mutant), increased resistance to the described stresses can be traced to normal functioning of the PHA cycle and not exclusively to the presence of the polymer (Kadouri *et al*., 2005).

There is increasing evidence that mobilization of previously accumulated PHAs

enables the PHAs-accumulated bacteria to enhance their resistance towards some bactericides and other challenges (Ruiz *et al*., 2001; Ruiz *et al*., 2004). *Pseudomonas* spp. 14-3, a strain that accumulates large quantities of PHB when grown on octanoate, was isolated from antarctic environments, a habitat normally exposed to extreme conditions. Compared with *P. oleovorans*, *P. veronii*, and *P. fluorescens*, other *Pseudomonas* spp. 14-3 showed increased tolerance to both thermal and oxidative stress (H_2O_2 exposure) (Ayub *et al*., 2004).

E. coli strains engineered with elevated PHB production or degradation exhibit altered stress survival capability. When strains were incubated at 65°C, the PHB production strain and PHB degradation strain exhibited an increased heat resistance compared with the parent strain. After 60 min of heating, about 30% of the cells remained alive compared to only 1.8% for the control strain. Likewise, a PHB production strain and a PHB mobilization strain showed an increased tolerance to UV irradiation compared to wild type *E. coli*.

Acid resistance of the three strains was also studied: *E. coli* DH5α with PHB mobilization exhibited the highest survival rate among the three strains. By 20 min of acid treatment, cell viability in *E. coli* DH5α with PHB mobilization, *E. coli* DH5α with PHB production and *E. coli* DH5α control was reduced to 34.5%, 25.6% and 11.6% of the initial number of inoculated cells, respectively. *E. coli* DH5α with PHB mobilization also exhibited the highest survival rate when treated by a glucose-induced osmotic pressure (Wang *et al*., 2009).

The mechanisms by which the PHA cycle favours stress alleviation are not yet fully understood. Early work on *R. eutropha* suggested an association of PHA utilization with respiration and oxidative phosphorylation (cited in Kadouri *et al*., 2005). It was found that rises in ATP and guanosine tetraphosphate (ppGpp) levels were concomitant with PHA degradation. This phenomenon was only observed in wild type *P. oleovorans* and not in a PHA depolymerase-deficient strain unable to degrade the polymer (Ruiz *et al*., 2001).

The effector ppGpp was shown to increase mRNA translation of the central stationary phase regulator *rpoS* (Brown *et al*., 2002). The RpoS of RNA polymerase is the master regulator of general stress responses in *E. coli* and related bacteria. RpoS activates the expression of genes involved in cell survival and provides cross-protection to various environmental insults such as ethanol, H_2O_2, high temperature, and high salt concentration (Lange and Hengge-Aronis, 1991; Sarniguet *et al*., 1995; Ramos-Gonzalez and Molen, 1998;

Ruiz *et al*., 2001). RT-PCR studies showed an increase in *rpoS* expression in wild type *A. hydrophila* cells compared to a *phbC* mutant when exposed to high temperature (48^{o}C) and changing NaCl concentrations (Zhao *et al*., 2007).

In addition, Peralta-Gil *et al*. (2002) showed that one of the promoters that controls PHA synthesis (*phbBAC* operon) in *A. vinelandii* is regulated by RpoS. It seems that to respond properly to diverse stresses, PHA-producing bacteria require the *rpoS* gene product, which induces expression of many genes, including the ones responsible for PHA depolymerization, and allows the organism to mediate changes in cellular physiology and structure and to adapt, resist, and survive under stress conditions. While stress response players such as *rpoS* and ppGpp are central in determining the type and strength of the response by redirecting cell resources to the synthesis of the appropriate effectors, the PHA cycle could be responsible for providing the fuel necessary for this response and therefore could also determine its intensity (Kadouri *et al*., 2005).

Some bacteria produce spores and cysts as a survival strategy, and some evidence suggests that spore formation and germination may be linked with PHA biosynthesis and utilization. In *Bacillus cereus* cells that had accumulated PHA, the polymer disappeared after sporulation while the degradation products were incorporated into the spore. In that case PHA may serve as a carbon and energy reserve for sporulation (Nakata, 1966). Moreover, López *et al*. (1995) observed that in a PHA-negative mutant of *Bacillus megaterium*, sporulation occurred immediately after exposure to river water, while survival of vegetative cells was clearly decreased as compared to wild type. This result suggests that in an oligotrophic environment, cells depleted of intracellular carbon sources may be committed to earlier sporulation than normal cells.

PHB also plays an important role in encystment of the genus *Azotobacter*. Encystment is the process of cysts forming under nutrient-limiting conditions. PHAs serve as a carbon and energy source which encourages the extensive formation of cysts in *Azotobacter* (Stevenson and Socolofsky, 1966).

In a very special case, PHB serves as an oxidizable substrate that provides respiratory protection to nitrogenase of Azotobacteriaceae. The nitrogenase system of *Azotobacter* is very sensitive to oxygen. High concentration of oxygen is inhibitory to this strain. Mobilization of PHB may increase the respiratory activity while reducing the concentration

of oxygen, thus protecting the nitrogenase system (Senior *et al*., 1972).

It has been shown that PHA accumulation plays a major role in some bacteria that live in close association with eukaryotes. In rhizobia, bacteria that are characterized by a free-living and a symbiotic stage with a eukaryotic host, the impact of the bacteria-host relationship on PHA accumulation varies considerably (Kadouri *et al*., 2005). Rhizobia with disrupted PHB synthesis genes have been shown to be less competitive for nodulation and reproduce less under starvation than wild-type cells (Aneja *et al*., 2005).

Cai *et al*. (2000) found that *R. meliloti* mutants unable to synthesize or degrade PHB reproduced to 5.1-fold their inoculum population size when starved, while wild-type reproduced to 9.7-fold their inoculum population size. The increased reproduction of the wild-type Rhizobia reflects the reproductive benefit of PHB accumulation. The competitive abilities of *S. meliloti* mutant strains containing lesions in the PHB synthesis (*phbC*) and degradation (*bdhA*) pathways were compared. While the *bdhA* mutant showed no noticeable symbiotic defects on alfalfa host plants when inoculated alone, mixed inoculation experiments revealed the mutant to be less competitive than the wild type in terms of nodule occupancy. Long-term survival of the *bdhA* mutant on a carbon-limiting medium was not affected. However, when subjected to competition with the wild-type strain in periodic subculturing through alternating carbon-limiting and carbon-excess conditions, the *bdhA* mutant performed poorly. A more severe defect in competition for growth and nodule occupancy was observed with a mutant unable to synthesize PHB (*phbC*). These results indicate that the ability to efficiently deposit cellular PHB stores is a key factor influencing competitive survival under conditions of fluctuating nutrient carbon availability, whereas the ability to use these stores is less important (Aneja *et al*., 2005).

PHA accumulation was also involved in promoting long-term survival of *Legionella pneumophila* under starvation conditions. Interestingly, PHA accumulation was promoted during bacterial growth under iron limitation in the amoeba host, which therefore may induce a PHA-rich phenotype, rendering the bacteria more fit for extracellular survival in low-nutrient environments. Chemostat-grown *L. pneumophila* cells survived in a culturable state for at least 600 days when incubated at 24°C in a low-nutrient tap water environment. Nile red spectrofluorometry and flow cytometry demonstrated that PHB reserves were utilized during starvation (James *et al*., 1999).

1.3.3. The 3-Hydroxybutyrate Dehydrogenase Enzyme

The enzyme 3-hydroxybutyrate dehydrogenase (EC 1.1.1.1.30, HBDH) belongs to the family of short-chain dehydrogenases/reductases (SDRs). It catalyses the oxidation-reduction reaction between β-hydroxybutyrate and acetoacetate in the PHB cycle and has a key role in the synthesis and degradation of PHB. HBDH enzyme has been found in mammals to be bound to membranes in the mitochondrion where it catalyses the degradation of β-hydroxybutyrate to acetate during starvation periods or diabetes (Kaminsky, 1985).

In contrast to the mammalian enzymes (McIntyre *et al*., 1978), HBDHs from bacteria are cytosolic enzymes (Tal *et al*., 1990), are not preceded by a leader peptide, and lack the C-terminal 28 residues which harbor the phospholipid binding site essential for mammalian enzyme function (Kruger *et al*., 1999). Enzymes of the SDR family span several enzyme classes, with NAD+- and NADP+-dependent oxidoreductases representing the majority (Kallberg *et al*., 2002). All of these dehydrogenases function without metal ions, their coenzymes binding to a Rossmann fold dinucleotide-binding motif (Rossmann *et al*., 1974).

Highly conserved amino acid sequence regions include the N-terminal Gln-X_3-Gln-X-Gln motif of the Rossmann fold and the active site, with a catalytic tetrad consisting of the residues asparagine, serine, tyrosine, and lysine (Oppermann *et al*., 2003). SDR enzymes are one-domain enzymes, so the coenzyme and the substrate bind in the same deep and mainly hydrophobic cleft, with the NAD+ at the bottom (Feller *et al*., 2006).

Sequence comparison of 44 bacterial HBDHs shows the residues Gln91, His141, Lys149, Lys192, and Gln193 to be strictly conserved. Site-directed mutagenesis of these amino acids to alanine and subsequent kinetic characterization of the mutated enzymes provided insight into the importance of these residues for substrate recognition and catalysis. Simulations based on a three-dimensional structure model of a complex between *P. putida* Bdh and its coenzyme provided deeper insight into the binding of the ligands at the molecular level. They showed the residues Gln91, His141, Gln193, and especially Lys149 to be involved in a hydrogen-bonding network with the carboxylate group of the substrate (Feller *et al*., 2006).

Both the bacterial and mammalian HBDH proteins are homotetramers (McIntyre *et al*., 1983; Nakada *et al*., 1981; Tal *et al*., 1990). For example, HBDH purified from *A.brasilense* Cd is a tetramer with identical subunits and a total molecular mass of 100 kDa (Tal *et al*., 1990). HBDH is inhibited by NADH, NADPH, α-oxoglutarate, adenosine phosphates, pyruvate, oxaloacetate, 2-oxoglutarate (Tal *et al*., 1990), Acetyl CoA, D-lactate, 2-hydroxybutyrate, sulfhydryl reagents such as *p*-chloromercuribenzoic acid, 5,5'-dithiobis (2-nitrobenzoic acid), and $HgCl_2$ (Nakada *et al*., 1981). HBDH from *Rhodopseudomonas spheroides* is extremely sensitive to mercurials, but can be protected by $NADH_2$ or Ca^{2+} ions (Bergmeyer *et al*., 1967).

Although the organization of the HBDH-encoding gene (*bdhA*) has been found to vary in different bacteria, it is mostly found in operons with other genes. In *Rhizobium* (*Sinorhizobium*) *meliloti*, *bdhA* has an open reading frame of 777 bp that encodes a polypeptide of 258 amino acid residues (molecular weight 27,177). *bdhA* is the first gene transcribed in an operon that also includes *xdhA* and *xdhB*, encoding xanthine oxidase/ dehydrogenase. Transcriptional start site analysis by primer extension identified two transcription starts: S1, a minor start site, was located 46 to 47 nucleotides upstream of the predicted ATG start codon, while S2, the major start site, was mapped to a location 148 nucleotides from the start codon. Analysis of the sequences immediately upstream of S1 and S2 failed to reveal the presence of any known consensus promoter sequences. Although an RpoN consensus sequence was identified in the region between S1 and S2, a corresponding transcript was not detected, and an *rpoN* mutant of *R. meliloti* was able to utilize 3HB as a sole carbon source. Studies with a strain carrying a *lacZ* transcriptional fusion to *bdhA* demonstrated that gene expression is growth phase-associated (Aneja and Charles, 1999). The expression of the *bdhA-xdhA2-xdhB2* mixed-function operon in *R. meliloti* is biotin-inducible. *R. meliloti* responds to external biotin signals from alfalfa plants through the *bioS* regulatory locus. Immunogold labeling and electron microscopy revealed that the BioS protein is located within the *R. meliloti* cytoplasm. Under biotin-limiting conditions the *R. meliloti* cell lumen was filled with PHB granules. A *bdhA-lacZ* fusion revealed an overall 3.6-5.2-fold increase in *bdhA* transcription in the presence of added biotin, and comparison of the *bdhA* and the *bioS* promoter regions identified several common motifs (Hofmann *et al*., 2000).

In *B. subtilis* the *bdhA* homologue *yxjF* is positioned beside two loci designated *yxjE* and *yxjD*, arranged in the order *yxjDEF* (Kunst *et al*., 1997). On the basis of sequence similarity analyses, *yxjE* and *yxjD* have been proposed to encode the two subunits of 3-oxoadipate CoA transferase, an enzyme that is able to catalyze the activation of acetoacetate to acetoacetyl-CoA. *yxjF* and *yxjE* are interrupted by a mere 16 nucleotides, making the existence of an independent promoter in this region highly unlikely (Aneja and Charles, 1999).

L. pneumophila bdhA (*lpg2316*) encodes a 260-amino-acid (28-kDa) protein with full-length protein homology to BdhA of *Sinorhizobium* spp. strain NGR234. *Sinorhizobium* spp. strain NGR234 *bdhA* mutants are unable to utilize PHB and, interestingly, show symbiosis defects with Leucaena host plants (Aneja and Charles, 2005). *L. pneumophila* BdhA contains the amino acids S142, Y155, and K159, usually conserved in 3HB dehydrogenases and likely required for catalysis. Furthermore, it is characterized by cytoplasmic localization and the lack of signal peptide (Aurass *et al*., 2009).

Expression of *bdhA* was upregulated in a PmrA/PmrB-dependent manner, mainly at the exponential phase of growth, suggesting that *L. pneumophila* may be using this TCS to couple its differentiation to metabolic state (Al-Khodor *et al*., 2009). Examination of the *L. pneumophila* Philadelphia-1 *bdhA* locus revealed that the surrounding open reading frames are organized in the same orientation. Operon prediction analyses suggested a transcriptional unit of *bdhA* together with the next downstream open reading frame, *lpg2317* (Aurass *et al*., 2009). RT-PCR revealed that *bdhA* and *lpg2317* are indeed cotranscribed and therefore form an operon. *lpg2317*, which was designated *patD* (patatin-like protein D), encodes a 386-amino-acid (44-kDa) putative phospholipase A.

Aurass *et al* (2009) found that the *L. pneumophila bdhA-patD* mutant possessed an increased number of PHB granules. Double amounts of PHB granules per bacterial cell at each time point were detected compared to the wild type. The *bdhA-patD* mutant possessed about 1 granule per cell, while wild-type bacteria contained ~ 0.5 granules per cell, suggesting that the operon is involved in PHB utilization. Furthermore, the *L. pneumophila bdhA-patD* mutant showed a severe replication defect in amoebae and U937 macrophages, indicating that the *bdhA-patD* operon is a virulence determinant of *L. pneumophila*.

The infection defect observed in the *bdhA-patD* mutant is very comparable with a

Dot/Icm secretion system mutant, suggesting that PHB metabolism is essential for the life style of *L. pneumophila* and intracellular propagation, and that provision of energy by PHB cleavage is an essential determinant to efficiently energize the Dot/Icm secretion system or another essential component. However, it is currently not known whether the mutant is defective in the export of effector proteins.

PHB depolymerases characteristically contain a catalytic triad of serine (embedded in a G-XS-X-G motif)/aspartate (or glutamate)/histidine, which is typical for esterase-lipase superfamily enzymes, and are alpha/beta folded (Papageorgiou *et al*., 2008). Most of those structural features, such as a serine/aspartate catalytic dyad and the alpha/beta folding, have been found for patatin, which is a nonspecific lipid acyl hydrolase found in potato and some other plants (Rydel *et al*., 2003). Thus, PatD is structurally closely related to PHB depolymerases and may fulfill a similar function under PHB-mobilizing conditions. Interestingly, within the four sequenced *L. pneumophila* (Philadelphia-1, Paris, Lens, and Corby) (Cazalet *et al*., 2004; Chien *et al*., 2004; Glockner *et al*., 2008) genomes, no genes coding for homologs of known PHB depolymerases were found.

Another scenario of concerted *bdhA-patD* action is possible. Bacterial and eukaryotic lipid inclusions are surrounded by a phospholipid monolayer with embedded amphiphatic proteins, the phasines. Phasines support the solubilization of granules in hydrophilic environments, as the cytoplasm, and they also avoid an aggregation of granules (Griebel *et al*., 1968). Phospholipase A partially destructs the granule phospholipid layer, making lipids accessible for degrading enzymes such as depolymerases and consequently allowing PHB metabolization (Noll *et al.*, 2000). Accordingly, May *et al*. (1998) previously demonstrated that a plant patatin-like protein of *Cucumis sativus* specifically localized to lipid granules for support of lipid mobilization during seed germination. Whether PatD directly facilitates PHB utilization in *L. pneumophila* requires further work (Aurass *et al*., 2009).

1.4. Hypothesis and Objectives

Since Legionnaires' disease is transmitted by contaminated water aerosols and there is no human-to-human transmission, there was a strong motive to study VBNC *L. pneumophila* in water, particularly because *L. pneumophila* in man-made water systems is often monitored by growth and enumeration on agar medium (England *et al*., 1982; Hussong *et al*., 1987).

The VBNC *L. pneumophila* remains largely uncharacterized. Therefore, I postulate 3 hypotheses:

Hypothesis 1. VBNC *L. pneumophila* displays ultrastructural characteristics that are distinct from those of culturable *L. pneumophila*.

Hypothesis 2. VBNC *L. pneumophila* cells represent an important differentiated form within the developmental cycle of *L. pneumophila*.

Hypothesis 3. Specific gene products play a role in the differentiation of *L. pneumophila* into VBNC forms.

Therefore, the objectives of the present study are (i) to study the morphology, physiology, and biochemical characteristics of VBNC forms produced in water (to test hypotheses 1 and 2); (ii) to compare the effect of dd- and tap-water on the production and ultrastructural traits of VBNC *L. pneumophila* (to test hypothesis 1)*;* (iii) to identify the protein profile that characterizes VBNC forms produced in water (to test hypotheses 2 and 3); (iv) to study the role of *bdhA* gene (encoding the enzyme 3-hydroxybutyrate dehydrogenase) in *L. pneumophila* VBNC cells formation in water (to test hypothesis 3).

1.5. Overview of My Results

In the present study, I determined that *L. pneumophila* produces VBNC forms with different characteristics, depending on the developmental form used to produce them and that VBNC forms are morphologically and (or) physiologically different from culturable *L. pneumophila*, supporting my Hypothesis 1. I also provided the first quantitative method to determine the number of resuscitated VBNC cells in the amoeba *A. castellanii*. In addition, I showed that the VBNC cells are environmentally fit forms, as evident from their resistance to digestion inside the ciliate food vacuoles and detergent-mediated lysis. These data suggest a new pathway in the *Legionella* life cycle wherein VBNC cells can be ingested and expelled by the ciliate *Tetrahymena*, supporting that VBNC are not only part of the *L. pneumophila* life cycle, but also a part of the *L. pneumophila* developmental cycle (Hypothesis 2).

Protein analysis by SDS-PAGE and shotgun proteomics revealed VBNC cell-specific proteins, including 3-hydroxybutyrate dehydrogenase (BdhA), which catalyses the oxidation of β-hydroxybutyrate to acetoacetate. A *bdhA* mutant showed an early loss of culturability and a dramatic decrease in viability. Thus my results obtained with the *bdhA* mutant show

that BdhA plays a role in the production of VBNC forms and support my Hypothesis 3.

CHAPTER 2. MATERIALS AND METHODS

2.1. Bacterial Strains and Growth Conditions

The bacterial strains used in this study are listed in Table 2.1.

2.1.1. Growth of *L. pneumophila*

All *L. pneumophila* strains were grown at 37^{o}C on buffered charcoal yeast extract (BCYE) agar or in buffered yeast extract (BYE) broth (Pasculle *et al*., 1980). BCYE contained 10 g/L yeast extract, 6 g/L (2-[2-amino-2-oxoethyl]-amino) ethanesulfonic acid (ACES), 1 g/L α-ketoglutaric acid, 15 g/L agar, and 1.5 g/L activated charcoal, with the pH adjusted to 6.6 using 6 N KOH. After autoclaving, 0.4 g/L L-cysteine (filter sterilized, pH adjusted to 6.6 with 1 N KOH) and 0.25 g/L ferric pyrophosphate (filter sterilized) were added to the media. BYE broth contained the same components as BCYE agar except that no charcoal and agar were added. When needed, media was supplemented with sucrose and antibiotics at the following concentrations: 50 g/L sucrose, 100 μg/ml streptomycin, 25 or 75 μg/ml kanamycin, 3 or 5 μg/ml chloramphenicol, and 100 μg/ml erythromycin.

L. pneumophila strain JR32 was routinely grown from frozen stocks kept at -80°C on BCYE agar plates containing 100 μg/ml streptomycin, and the plates were incubated for 3 days at 37°C in 5% CO_2. To obtain a homogeneous population of Stationary Phase Forms (SPFs), single colonies from BCYE agar plates were inoculated into 30 ml BYE broth and incubated at 37°C with shaking (200 rpm) until an OD_{620} of 3.5 was reached, which usually required an overnight (O/N) incubation. *L. pneumophila* stocks were frozen at -80°C in 10% dimethylsulphoxide (DMSO) for long term storage.

Measurement of *L. pneumophila* growth was performed by monitoring the change in OD_{620} at regular intervals for up to 77 h.

2.1.2. Growth of *Escherichia coli*

E. coli strains DH5α and XL1-Blue were used for plasmid propagation and long-term plasmid storage. *E. coli* cells were propagated at 37°C in LB (Luria-Bertani) broth (5 g/L yeast extract, 10 g/L tryptone, and 10 g/L NaCl) or on solid LB medium-containing 1.5% agar (Bertani, 1951). When needed, ampicillin (100 μg/ml), tetracycline (10 μg/ml),

Table 2.1. Bacterial and protozoan strains used in this study

Bacterial or Protozoal strain	Relevant properties	Reference or Source
E. coli		
DH5α	F^-Φ80*lacZ*ΔM15Δ(*lacZYA-argF) U169 supE44 hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Clontech
DH5α pCDP05	DH5α contains pCDP05 (Mini-Tn*10*, Km^r, Cm^r, Suc^S)	This study
DH5α pMMB207C	DH5α containing plasmid pMMB207C (Cm^R)	Dr. Audrey Chong (Chong, 2007)
XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′ *proAB lacIqZΔM15* Tn*10* (Tet^R)]	Stratagene
XL1-Blue pBluescript *bdhA::aphA3*	XL1-Blue contains pBluescript *bdhA::aphA3* (Amp^R, Tet^R, Km^R)	This study
XL1-Blue pBluescript *bdhA::ermAM*	XL1-Blue contains pBluescript *bdhA::ermAM* (Amp^R, Tet^R, Erm^R)	This study
XL1-Blue pMMB207C	XL1-Blue contains pMMB207C (Cm^R)	This study
XL1-Blue pBdhA1	XL1-Blue contains pBdhA1 (Cm^R)	This study
XL1-Blue pBdhA2	XL1-Blue contains pBdhA2 (Cm^R)	This study
XL1-Blue pBdhA3	XL1-Blue contains pBdhA3 (Cm^R)	This study
L. pneumophila		
JR32	Salt sensitive isolate of AM5011 (AM5011:Philadelphia 1, serogroup1, Sm^R, restriction deficient, modification positive)	Dr. Howard Shuman (Columbia University), (Sadosky *et al*., 1993)

Table 2.1. Continuation

Bacterial or Protozoal strain	Relevant properties	Reference or Source
JR32 N	JR32 mini-Tn *10* mutant (Sm^R, Km^R)	This study
JR32 *bdhA* mutant	JR32 *bdhA:: aphA3* (Sm^R, Km^R)	This study
parent207C	JR32 contains pMMB207C (Sm^R, Cm^R)	This study
bdhA207C	JR32 *bdhA* mutant contains pMMB207C (Sm^R,Km^R, Cm^R)	This study
Comp 1P	JR32 *bdhA* mutant contains pBdhA1 (Sm^R,Km^R, Cm^R)	This study
Comp 3P	JR32 *bdhA* mutant contains pBdhA3 (Sm^R,Km^R, Cm^R)	This study
Comp 4P	JR32 *bdhA* mutant contains pBdhA4 (Sm^R,Km^R, Cm^R)	This study
Protozoal Strains		
A. castellanii strain Neff		Dr. David Spencer, Dalhousie University, (Anderson *et al*., 2005)
*Tetrahymena tropicali*s		Dr. Sharon Berk (Berk *et al*., 2008)
Tetrahymena thermophila		Dr. David Spencer, Dalhousie University

Amp^R, Km^R, Cm^R, Erm^R, Sm^R indicate resistance markers to ampicillin, kanamycin, chloramphenicol, erythromycin, and streptomycin, respectively. Suc^S indicate sensitivity to sucrose due to *sacB* (encoding levansucrase).

kanamycin (50 μg/ml), chloramphenicol (25 μg/ml), or erythromycin (300 μg/ml) were added to LB medium. Also, sucrose was added at a concentration of 50 g/L when necessary. LB medium was stored at 4°C and antibiotics were added prior to use. *E. coli* strains generated in this study were stored at –80°C in 25% (v/v) glycerol.

2.2. Protozoal Strains and Growth Conditions

Protozoal strains used in this study are listed in Table 2.1.

2.2.1. Growth of *Acanthamoeba castellanii*

Axenic cultures of *A. castellanii* strain Neff (Anderson *et al*., 2005), a gift from D. Spencer and M. Gray (Dalhousie University), were grown as trophozoites in Neff's medium (15 g/L glucose, 7.5 g/L yeast extract, 7.5 g/L proteose peptone, 2 mM KH_2PO_4, 1 mM $MgSO_4$, 0.05 mM $CaCl_2$, 0.1 mM ferric pyrophosphate, 100 μl/L of 10000 x stock Neff' vitamin mix; pH 7) (Cursons *et al*., 1980; Neff, 1957) in 75 cm^2 tissue culture flasks at 37°C. For infection with *L. pneumophila*, trophozoites were allowed to grow in 75 cm^2 tissue culture flasks at 37°C until they formed a monolayer, which was examined under a light microscope. The monolayers were gently washed with, and kept in, modified Neff's medium (Neff's medium without proteose peptone, yeast extract, and multi-vitamin mix).

2.2.2. Growth of *Tetrahymena* Species

*Tetrahymena tropicali*s and *T. thermophila* were routinely grown in Plate Count Broth (PCB) (5 g/L yeast extract, 10 g/L tryptone, 2 g/L glucose; pH 7) at 25°C, as previously described (Berk *et al*., 2008). Briefly, 1 ml of *Tetrahymena* culture was subcultured into 40 ml of fresh PCB broth every two weeks. Before use in feeding experiments, *Tetrahymena* cells were gradually transferred from PCB to Tris-buffered Osterhout's solution (NaCl (420 mg/liter), KCl (9.2 mg/liter), $CaCl_2$ (4 mg/liter), $MgSO_4 \cdot 7H_2O$ (16 mg/liter), $MgCl_2 \cdot 6H_2O$ (34 mg/liter), and Tris base (121 mg/liter) pH 7) (Osterhout, 1906; Provasoli, 1958). *Tetrahymena* cells were pelleted by centrifugation at 500 x *g* for 10 min at 25°C. Half of the supernatant fluid was carefully removed and Osterhout's solution was added to the remaining culture to obtain 50% PCB media and 50% Osterhout's solution. The cell suspensions were allowed to stand for 10 minutes. The process was

repeated two more times and the *Tetrahymena* cells were then resuspended to 10^4 cell/ml in Osterhout's solution (determined by direct count of Lugol's iodine-fixed samples by using 0.2 mm gap hemacytometer).

2.3. Production of *L. pneumophila* MIFs from *A. castellanii*

BCYE agar-grown *L. pneumophila* were resuspended in Modified Neff's medium to an OD_{620} of 1 and used to infect amoeba trophozoites at a ratio of one bacterium per trophozoite. The culture was incubated at 37°C for 5 days. After incubation, when all infected trophozoites had been lysed, MIFs were harvested by centrifugation for 6 min at 4500 x *g* at 25°C.

2.4. Production of VBNC Bacterial Cells

VBNC cells were first produced by incubating SPF bacteria in sterile distilled deionized water (dd-water) at different cell densities and temperatures. Once the effect of bacterial concentration and temperature was determined, VBNCs were subsequently produced by incubating SPFs or MIFs at a cell density of 1 x 10^8 cfu/ml in glass bottles containing 500 ml of dd-water or tap water at 45°C. Absence of chlorine in the autoclaved tap water was confirmed using the Chlorine Color Disc Test Kit (Hach, Model CN-66). The bacterial inoculum was routinely prepared as follows: SPFs or MIFs were pelleted by centrifugation at 4500 x *g* for 6 min, washed 3 times in the corresponding water type, and resuspended to an OD_{620} of 1. Next, 50 ml of each bacterial suspension was mixed with 450 ml of the corresponding sterile dd- or tap water in 500 ml bottles (VWR Laboratory Glassware), followed by incubation at 45°C in a water bath (HAAKE SWB20). Bacterial culturability in 0.1 ml samples was monitored by dilution plating on BCYE agar plates, using the corresponding water type as a diluent. For time points with low culturability, 10 ml samples were centrifuged at 4500 x *g* for 6 min at 25°C and resuspended in 1 ml of dd-water before plating on BCYE. Experiments were routinely conducted in sets of three independently prepared bottles.

Viability was monitored using the LIVE/DEAD BacLight vital stain (Invitrogen), which utilizes a mixture of the stains SYTO 9 and propidium iodide to evaluate cell membrane integrity, as per the manufacturer's instructions (Chang *et al*., 2009; Ohno *et al*.,

2003; Alleron *et al*., 2008). Briefly, 330 µl samples were mixed with l µl of the prepared BacLight dye, incubated at room temperature in the dark for 15 min, and mounted on glass microscope slides covered with 22 x 22 mm coverslips for observation in a BX61 Olympus fluorescence microscope. Controls were prepared using 72 h old *Legionella* cells. Total numbers of live (green fluorescent) and dead (red fluorescent) bacterial cells were estimated from a count of ~ 4000 cells from at least 10 randomly chosen microscopic fields according to the internal standard method described by Mallette (Mallette, 1969), using a calibrated suspension of 1 µm diameter microbeads as reference (Carboxylate- modified 1 µm polystyrene fluorescent beads, Molecular Probes, Invitrogen).

2.5. Effect of pH on *L. pneumophila* Viability

Glass bottles containing 100 ml tap water at pH 6.6, dd-water at pH 4.4, tap water at pH 4.4, and dd-water buffered to pH 6.6 with 10 mM ACES were inoculated with SPF bacteria at a final concentration of 1 x 10^8 cfu/ml. The suspensions were incubated at 45°C. Viability and cfu/ml were monitored as above until loss of culturability was reached. These experiments were done in duplicate.

2.6. Addition of Salts to Buffered dd-Water

Salts at concentrations shown in Table 2.2 were individually dissolved in 100 ml of dd-water buffered to pH 6.6 with 10 mM ACES. Salts were chosen to provide one or more of the ions known to be present in our local tap water, according to an analysis provided by the Halifax Regional Water Commission (Halifax Regional Water Comission, 2005/06). After inoculation of the buffered salt solutions with SPF bacteria (at a final concentration of 10^8 cfu/ml), duplicate bottles were incubated at 45°C and culturability and viability were monitored as described above until complete loss of culturability was reached.

2.7. Giménez Staining

Giménez staining, which uses carbol fuchsin as the primary stain and malachite green as the secondary stain, was performed exactly as reported by McDade (McDade, 1979). Bacteria that are stained red are referred to as Giménez-positive and those stained green, blue green, or gray are referred to as Giménez-negative.

Table 2.2. Concentrations of ions added to dd-water samples in which SPF *L. pneumophila* was inoculated.

Ions	final concentration (mg/L)	salts used
Aluminum	0.064	$KAl(SO_4)_2$
Chloride	7.3	NaCl
Fluoride	0.70	NaF
Iron	0.025	$FeCl_3$
Manganese	0.020	$MnCl_2.4H_2O$
Nitrate	0.05	$Ca(NO_3)_2.4H_2O$
Sodium	10.0	Na_2SO_4
Sulphate	12.0	$FeSO_4$

2.8. Electron Microscopy (EM)

EM was performed using the standard protocols reported previously (Garduno *et al*., 1998a; Garduno *et al*., 1998c). Briefly, for transmission EM of ultrathin sections, samples were fixed in glutaraldehyde, postfixed in OsO_4, *in-bloc* stained with aqueous uranyl acetate, dehydrated in acetone, and embedded in epoxy resin. Ultrathin sections were poststained with uranyl acetate-lead citrate before observation. Sample processing was performed by Mary Ann Trevors at the Electron Microscopy Unit at Dalhousie University.

For transmission EM of negatively-stained bacteria, a grid was floated over a drop of bacterial cell suspension in water (usually a direct sample from VBNC bacteria) for 20 minutes, after which the grid was stained for 30 sec on a drop of saturated unbuffered ammonium molybdate. Stained specimens (ultrathin sections or negatively stained bacteria) were observed in a JEOL JEM-1230 transmission electron microscope and images were captured with an ORCA-HR (2K by 2K, Hamamatsu) and saved as TIFF files with a resolution of 525 pixels/in.

2.9. Susceptibility to Lysis by Detergent

Susceptibility of *L. pneumophila* to lysis by detergent was tested by making suspensions of 10^9 bacteria/ml in SDS (10 mg/ml in 10 mM Tris, pH 7.5) and measuring the OD_{620} at regular intervals. A graph of OD versus time was made, and the time in minutes required to reach half of the initial OD_{620} was reported as the detergent lysis index.

2.10. VBNC Bacteria Resuscitation Attempts

VBNC cells were used to infect *A. castellanii*, U937-derived macrophages, or HeLa cells (at a VBNC bacteria to cell ratio of 1) to determine whether they could initiate infection and recover culturability.

Monolayers of *A. castellanii* trophozoites were grown in 25 cm^2 cell culture flasks (Falcon) and inoculated with VBNC cells prepared in either sterile tap water or dd-water (according to the microcosm used to produce VBNC cells). The inoculum was centrifuged onto the amoeba (500 x *g* at 25°C for 10 min) to enhance contact. Flasks were then incubated at 37°C for 12 days. HeLa cells were routinely grown in 25 cm^2 cell culture flasks (Falcon) in Dulbecco modified Eagle's medium (DMEM; high-glucose formulation)

supplemented with 10 % (v/v) newborn calf serum and an antibiotic-antimycotic mixture (all from GIBCO). The VBNC inoculum was prepared in DMEM, centrifuged as above onto the HeLa cells, and flasks were then incubated at 37°C in 5% CO_2 for at least 5 days. U937 cells were grown in suspension in RPMI 1640 medium, supplemented with 5% (v/v) fetal bovine serum and the antibiotic-antimycotic mixture (GIBCO Laboratories). To obtain adherent macrophage-like cells, U937 cells were treated with 20 ng/ml phorbol myristoyl acetate (Sigma) and transferred to 25 cm^2 flasks at cell density of 5 x 10^6/flask. Following differentiation, adherent cells were infected with a VBNC inoculum prepared in RPMI-1640. The inoculum was centrifuged as above, and the flasks were incubated at 37°C in 5% CO_2 for at least 5 days. Aliquots (100 µl) of the infected cultures were taken at different time points post-infection, serially diluted and spotted on BCYE agar plates for colony formation at 37°C.

In addition to the infection assay, the effect of conditioned media on the resuscitation of VBNC cells was also assessed. Attempts were made to resuscitate an inoculum of 5 x 10^8 VBNC cells in 30 ml of shaken (200 rpm) fresh BYE, spent BYE of an overnight *L. pneumophila* culture, or in cell-free spent modified Neff's medium in which *A. castellanii* had been infected with *L. pneumophila*. The latter was produced as the supernatant of an infected culture by centrifugation (4500 x *g* at 25°C for 6 min), after which the supernatant was sterilized by filtration through a 0.2µm filter. Culturability was monitored by plating 100 µl on BCYE at regular intervals for up to one year.

2.11. Quantification of Resuscitated VBNC Cells in Amoeba

To determine the number of VBNC cells that were able to resuscitate in amoeba, 1 ml of Neff's medium was placed onto a BCYE agar plate and 100 µl of a suspension of *A. castellanii* cysts was added, followed by the addition of 1 ml of VBNC cells. The VBNC samples were prepared by either diluting or concentrating the suspension of the VBNC bacteria (originally inoculated with 10^8 cfu/ml). The 1 ml sample added to the BCYE agar plates contained a range of bacterial cell equivalents from 10^2 to 10^9. After mixing the components (Neff's medium, cyst inoculum, and sample) by gentle rotation, plates were incubated in the upright position at 37°C in 5% CO_2 for 24 h. After that, the samples were dried and the plates were inverted and incubated for four additional days, and colonies were

counted.

2.12. Feeding Experiments with *Tetrahymena* Species

VBNC cells were washed and resuspended in Osterhout's solution. *Tetrahymena* were prepared in 5 ml of Tris-buffered Osterhout's solution in 25 cm^2 cell culture flasks and fed the VBNC cells at a bacteria to ciliate ratio of 10000:1. After a 24 h incubation at 25°C, pellets obtained by centrifugation (500 x *g*, 10 min, 25°C) were prepared for culturability, transmission EM, Giménez staining and BacLight viability staining, as described above.

2.13. Molecular Techniques

2.13.1. Agarose Gel Electrophoresis

Electrophoresis of DNA was carried out at 120 V in 200 ml of Tris-acetate-ethylenediamine tetraacetic acid (TAE) buffer (40 mM Tris base, 20 mM acetic acid, 1 mM EDTA; pH 8.0) containing10 µl ethidium bromide (10 mg/ml). DNA samples were analyzed on 0.8% or 2% (w/v) agarose gels. One kilobase (kb) or 100 basepair (bp) DNA ladders (NEB) were used as standards. Gel images were captured using a Bio Doc-It imaging system (UVP, USA).

2.13.2. Isolation of DNA Fragments from Agarose Gels

Agarose gels were viewed at 302 nm UV (Spectroline transilluminator, Westbury, NY) and gel slices containing the desired DNA fragments were cut from the gel with a clean scalpel. The gel slices were placed in a dialysis bag (Fisher Scientific, Nepean, ON) with 0.5 ml of TE buffer (10 mM Tris-HCl, pH 7.6; 1 mM EDTA, pH 8), which was placed in TAE buffer and electrophoresed at 100 V for 1 hour. An Elutip-D minicolumn (Whatman Inc. Florham Park, NJ) was washed with 1 ml of high salt buffer (1 M NaCl, 20 mM Tris, and 1 mM EDTA; pH 7.4), followed by 3 ml of low salt buffer (20 mM NaCl, 20 mM Tris, and 1 mM EDTA; pH 7.4) to equilibrate the column. The electro-eluted DNA (0.5 ml) was transferred from the dialysis bag to a 1.5 ml microcentrifuge tube (Eppendorf). The dialysis bag was washed with an additional 0.5 ml of low salt buffer, which was pooled with the eluted DNA and applied to the column. The bound DNA was washed with 3 ml of low salt buffer and eluted with 0.5 ml of high salt buffer into a 1.5 ml microcentrifuge tube. The DNA

was precipitated with 1 ml of 95% (v/v) ethanol containing 2.5% (w/v) potassium acetate (KAc), washed with 1 ml of 70% (v/v) ethanol, vacuum dried and dissolved in TE or dd-water.

2.13.3. Plasmid Isolation from *E. coli*

Plasmids used in this study are listed in Table 2.3. Plasmid pCDP05 (a gift from Dr. Nicholas P. Cianciotto), used for creating the transposon library of *L. pneumophila*, was electroporated into *E. coli* DH5α and subsequently isolated using a QIAprep Spin Miniprep kit (QIAGEN). Briefly, 5 ml of *E. coli* cultures were grown in LB media containing 30 µg/ml chloramphenicol and 50 µg/ml kanamycin and harvested by centrifugation (4500 x *g*, 10 min, 25^{o}C). Plasmid purification was carried out according to the manufacturer's instructions, and the plasmid was confirmed by restriction analysis using EcoRI and NotI.

Other plasmids were isolated from *E. coli* transformants by a standard alkaline lysis method as described by Birnboim and Doly (1979). Briefly, *E. coli* cultures were grown in 1.5 ml LB broth and the cells were harvested by centrifugation for 2 min at 11000 x *g*. The culture supernatant was discarded and the pellet was resuspended in 100 µl GTE (50 mM glucose, 25 mM Tris pH 8, 10 mM EDTA), 186 µl dd-water, and 1 µl RNase A (10 mg/ml) (Sigma). Subsequently, 10 µl of 20 % (w/v) sodium dodecyl sulphate (SDS) was added to the cell suspension, while 4 µl of 10 M NaOH was added to the lid of the microcentrifuge tube. The tube was inverted gently to mix and then incubated at 25^{o}C for 5 min. One hundred fifty µl of KAc (3 M K^{+} and 5 M Ac^{-}) solution was added to the cell suspension and the tube was inverted to mix, followed by a 10 min incubation on ice. The mixture was centrifuged at 14000 x *g* for 5 min at 4^{o}C and the supernatant was vortexed with 500 µl chloroform for 1 min. The mixture was centrifuged for 5 min at 14000 x *g* at 4^{o}C, and the aqueous layer was transferred to a new tube with two volumes of 95% (v/v) ethanol containing 2.5% (w/v) KAc. The mixture was incubated at -80^{o}C for a minimum of 30 min before centrifugation for 10 min at 14000 x *g* at 4^{o}C to pellet the DNA. The DNA pellet was washed with 70% (v/v) ethanol, vacuum dried and dissolved in 10-20 µl TE buffer containing 0.1 µl RNase. The alkaline lysis method was also adopted for the isolation of plasmids from *Legionella*.

Table 2.3. Plasmids used in this study

Plasmids	Relevant properties	Reference or Source
pCDP05	Plasmid containing mini-Tn*10* and conferring sucrose sensitivity, resistance to kanamycin and chloramphenicol	Dr. Nicholas P. Cianciotto
pBluescript II KS (-)	High-copy cloning vector conferring resistance to ampicillin	Stratagene
pBluescript *bdhA::aphA3*	pBluescript II KS (-) containing *bdhA::aphA3*	This study
pBluescript *bdhA::ermAM*	pBluescript II KS (-) containing *bdhA::ermAM*	This study
pMMB207C	pMMB207 with Δ*mobA* (pMMB207:RSF1010 derivative, IncQ, Cm^R, P*tac*, *oriT*)	(Chen, 2004)
pBdhA1	pMMB207C harboring parent copy of *bdhA* and 1 upstream hypothetical promoter	This study
pBdhA3	pMMB207C harboring parent copy of *bdhA* and 3 upstream hypothetical promoter	This study
pBdhA4	pMMB207C harboring parent copy of *bdhA* and 4 upstream hypothetical promoter	This study

Cm^R indicate resistance marker to chloramphenicol.

2.13.4. Isolation of Genomic DNA from *L. pneumophila*

Genomic DNA was prepared from 10 ml *L. pneumophila* cultures. Cell pellets were resuspended in 350 µl of the supernatant fluid and a 100 µl aliquot of the resulting cell suspension was mixed with 600 µl of TE buffer, followed by the addition of 6 µl of pre-warmed 10% (w/v) SDS. The samples were mixed by inverting the tube several times, and then 4 µl of RNase A was added prior to incubation at 37^{o}C for 30 min. Next, 8 µl of Proteinase K stock (20 mg/ml, Fermentas) was added and the tubes were incubated for an additional 45 min at 50-55^{o}C. The DNA was then cleaned by phenol chloroform extraction by mixing with 350 µl of phenol and 350 µl of chloroform, followed by centrifugation for 3 min at 13000 x *g*. The resulting aqueous layer was transferred to a new tube, and the process was repeated 2 -3 times to ensure removal of proteins. This was followed by the addition of 350 µl of chloroform, mixing, centrifugation for 3 min at 13000 x *g*, and removal of the top aqueous layer into a new tube. One-tenth volume of 5 M NaCl and two volumes of cold 95% (v/v) ethanol were added. The DNA was precipitated at -20^{o}C for 30 min, and centrifuged for 10 min at 13000 x *g*. The DNA pellet was rinsed with 100 µl 70% (v/v) ethanol, air dried for 5-10 min, and resuspended in 100 µl sterile dd-water and incubated at 4^{o}C overnight to allow solubilization.

2.13.5. Quantification of DNA of Plasmids and PCR Products

The quantity of DNA was estimated by comparison to DNA ladder standards (NEB) with known molecular weights for each band in the ladder. Briefly, 1 µl of 1:20, 1:50, and 1:100 dilutions of the genomic DNA and 10 µl of a standard 1 kb DNA ladder were electrophoresed on a 0.8% agarose gel. The intensity of the DNA bands was analysed using Image J software (NIH, http://rsb.info.nih.gov/ij).

2.13.6. Polymerase Chain Reaction (PCR)

Typical cycling conditions used for PCR with *Taq* DNA polymerase (NEB) were: Step 1- 95^{o}C for 5 min, Step 2- 94^{o}C for 30 s, Step 3- the appropriate annealing temperature of the primers for 30 s, Step 4- 72^{o}C for 1 min per 1 kb of amplicon, Step 5- repeat steps 2 to 4 for a total of 30 cycles, Step 6- 72^{o}C for 5 min.

PCR using *Taq* DNA polymerase was carried out with the buffer provided by the supplier (NEB), according to the manufacturer's directions. Each PCR reaction contained 10 µl 1 X PCR ThermoPol buffer (NEB) plus 1.5 µl of 10 mM dNTP (Invitrogen), 5 µl of each primer (15 pmoles/µl), 2.5 U of *Taq* DNA polymerase (NEB), 1 µl of template DNA and the appropriate volume of dd-water required to bring the reaction volume to 100 µl.

PCR using Phusion DNA polymerase (Finnzymes) was used to amplify the kanamycin resistant cassette from pDL276 using the buffer and conditions recommended by the supplier. Each reaction contained 10 µl of 5 X Phusion HF buffer, 1 µl of 10 mM dNTP (Invitrogen), 2.5 µl of each primer (100 ng/µl), 0.6 U of Phusion DNA polymerase, 1 µl of template DNA and the appropriate volume of dd-water required to bring the reaction volume to 50 µl. Phusion DNA polymerase was used to produce PCR products with blunt ends.

Typical conditions used for PCR amplification using Phusion DNA polymerase were: Step 1- 98^{o}C for 30 s, Step 2- 98^{o}C for 5-10 s, Step 3- the appropriate annealing temperature for 10-30 s, Step 4- 72^{o}C for 20 s per 1 kb of amplicon, Step 5- repeat steps 2 to 4 for a total of 35 cycles, Sep 6- 72^{o}C for 5-10 min.

Colony PCR screening of *L. pneumophila* transformants was performed using *Taq* DNA polymerase. Briefly, individual colonies were picked using sterile glass rods into 50 ul volumes of TE buffer. One µl of the cell suspension was used as template for PCR. Alternatively, DNA was prepared from a single bacterial colony by putting the colony into an 1.5 ml tube containing 100 µl of TE buffer, 100 µl chloroform, and 100 µg glass beads (VWR beads silica molbio 400M, Radnor, PA), vortexed for 1 min then centrifuged at 14000 x *g* at 4^{o}C for 5 min. The DNA was precipitated with 95% (v/v) ethanol containing 2.5% KAc, washed with 70% (v/v) ethanol, vacuum dried, and resuspended in 15 µl of TE buffer. One µl of the DNA was used as the template for PCR.

The PCR primers used throughout this study are listed in Table 2.4. Oligonucleotides used as 'primers' in PCR were purchased from either Integrated DNA Technologies, Inc. (IDT) (Coralville, IA) or Alpha DNA (Montreal, QC). The melting temperatures (Tm) of primers were designed to be between 50-60°C. When the amplified DNA fragment was to be cloned, restriction enzyme sites were added to the 5' ends of the primers and were themselves preceded at their 5' end by 4 Cs or TAC. This was done to maximize the efficiency of restriction enzyme digestion.

Table 2.4. Primers used in this study

Primer	Sequence (5' to 3')	Target
SL609	TGAgatatcCCGGGCCCAAAACCCGTTTGA (EcoRV)	Forward primer for erythromycin cassette
SL729	TACggatccAGCGACTCATAGAATTATTT (BamHI)	Reverse primer for erythromycin cassette
SL801	TACggatccGCAAGGAACAGTGAATTGGA (BamHI)	Forward primer for kanamycin cassette
SL823	TACGGTACCCAGTTGCGGATGTACTTCAG	Reverse primer for kanamycin cassette
SL867	CTCTGGCTTCAAGTTTTGTT	Forward primer to amplify *sgo1171::ermAM* fragment
SL870	ACAACTATCAAGCTATGTACT	Reverse primer to amplify *sgo1171::ermAM* fragment
SL960	CCCCgcggccgcCAAGTTGGAACAGCAGTTGG (NotI)	Forward primer for *bdhA* upstream region
SL961	CCCCgatatcACATCAACCCCTCCAAAGTG (EcoRV)	Reverse primer for *bdhA* upstream region
SL962	CCCCggatccTGAATCGAATGCGCTTACAG (BamHI)	Forward primer for *bdhA* downstream region
SL963	CCCCctcgagTGATATGCCACCATCCCAGT (XhoI)	Reverse primer for *bdhA* downstream region
3hbd-Q F	TGTCCGTACTCCTCTCGTTG	Forward primer for *bdhA*
3hbd-Q R	ACATCGTCTGTGGTGGTGAA	Reverse primer for *bdhA*
3hbdF	AAAGAAGGGGCAAAGGTAGC	Forward primer for *bdhA* used to study its expression in the VBNC state
3hbdR	GAATACCGGCATTGCTGACT	Reverse primer for *bdhA* used to study its expression in the VBNC state

Table 2.4. Continuation

Primer	Sequence (5' to 3')	Target
HspC2F	CCTGGTATGGGTGAAGAAGA	Forward primer for *hspC2* used to study its expression in the VBNC state
HspC2R	AGGCAGTCGCTTTATCCAC	Reverse primer for *hspC2* used to study its expression in the VBNC state
Lpg0634F	TTCCTGCAAGTCCTTTCGTT	Forward primer for *lpg0634* used to study its expression in the VBNC state
Lpg0634R	TCCCAGGATTTGAATTGCTC	Reverse primer for *lpg0634* used to study its expression in the VBNC state
SL956	CCCCgcggccgcACTGATTATAGGCGG CTTGC (NotI)	Forward primer for *rpoS* upstream portion
SL957	CCCCgatatcTGGCTCAGACCATTCCT CTT (EcoRV)	Reverse primer for *rpoS* upstream portion
SL959	CCCCctcgagTGGCAGCGAGCATACT ATAA (XhoI)	Reverse primer for *rpoS* downstream portion
RpoS-Q F	TTTATCTCAGAGCGGCAAGG	Forward primer for *rpoS*
RpoS-Q R	CTCCAAAGGCTTATCCACCA	Reverse primer for *rpoS*
SL971	GTTCCCACCTCAATGGATGA	Forward primer for Transmembrane gene
SL972	AATTCCGTTTCATCGTGCTC	Reverse primer for Transmembrane gene
SL982	TGCTTGCCATTCATTTGG	Forward primer for *bdhA* internal region (280 bp)

Table 2.4. Continuation

Primer	Sequence (5' to 3')	Target
SL983	TTCTTTTGCTTGCTCTGGAA	Reverse primer for *bdhA* internal region (280 bp)
SL1004	TACggatccTTCCTTAATCATTTTAGC GATCA (BamHI)	Forward primer for *bdhA::aphA3* complementation including 3 candidate promoters upstream of start codon
SL1018	TACggatccACAAAAGGAAGTTTACT ATGAAAAAGA (BamHI)	Forward primer for *bdhA::aphA3* complementation including 4 candidate promoters upstream of start codon
SL1019	ATTACCTCCCTTggatccAACTCTCT (BamHI)	Forward primer for *bdhA::aphA3* complementation including 1 candidate promoters upstream of start codon
SL1005	TACctgcagTCGAGTCATGGTTGTTTA CTCC (PstI)	Reverse primer for *bdhA::aphA3* complementation
Tn*10* up	CAGGAATCGAATGCAACCGGC	Forward primer for mini-Tn*10*
Tn*10* down	CAAAGCCGCCGTCCCGTCAAG	Reverse primer for mini-Tn*10*

Lower case sequences indicate the restriction site named in parentheses.

For cloning, the PCR products were extracted with chloroform prior to restriction endonuclease digestion.

When the PCR product was to be used for electroporating competent *L. pneumophila* cells, the PCR product was precipitated with 95 % (v/v) ethanol containing 2.5 % (w/v) KAc. The pellet was washed 3 times with 70 % (v/v) ethanol and dissolved in dd-water.

2.13.7. Preparation of Competent *E. coli* Cells

LB medium (2 ml) containing 10 µg/ml tetracycline was inoculated with 10 µl frozen *E. coli* XL1-Blue stock and incubated overnight at 37°C with shaking at 200 rpm. After that, LB (45 ml) containing 10 µg/ml tetracycline was inoculated with 1 ml of overnight culture and incubated until an OD_{600} ~ 0.35 was reached. The culture was centrifuged at 10000 x *g* for 10 min at 4°C and the supernatant was removed. The cell pellet was washed with 50 ml cold transformation buffer 1 (Tfm1: 10 mM Tris and 150 mM NaCl; pH 7.5) and centrifuged at 10000 x *g* for 10 min. The supernatant fluid was removed and the cells were resuspended in 50 ml of transformation buffer 2 (Tfm 2: 50 mM $CaCl_2$), followed by a 45 min incubation on ice. The cell suspension was centrifuged again and resuspended in 3 ml of Tfm2 and 2 ml of glycerol. Aliquots were stored at -80°C.

For the preparation of electro-competent *E. coli* DH5α cells, 50 ml of LB broth was inoculated with 500 µl of an overnight culture and incubated for 2-3 h. The culture was centrifuged at 4500 x *g* for 10 min at 4°C and the pellet was washed with 40 ml of 10 % cold glycerol. This was followed by centrifugation and the glycerol was discarded. Twenty millilitres of 10 % cold glycerol was then added and centrifuged. The pellet was then washed in 1.5 ml of 10 % cold glycerol and centrifuged at 6000 x *g* for 1 min at 4°C. Finally, 120 µl of 10 % cold glycerol was added to the pellet, divided into aliquots of 40 µl and stored at -80°C.

2.13.8. Transformation of *E. coli*

In an Eppendorf tube on ice, 200 µl of *E. coli* XL1-Blue competent cells were mixed with 100 µl of transformation buffer 3 (tfm3: 10 mM Tris, 50 mM $CaCl_2$, and 10 mM $MgSO_4$, pH 7.5) and 10 µl of ligated DNA and incubated on ice for 45 min. The cells were then heat shocked at 37°C for 2 min, followed by incubation at 25°C for 10 min. LB (500 µl)

was added to the cell suspension and incubated at 37°C for 1 h. The cells (200 µl) were then plated on selective LB agar containing the appropriate antibiotics.

For transformation of electro-competent *E. coli* DH5α cells, 5 µl of plasmid DNA in dd-water (at ~ 1 µg/µl) was added to 40 µl of thawed electro-competent *E. coli* DH5α cells and incubated on ice for 10 min. The DNA/cell mixture was transferred to a pre-chilled 1 mm gap electroporation cuvette (BioRad). The electroporation conditions were 2.4 kV, 25 µF and 400 OHMS (4.3 milliseconds (ms) was the typical time constant). The cells were then transferred to 750 µl of pre-warmed LB broth and incubated at 37°C for 1 h with shaking at 200 rpm. Different volumes of the cells (100 µl, 200 µl, and 495 µl) were then plated onto LB agar with the appropriate selection.

2.13.9. Preparation of Electrocompetent *L. pneumophila* Cells

One colony of *L. pneumophila* strain JR32 was suspended in 500 µl sterile dd-water, spread on BCYE agar, and incubated overnight to form a lawn. The lawn of *L. pneumophila* was harvested (by pipetting) into 20 ml of sterile dd-water and centrifuged at 4500 x *g* for 10 min at 4°C. The cells were resuspended in 50 ml of cold 10% glycerol, centrifuged at 4500 x *g* for 10 min at 4°C and the pellets were washed again with 50 ml of cold 10% glycerol, and finally resuspended in 500 µl of cold 10% glycerol. The cells were stored at -80°C in 40 µl aliquots.

2.13.10. Electroporation of *L. pneumophila*

Electrocompetent *L. pneumophila* were thawed on ice. Five microliters of plasmid or constructed linear DNA fragments (at ~ 1 µg/µl) were added to the thawed cells and incubated on ice for 10 min. The DNA/cell mixture was transferred to a pre-chilled 1 mm gap electroporation cuvette. The electroporation conditions were 2.4 kV, 25 µF and 400 OHMS (4.3 ms was the time constant). The cells were then transferred to 750 µl of pre-warmed BYE and incubated at 37°C for 2 h at 100 rpm. The cells were then plated onto BCYE agar with the appropriate selection. The plates were incubated at 37°C and 5% CO_2 for 5-7 days. When needed, colonies were replica-plated and screened by colony PCR, as described above.

2.13.11. Construction of Mini-Tn*10* Insertion Library in *L. pneumophila*

A diagram illustrating the approach used in the construction of a mini-Tn*10* mutant library is shown in Fig.2.1. After electroporation of competent *L. pneumophila* JR32 with pCDP05 and incubation in BYE, 100 µl of the culture was plated on BCYE agar containing streptomycin, kanamycin, and sucrose. The plates were incubated for 5-7 days at 37^{o}C and 5% CO_2, and individual transformants were transferred into 96-well plates containing BYE with streptomycin and kanamycin and incubated overnight.

To identify transposon mutants that exhibited changes in culturability, and to confirm the absence of the carrier plasmid, the transformants were replica-plated into three sets of plates: (i) 96-well plates containing monolayers of *A. castellanii* in sterile tap water (200 µl/well), (ii) BCYE plates containing streptomycin and chloramphenicol, and (iii) BCYE plates with streptomycin, kanamycin, and sucrose. The plates containing the infected *A. castellanii* were incubated at 37^{o}C for 5 days, and each well was subsequently replica-plated onto BCYE agar containing streptomycin, kanamycin, and sucrose. This was considered as time 0 for the assessment of the loss of culturability. In parallel, 50 µl from each well of the plates containing the infected *A. castellanii* was transferred into new 96-well plates containing 150 µl/well of sterilized dd-water. The plates were sealed with sterile adhesive sealing films (Excel Scientific Inc.) and incubated in humidity at 45^{o}C. The culturability was checked regularly by replica-plating on BCYE plates containing streptomycin, kanamycin, and sucrose. Transformants that exhibited a difference in the extent of culturability compared to the parent strain were selected for analysis of the production of VBNC cells, as described above.

The replica-plating on (ii) and (iii) was to confirm the absence of the carrier plasmid pCDP05 and the insertion of the mini-Tn*10* into *L. pneumophila* genome. Further confirmation of the insertion of the mini-Tn*10* was performed by PCR amplification of the mini-Tn*10* using (Tn*10* up/Tn*10* down) primers.

2.13.12. Construction of *L. pneumophila bdhA::aphA3* Mutant (*bdhA* Mutant) Strain

Two strategies were used in an attempt to mutate the *bdhA* gene of JR32. Initially, we attempted to inactivate the *bdhA* gene by inserting the erythromycin resistance cassette, *ermAM,* into the *bdhA* open reading frame. To do this, pBluescript *bdhA::ermAM* was

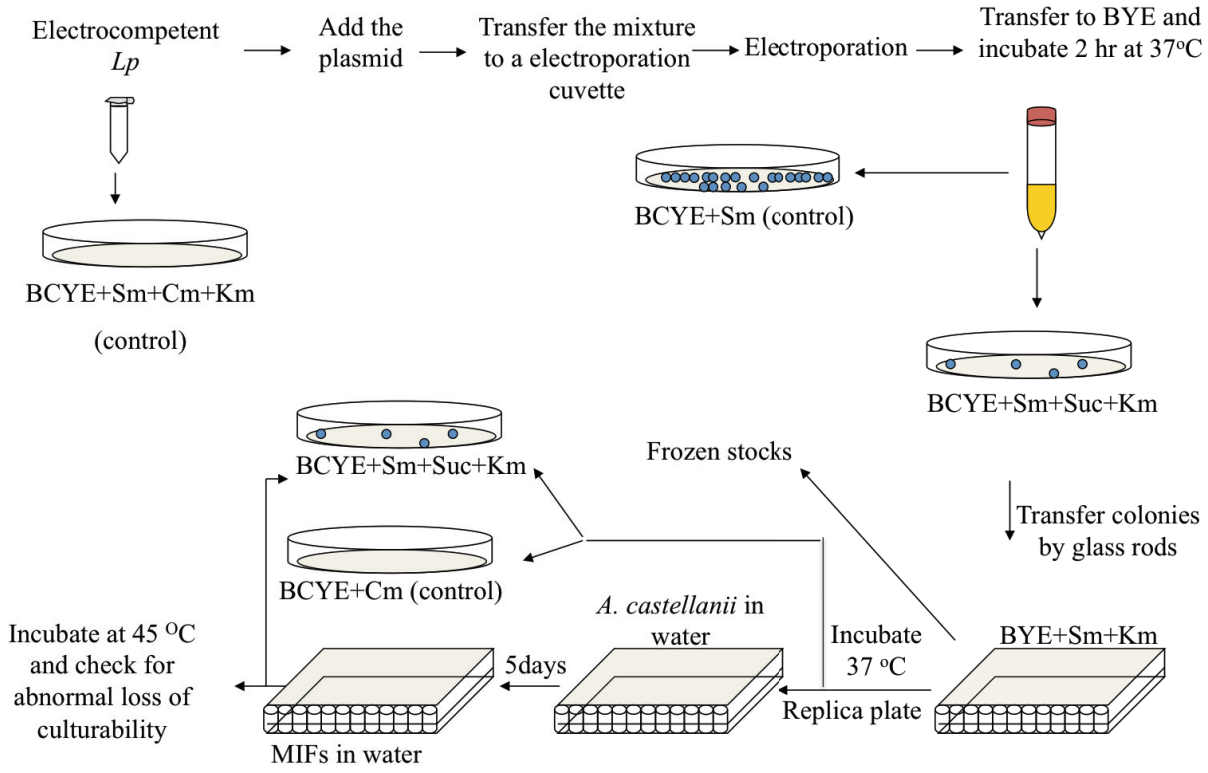


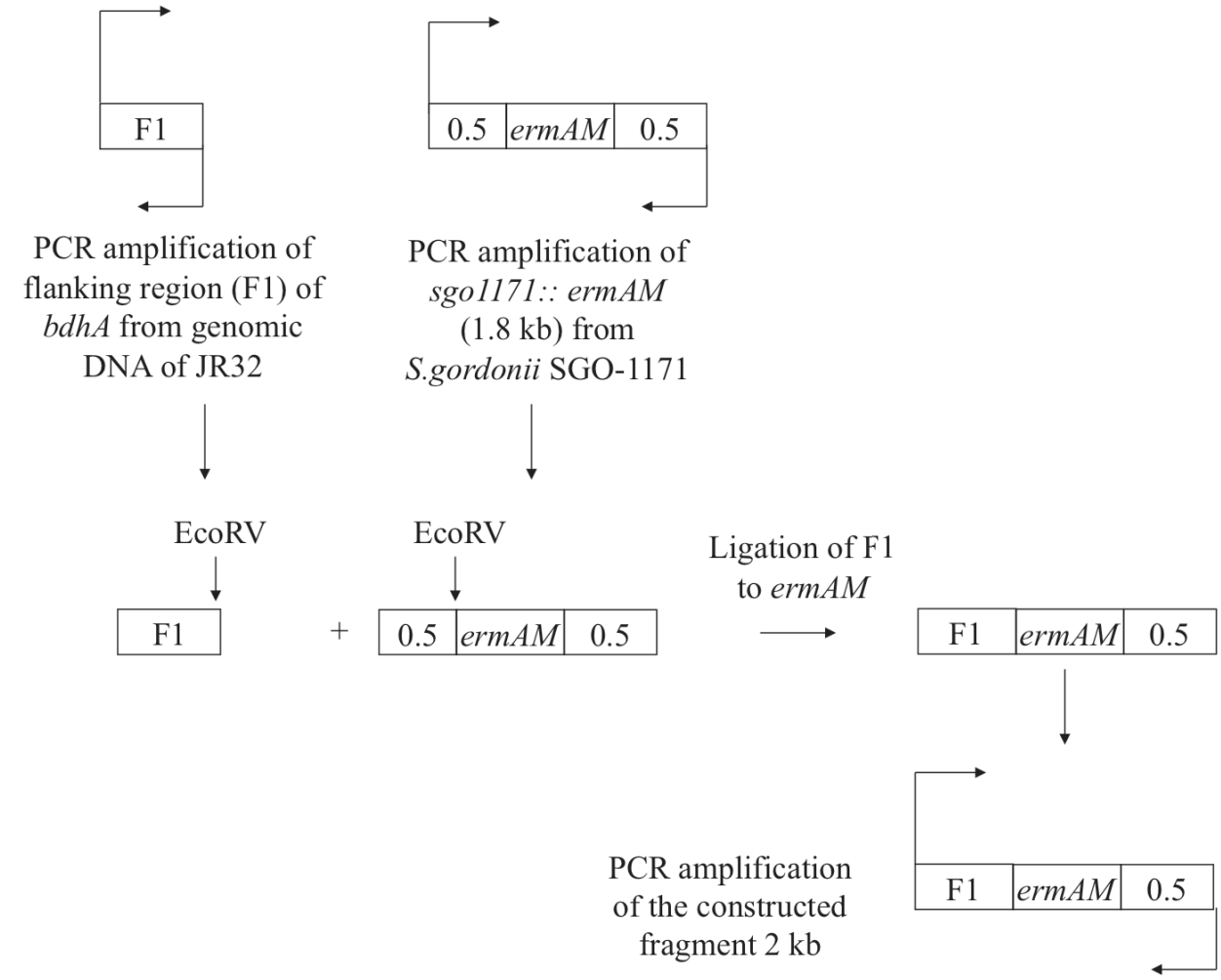
Fig. 2.1. Diagram illustrating the method used to create a mini-Tn*10* insertion library in *L. pneumophila.*

constructed in two steps. The first step was to construct the *bdhA* upstream-*ermAM* fragment. An upstream (F1) (~700 bp) fragment of *bdhA* was amplified by PCR from *L. pneumophila* JR32 chromosomal DNA with the primer pair SL960/SL961. A 1.8 kb fragment of *sgo1171::ermAM* was PCR amplified from *Streptococcus gordonii* SGO-1171 (Lee, unpublished) using the primer pair SL867/SL870. The *bdhA* upstream and *sgo1171::ermAM* PCR products were digested with EcoRV (Fermentas). The digested DNA was electrophoresed on a 0.8% agarose gel, and the 700 bp *bdhA* upstream and the 1.3 kb *ermAM* fragments were excised from the gel and ligated using T4 DNA ligase (NEB). The ligation mixture contained 8.5 µl DNA, 1 µl 10 X T4 DNA ligase buffer, and 0.5 µl T4 DNA ligase (400 U, NEB). The ligation mixtures were incubated overnight at 25^{o}C. One microlitre of the ligation mixture was used as the template for PCR amplification of the ligated *bdhA* upstream-*ermAM* fragment using the primers SL960/SL870, yielding a 2 kb fragment (Fig. 2.2A).

The second step was to construct *bdhA* downstream-pBluescript. The downstream (F2) (~700 bp) fragment of *bdhA* was amplified by PCR from *L. pneumophila* JR32 chromosomal DNA with the primer pair SL962/SL963. The *bdhA* downstream fragment and pBluescript were both digested with BamHI and XhoI (Invitrogen). The digested DNA was electrophoresed on a 0.8% agarose gel, and the 700 bp *bdhA* downstream and 3 kb pBluescript fragments were excised from the gel and ligated using T4 DNA ligase (Fig. 2.2B). *E. coli* XL1-Blue was transformed with the ligation mixture and plated on LB agar containing tetracycline, ampicillin, X-gal (200 µl of 10 mg/ml), and IPTG (4 µl of 0.1 M), and incubated overnight at 37°C. White colonies were picked up and grown on LB agar containing ampicillin.

Finally, the resulting 3.7 kb plasmid was ligated to the *bdhA* upstream-*ermAM* construct. The plasmid DNA and *bdhA* upstream-*ermAM* fragment were both digested with BamHI (Invitrogen) and NotI (NEB). The digested DNA was electrophoresed on a 0.8% agarose gel. The 1.5 kb *bdhA* upstream-*ermAM* fragment and 3.7 kb constructed plasmid were excised from the gel and ligated using T4 DNA ligase (Fig. 2.2B). *E. coli* XL1-Blue was transformed with the ligation mixture and plated on LB agar containing erythromycin and incubated overnight. Transformants were grown on LB broth containing ampicillin. The constructed plasmid (pBluescript *bdhA::ermAM*) was isolated by alkali lysis. Restriction

A


F1
0.5 ermAM 0.5
PCR amplification of flanking region (F1) of bdhA from genomic DNA of JR32
PCR amplification of sgo1171:: ermAM (1.8 kb) from S.gordonii SGO-1171
EcoRV
EcoRV
F1
+
0.5 ermAM 0.5
Ligation of F1 to ermAM
F1 ermAM 0.5
PCR amplification of the constructed fragment 2 kb
F1 ermAM 0.5

Continuation

B

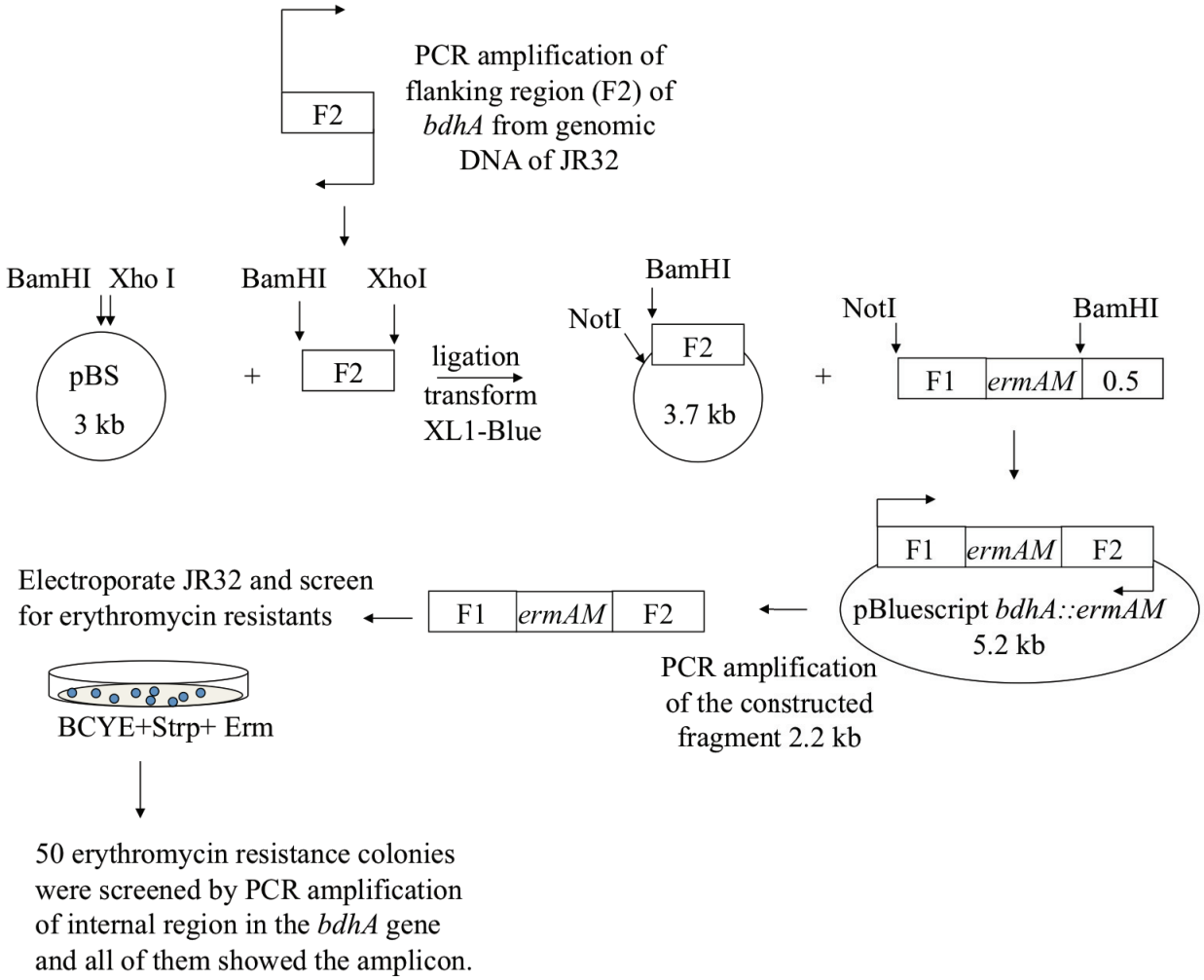


Fig.2.2. Diagram depicting the construction of pBluescript *bdhA*::*ermAM*.

analysis was performed to confirm the correct size (5.2 kb) of the plasmid. PCR amplification of the *bdhA* upstream-*ermAM*-*bdhA* downstream fragment was performed using the primer pair SL960/SL963 and 1 µl of pBluescript *bdhA::ermAM* plasmid as template. The PCR product was precipitated with ethanol and resuspended in 50 µl dd-water and confirmed by restriction analysis (2.2 kb).

The 2.2 kb *bdhA* upstream-*ermAM*-*bdhA* downstream fragment was used to electroporate competent *L. pneumophila* JR32 cells, and the transformants were selected on BCYE agar plates containing erythromycin incubated for up to 6 days at 37^{o}C with 5% CO_2. To confirm the insertion of the *bdhA* upstream-*ermAM*- *bdhA* downstream cassette in *bdhA* gene, chromosomal DNA was isolated from *bdhA* transformants and analyzed by PCR using the SL609/SL729 primer pair, and later SL960/SL729; SL609/SL963; SL960/SL963; SL609/SL972 primer pairs. This was followed by RT-PCR analysis using the primer pairs SL971/SL972 and 3hbd-QF/3hbd-QR.

More transformants were screened by PCR using 3hbd-QF/3hbd-QR and results revealed that *bdhA* gene was still intact. Thus, the method to mutate the *bdhA* gene by inserting the *ermAM* cassette failed to produce a *bdhA* mutant. In light of this, a decision was made to substitute the erythromycin resistance cassette, *ermAM,* with a kanamycin resistance cassette, *aphA3*, in the mutant construction.

The 1 kb kanamycin resistance cassette *aphA3* was amplified from pDL276 plasmid DNA (Dunny *et al*., 1991) using the primer pair SL801/SL823 and then chloroform-extracted. To construct pBluescript *bdhA::aphA3*, *ermAM* was excised from pBluescript *bdhA::ermAM* by digestion with EcoRV and BamHI, while *aphA3* was digested with BamHI. The digested DNA was electrophoresed on a 0.8% agarose gel. The 1 kb *aphA3* fragment and 4.4 kb digested plasmid were excised from the gel and ligated using T4 DNA ligase, yielding pBluescript *bdhA::aphA3* (Fig. 2.3). *E. coli* XL1-Blue was transformed with pBluescript *bdhA::aphA3*, plated on LB agar containing kanamycin, and incubated overnight at 37°C. Transformants were grown in LB broth containing kanamycin, and pBluescript *bdhA::aphA3* was isolated by alkali lysis. Restriction analysis was performed to confirm the correct size (5.4 kb) of the plasmid. PCR amplification of the *bdhA* upstream-*aphA3*-*bdhA* downstream fragment was performed using the primer pair SL960/SL963 and 1 µl pBluescript *bdhA::aphA3* as a template.

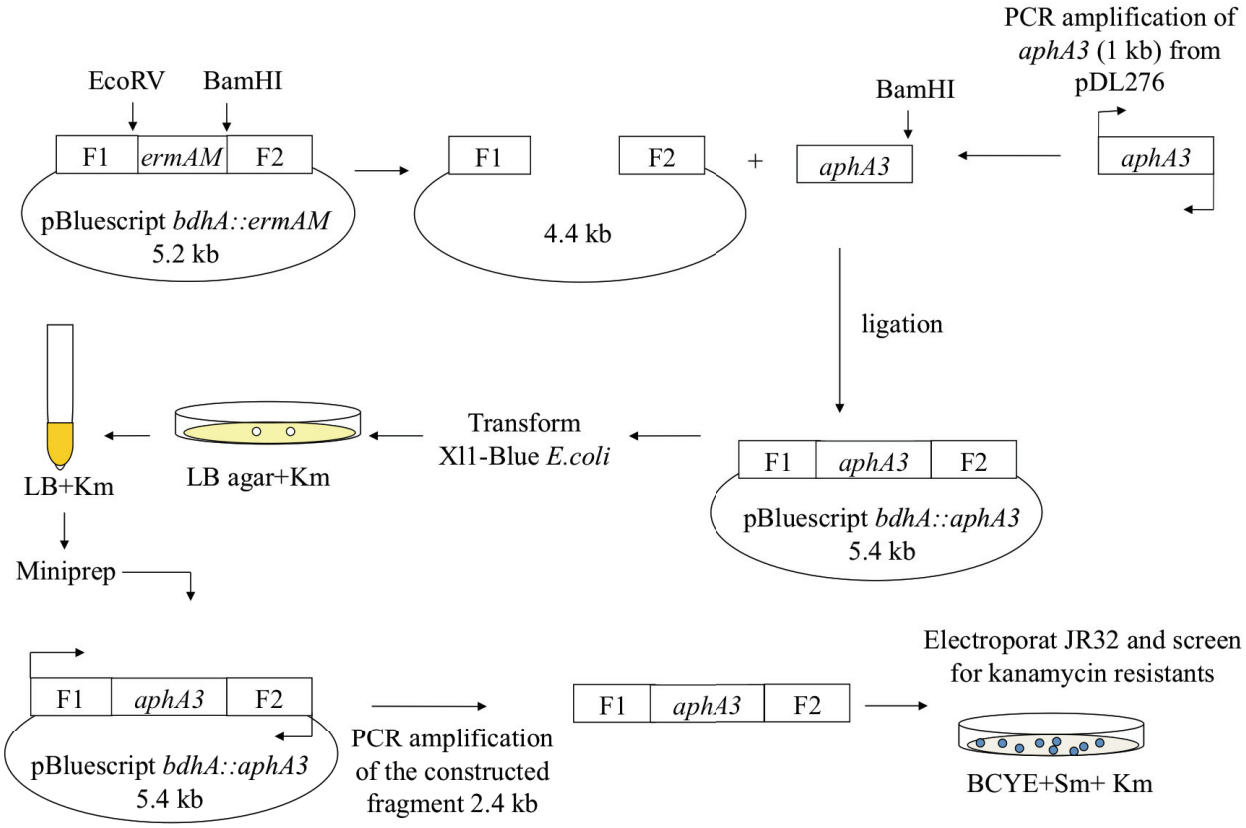


Fig.2.3. Construction of pBluescript *bdhA::aphA3*. pBluescript *bdhA::aphA3* was constructed by replacing the *ermAM* with kanamycin resistance cassette *aphA3*.

The 2.4 kb *bdhA* upstream-*aphA3*-*bdhA* downstream fragment was used to electroporate competent *L. pneumophila* JR32 cells. The transformants were plated on BCYE agar plates containing kanamycin and the plates were incubated for up to 6 days at 37^{o}C with 5% CO_2. Chromosomal DNA was isolated from the transformants and analyzed by PCR to confirm the insertion of *aphA3* in the *bdhA* gene using different primer pairs (3hbd-QF/3hbd-QR, SL801/SL823, SL801/SL960, SL960/3hbd-QR, 3hbd- QF/SL972, SL823/SL972, SL956/RpoS-QR, SL982/SL983, RpoS-QF/RpoS-QR, SL960/SL963, SL823/SL963, SL960/SL983, and SL982/SL972). RNA was isolated from the mutant to confirm absence of *bdhA* transcript by RT-PCR using the following primer pairs: SL965/SL959, SL965/SL957, SL972/SL972, and SL982/3hbd-QR.

2.13.13. Complementation of The *L. pneumophila bdhA* Mutant

The *bdhA* gene was analyzed for candidate promoter(s) using the Neural Network Promoter Prediction software (http://www.fruitfly.org/seq_tools/promoter.html). The analysis showed four putative promoters. The *bdhA* gene with the closest candidate promoter (34 bp upstream from start codon) was amplified using the primer pair SL1019/SL1005, with its three candidate promoters (the farthest one was 168 bp upstream from start codon) amplified using SL1004/SL1005 primers or with its four candidate promoters (the farthest one was 461 bp upstream from start codon) amplified using SL1018/SL1005 primers. The amplified products were digested with PstI and BamHI and cloned into pMMB207C, yielding pBdhA1, pBdhA3, and pBdhA4, respectively, and transformed into *E.coli* XL1-Blue. Transformants were verified to carry the recombinant plasmids by alkali lysis and restriction analysis. The transformants were further analyzed by PCR amplification of the cloned fragment using SL1019/3hbd-Q R, SL1004/3hbd-Q R, and SL1018/3hbd-Q R primer pairs.

Competent *L. pneumophila bdhA* mutant cells were electroporated with the recombinant plasmids and selected on BCYE containing kanamycin, streptomycin, and chloramphenicol. Transformants were then grown in BYE plus with kanamycin, streptomycin, and chloramphenicol, and verified to carry the recombinant plasmids by alkali lysis and restriction analysis. The transformants were further analyzed by PCR amplification of the cloned fragment using SL1019/3hbd-Q R, SL1004/3hbd-Q R, and SL1018/3hbd-Q R

primer pairs. Hence, the Comp 1P strain represents a *bdhA* mutant complemented with pMMB207C harboring the parent copy of *bdhA* and one upstream hypothetical promoter (pBdhA1), the Comp 3P strain was complemented with pMMB207C harboring the parent copy of *bdhA* and 3 upstream hypothetical promoters (pBdhA3), and the Comp 4P was complemented with pMMB207C harboring the parent copy of *bdhA* and 4 upstream hypothetical promoters (pBdhA4). As a control for any potential effects of the presence of pMMB207C, parent and *bdhA* mutant were electroporated with the empty plasmid pMMB207C, yielding parent207C and *bdhA207C* strains respectively.

2.13.14. RNA

2.13.14.1. Isolation

Total RNA was obtained by the Trizol method described by Milohanic *et al.* (2003), with modifications. Cells from 6 ml of stationary phase cultures were resuspended in 400 µl suspension solution (1 part 20% glucose, 1 part 25 mM Tris) and 60 µl 0.5 M EDTA. Aliqouts of 500 µl of citrate-buffered phenol and 400 mg glass beads were added to a 2 ml screw-cap tube containing the cell suspension. The cell suspension was shaken in a FastPrep machine (FastPrep FP120, Thermo Scientfic, Milford, MA) at speed 6 for 30 s, followed by 1 min on ice. The suspension was then centrifuged for 5 min at 10000 x *g* at 4^{o}C. The supernatant was transferred to a sterile 1.5 ml tube and 1 ml Trizol (Invitrogen) was added and mixed gently. The mixture was incubated for 5-10 min at 25^{o}C with inversions at regular intervals. Chloroform (100 µl) was then added, mixed gently, and incubated for 3 min at 25^{o}C. The mixture was centrifuged and the aqueous phase was transferred into a sterile 1.5 ml tube. Chloroform (200 µl) was added, mixed, and incubated for 5 min at 25^{o}C. The mixture was centrifuged again and the aqueous phase was saved. The RNA was precipitated with 500 µl of isopropanol and washed with 1 ml cold 70% (v/v) ethanol. The RNA was dissolved in 50 µl of 0.1% DEPC (diethylpyrocarbonate)-treated dd-water.

2.13.14.2. RNA Quantification and DNase Treatment

The concentration of RNA in each sample was determined by spectrophotometry. The sample (5 µl) was diluted in 1 ml of dd-water and absorbance was read at 260 nm and at 280 nm. RNA concentration (µg/ml) was calculated using the following formula: OD_{260} x dilution

factor x 40 µg/ml. To remove DNA, RNA (1 µg) was mixed with 1 µl of Amplification Grade DNase I (1 U/µl, Fermentas), 1 µl of 10 X DNase I reaction buffer and DEPC treated dd-water to 10 µl, followed by incubation at 25^{o}C for 15 min. EDTA (1 µl of 25 mM; pH 8) was added to each sample and incubated at 65^{o}C for 10 min. To verify that the RNA was free of DNA, aliquots of the DNase I treated RNA (1 µl) were tested by PCR using primers specific for the control gene (*rpoS*). The reaction mixture contained 1 µl RNA sample, 5 µl 10 X ThermoPol buffer, 2.5 µl of each SL956 and SL959 primers, 1 µM dNTP mixture, 2.5 U *Taq* DNA polymerase, and dd-water to 35.5 µl.

2.13.14.3. Complementary DNA Synthesis

Complementary DNA (cDNA) was synthesized from the purified DNA-free RNA using Superscript II reverse transcriptase (200 U/µl, Invitrogen), following the manufacturer's instructions. Random primers 1 µl (3 µg/µl, Invitrogen) and 1 µl of 10 mM dNTPs mixture were mixed with 9 µl of the RNA template and incubated at 65^{o}C for 5 min. The reaction mixture was incubated on ice for 2 min, followed by the addition of 4 µl of 5 X first strand buffer and 2 µl dithiothreitol (0.1 M DTT). The reaction mixture was incubated at 25^{o}C for 2 min. One microlitre of SuperScript II reverse transcriptase was added to the reaction mixture and incubated at 25^{o}C for 10 min, 42^{o}C for 50 min, and 70^{o}C for 15 min. To confirm that cDNA had been synthesized, 1 µl of cDNA and primers specific for the control gene (*rpoS*) were used in a PCR reaction. The PCR product (7 µl) was analyzed by agarose gel electrophoresis.

2.13.14.4. Reverse Transcriptase PCR (RT-PCR)

Amplification of cDNA was performed by standard PCR techniques. To examine the expression of *bdhA*, *hspC2*, and *lp0634* in the VBNC bacteria, PCR was performed using primer pairs 3hbdF/3hbdR for *bdhA*, HspC2F/ HspC2R for *hspC2*, and lpg0634F/ lpg0634R for *lpg0634*.

To confirm that the *bdhA* gene was not expressed in the *bdhA::aphA3* mutant, RT-PCR was performed using the primers SL982/3hbd-QR to amplify the internal 366 bp fragment of *bdhA*. To confirm the lack of any polar effect of the *bdhA::aphA3* mutation on the downstream gene, SL971/SL972 primers were used to amplify a 242 bp fragment of the

transmembrane protein gene (second gene in the *bdhA* operon). The PCR product (7 µl) was analyzed by agarose gel electrophoresis.

2.14. Proteomic Techniques Used with VBNC Cells

2.14.1. Extraction and Quantification of *L. pneumophila* Proteins

Culturable and VBNC *L. pneumophila* JR32 were suspended in 100 µl dd-water and lysed by sonication (10 bursts of 20 s each at an amplitude of 60 with 4 min of cooling between each burst) (Vibracell, Sonics and materials Inc. Danbury, CT, USA). The *L. pneumophila* lysates were diluted 1:10 with dd-water for protein quantification using the commercial Bradford reagents (Bio-Rad laboratories, Ltd., Messisauga, ON) (Bradford, 1976). A standard curve was generated using dilutions of BSA in dd-water ranging from 0.2 to 1.6 mg/ml. The concentrations of the proteins in the samples were calculated using the equation from the BSA standard curve.

2.14.2. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Polyacrylamide gels were prepared using a 5% stacking gel and a 9-17% gradient resolving gel. The 5% stacking gel was prepared per 13.5 ml as follow: 7.76 ml dd-water, 2.26 ml 30% acrylamide:bisacrylamide (29:1), 3.36 ml stacking buffer (1.5 M Tris-HCl ph 6.8, 10% SDS), 62.4 µl of 15% ammonium persulfate (APS), and 26.4 µl of N, N, N', N'-tetramethylethylenediamine (TEMED). The 9% resolving gel contained (per 24 ml): 10.72 ml dd-water, 7.2 ml 30% acrylamide:bisacrylamide (29:1), 6 ml resolving buffer (1.5 M Tris-HCl ph 8.8, 10% SDS), 80 µl of 15% ammonium persulfate (APS), and 12 µl of TEMED. The 17% resolving gel was prepared as follows (per 24 ml): 4.32 ml dd-water, 13.6 ml 30% acrylamide:bisacrylamide (29:1), 6 ml resolving buffer, 80 µl of 15% APS, and 12 µl of TEMED.

Protein samples were prepared for SDS PAGE by mixing with 5 X sample buffer (315 mM Tris-HCl pH 6.8, 50% glycerol, 10% SDS, 25% beta-mercaptoethanl, 0.01% bromophenol blue) at one-fifth of their total volume; SDS was added to a final concentration of 2% of the final volume. The samples were then boiled for 5 min and mixed by vortexing. This was followed by the addition of β-mercaptoethanol to 10% of the total sample volume,

followed by boiling for 5 min and vortexing. The samples were centrifuged at 10000 x *g* for 10 min and the supernatant was loaded onto the gel.

Ten micrograms of the protein samples and 25 µl of protein markers (NEB) (boiled for 5 min) were loaded into the gel wells. Electrophoresis was performed in SDS electrophoresis buffer (15 g/L Tris base, 72 g/L glycine, and 5 g/L SDS) at 15 mA until the dye front passed the stacking gel, after which the current was increased to 30 mA.

2.14.3. Silver Staining of The SDS-PAGE Gels

Silver staining was carried out as follow: i) overnight fixation in 50% (v/v) methanol and 10% (v/v) acetic acid solution; ii) 15 min fixation in 50% (v/v) methanol; iii) five consecutive 5-min washes with sterile dd-water; iv) sensitization for 1 min with 0.02% sodium thiosulfate; v) two washes for 1 min with sterile dd-water; vi) treatment with freshly prepared 0.2% silver nitrate in cold water for 25 min; vii) washing twice with sterile dd-water for 1 min; viii) gel development using 3 % sodium carbonate containing 0.025% formalin (this involved two steps: the developer was decanted when the solution turned yellow and then the second step lasted until desired band intensity was achieved); ix) 1.4% EDTA was used to stop the reaction for 10 min; and, x) the gel was rinsed twice with sterile dd-water for 1 min each. Finally, the gels were preserved in 1% acetic acid. Protein bands were excised in a laminar flow hood using sterile scalpel blades. Protein bands from duplicate lanes were combined and submitted for mass spectrometry (Proteomic core facility at Dalhousie University, Halifax, NS).

2.14.4. Shotgun Proteomic Analysis

Samples containing 500 µg of proteins from culturable and VBNC bacteria were dried by speed vacuuming at 35^{o}C (SPD speed vac, Thermo). Denaturing buffer (200 µl) (8M urea, 400 mM ammonium bicarbonate, 0.1% SDS) was added to each sample, vortexed, and sonicated for 30 min at 25^{o}C in a water bath sonicator (Brannsonic Ultra sonic Bath.). Next, 10 µl of 0.5 M DTT was added to each sample, mixed by pipetting and incubated at 60^{o}C for 30 min. The samples were then left to stand for 5 min at 25^{o}C. Twenty microlitres of 0.7 M iodoacetamide was added, mixed by pipetting, and incubated at 25^{o}C. After 30 min, 10 µl of 0.1 M $CaCl_2$ was added and the samples were diluted 5 fold with dd-water. After dilution, 5

µg of trypsin was added to each sample, after which the sample was vortexed and incubated in a shaker (200 rpm) at 37^{o}C overnight to digest the proteins. The next day, the samples were centrifuged for 15 min at 18000 xg at 4^{o}C and the supernatant was transferred into a new 1.5 ml tube. The pH of the samples was adjusted to < 3 using 99% (v/v) trifluoroacetic acid (TFA).

Solid phase extraction was performed to remove salts from the samples. This was done by passing the samples through a C18 column, which had been conditioned with 3 ml of 100% acetonitrile followed by 3 ml of 50% (v/v) acetonitrile and 0.1% (v/v) TFA and finally by 5 ml of 0.1% (v/v) TFA. Each sample was loaded into a separate column and allowed to flow slowly. The column was then washed with 5 ml of 0.1% (v/v) TFA. Finally, the samples were eluted with 1.2 ml of 50% (v/v) acetonitrile and 0.1% (v/v) TFA, and dried by speed vacuuming overnight. Subsequently, 450 µl of 50 mM triethylammonium bicarbonate buffer (TEAB) (Sigma) was added to each sample and the samples were sonicated for 15 min in a water bath sonicator. Protein samples prepared from culturable *L. pneumophila* were mixed with 8 µl of 37% (v/v) formaldehyde (Sigma), while protein samples from VBNC bacteria were mixed with 14 µl of (20% w/w in D_2O) (v/v) D_2 formaldehyde (Cambridge Isotope Laboratories Inc, USA). Each of the samples was then mixed with 20 µl of 6 M sodium cyanogen borohydrate (Fluka) and vortexed. Four hundred and sixty microliters from each sample was combined into one 1.5 ml tube and vortexed. The pH was adjusted to < 3 with 99% (v/v) TFA. The samples were desalted by solid phase extraction and speed vacuumed as described above. When ionic exchange chromatography was used, 250 µl of 10 mM ammonium formate buffer (buffer A) (NH_4 HCO_2: ACN 10 mM [75:25] pH 3) was added to resuspend the sample, followed by sonication in a water bath sonicator for 20 min. Peptides were eluted from the ionic exchange column (Mono S^{tm} 5/50 GL) with a gradient form from buffers A and B (ammonium formate buffer (NH_4 HCO_2: ACN [75:25] 600 mM pH 3)) into 40 1.5 ml tubes (each containing 1 ml of the eluted peptide). The eluted peptides were detected at 280 nm and dried by speed vacuuming.

Following drying by vacuuming, 40 µl of reconstitution solution (2% ACN and 0.1% formic acid in HPLC H_2O) was added to the samples, mixed by pipetting, and sonicated in a water bath for 20 min. All samples were analyzed by LC-MS/MS (Thermo-Dionex Ultimate 3000 RSLCnano system coupled to a Thermo VelosPRO-Orbitrap tandem mass

spectrometer). One microlitre of each sample was injected into a C18 column (Acclaim PepMAP, RSLC, 75um x 15cm, PN#164534) running a gradient of 3% ACN in 0.1% formic acid to 90% ACN in 0.1% formic acid in 45 minutes at a flow rate of 300 nL/min. Data obtained from an information dependent acquisition (IDA) method was analyzed using SEQUEST algorithm on Proteome Discoverer software package. An NCBI *Legionella* database was used for protein identifications with the peptide confidence filter setting at high.

Multiple Reaction Monitoring (MRM) analysis was used as a method to quantify the proteins of interest. This work was done on an ABSciex Qtrap5500 instrument coupled to a nano chromatographic LC system as described before. MRM is a highly specific and sensitive mass spectrometry technique that can selectively quantify compounds within complex mixtures. This technique uses a triple quadrupole MS that first targets the ion corresponding to the compound of interest with subsequent fragmentation of that target ion to produce a range of daughter ions. One or more of these fragment daughter ions can be selected for quantification purposes. Only compounds that meet both of these criteria (i.e., specific parent ion and specific daughter ions corresponding to the mass of the molecule of interest) are isolated within the mass spectrometer. By ignoring all other ions that flow into the mass spectrometer, the experiment gains sensitivity while maintaining exquisite accuracy.

Twelve proteins from the shotgun proteomic screen were chosen for further analysis using the MRM method in order to accurately quantify their levels in the MIF-VBNCCs compared to the culturable MIFs. Of these twelve proteins, seven (BdhA, acetyl CoA synthetase, RelA, GGDEF/EAL motif protein, EnhA, two small Hspc2 proteins) were chosen from the list of proteins with increased levels, three proteins (D-alanyl-D-alanyl carboxypeptidase, D-alanyl-D-alanyl ligase, and acetyl CoA reductase) were chosen from the list of proteins with decreased levels, and two proteins (pyruvate dehydrogenase and acyl CoA) were chosen from the list of proteins with unchanged levels as controls. Protein samples were processed as described for the shotgun proteomics screen method, but the ionic exchange chromatography step was omitted. Skyline software (https://brendanx-uw1.gs.washington.edu/labkey/wiki/home/software/Skyline/ page.view?name=default) was used to analyze the data.

2.15. 3-Hydroxybutyrate Dehydrogenase Enzyme Assay

The *L. pneumophila* parent, the *bdhA* mutant, and the complemented mutant were grown in 120 ml of BYE broth to the stationary phase (OD_{620} ~ 3). Bacterial cells were centrifuged at 5000 x *g* for 15 min at 4°C and the pellets were washed twice with 15 ml of washing buffer (20 mM Tris-HCl, 1 mM $MgCl_2$; pH 7.8). One gram of cells (wet weight) resuspended in 4 ml of sonication buffer (20 mM Tris-HCl, 1 mM $MgCl_2$, 10% glycerol, 10 mM β-mercaptoethanol; pH 7.8) was disrupted by sonication (10 bursts of 20 s each at an amplitute of 60 with 4 min of cooling between each burst). The sonicated cells were centrifuged at 21000 x *g* for 20 min at 0°C. The supernatant was stored at -80°C.

The protein concentrations in the samples were determined as follows. A standard curve was generated using dilutions of BSA in dd-water ranging from 5 to 100 µg/100 µl. Twenty µl of BSA or protein sample was mixed with 1 ml of a laboratory-made Bradford reagent (Bradford, 1976). The samples were then incubated for 5 min and the absorbance was measured at 595 nm.

For the 3-hydroxybutyrate dehydrogenase enzyme assay, crude lysateswere mixed with 93 mM Tris, 27 mM *D*-β-hydroxybutyrate, and 2 mM β –NAD (Sigma). The lysate was added last to start the enzymatic reaction. The total volume of the reaction was 1.5 ml and the reaction was incubated at 25°C. The amount of increase in A_{340nm}, which represented the increase in the NADH level, was monitored for 40 min. The enzymatic activity (units/mg) was calculated as follow: (ΔA_{340nm} /min test - ΔA_{340nm} /min blank) divided by 6.22 where the 6.22 represent millimolar extension coefficient of β –NAD at 340nm. Bacterial lysates boiled for 5 min was used as a negative control.

2.16. Statistical Analysis

The results from the culturability and viability experiments were analyzed by 2-way ANOVA. The number of inclusion bodies between the parent and *bdhA* mutant were analyzed by the Student's *t* test. A *P* value of ≤ 0·05 was considered to be significant.

CHAPTER 3. RESULTS- CHARACTERIZATION OF VBNC *L. PNEUMOPHILA* CELLS PRODUCED IN WATER

3.1. Production of VBNC Cells from SPFs and MIFs in dd- and Tap Water

The culturability and viability of SPFs and MIFs were monitored in dd-water and tap water at a cell density of 10^8 cfu/ml and a temperature of 45°C (Fig.3.1). Loss of culturability in the entire bacterial population was regarded as the criterion for entrance into the VBNC state. In tap water, both SPFs and MIFs reached the VBNC state in 45 days, albeit with different final mean percentages of viable cells: 20.1 ± 4.3% (n=3) viability for VBNC cells derived from stationary phase forms (SPF-VBNCCs) and 69.3 ± 4.2% (n=3) viability for VBNC cells derived from mature infectious forms (MIF-VBNCCs). In dd-water, SPFs reached the VBNC state in 18 days with a final mean viability of 5.0 ± 0.5% (n=3), whereas MIFs did so in 30 days with a final mean viability of 87.2 ± 10.8% (n=3). Over the sampling period and before complete loss of culturability, a dramatic decrease in the colony forming units for SPF bacteria in dd-water was observed, while the decrease in cfu was gradual for those in tap water. It is noteworthy that once the VBNC state was reached, the viability appeared to remain constant, with an asymptotic trendline (Fig.3.2). Therefore, *L. pneumophila* loses culturability faster in dd-water than in tap water, and MIFs cope better than SPFs with prolonged starvation and high temperature, particularly in the dd-water microcosm.

In response to starvation in tap water at 45°C—and before completely losing their culturability (between days 25 and 32 after inoculation)—SPFs exhibited a unique growth pattern on BCYE plates after being spotted for culturability assessment. Instead of forming isolated single colonies on BCYE that would decrease in number proportionally to the dilution factor, SPFs from tap water produced lawns that covered the entire area of the original inoculum spot. Moreover, the lawns often showed a double-ringed growth pattern, reminiscent of the consolidation rings of swarming bacteria (Fig. 3.3A, arrows). This growth pattern was hypothesized to be the result of surface motility. Using negative stain transmission EM (TEM) of SPFs taken directly from the tap water bottles (Fig. 3.3B) and of bacteria sampled from the BCYE lawns (Fig. 3.3C), bundles of flexible hair-like structures were observed that are compatible with flexible pili and/or cellulose fibers (White *et al.,*

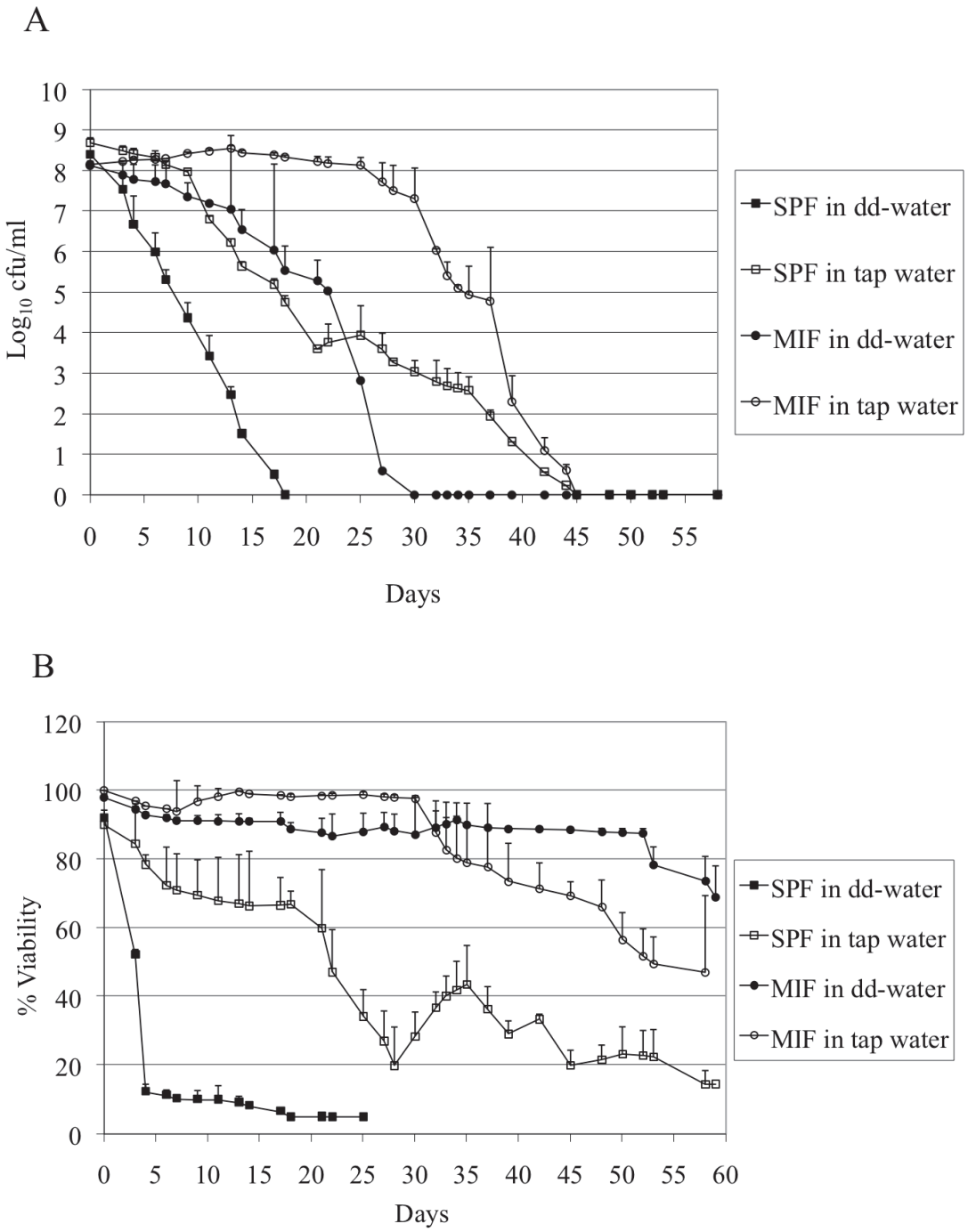


Fig. 3.1.The time taken to lose culturability in dd-water or tap water at 45°C, as well as the viability of VBNC cells, depends on the developmental form used to produce VBNC cells. Panel (A) Loss of culturability of SPFs and MIFs as a function of time in the two water microcosms tested. SPFs and MIFs lose culturability faster in dd-water than in tap water. Panel (B) Percent viability of SPFs and MIFs (as determined by the fluorescent vital stain BacLight) as a function of time. MIFs always showed a much higher viability for MIF-VBNCCs in dd- and tap-water respectivly than SPFs for SPF-VBNCCs in dd- and tap-water, respectively. Results are means ± SD (n=3).

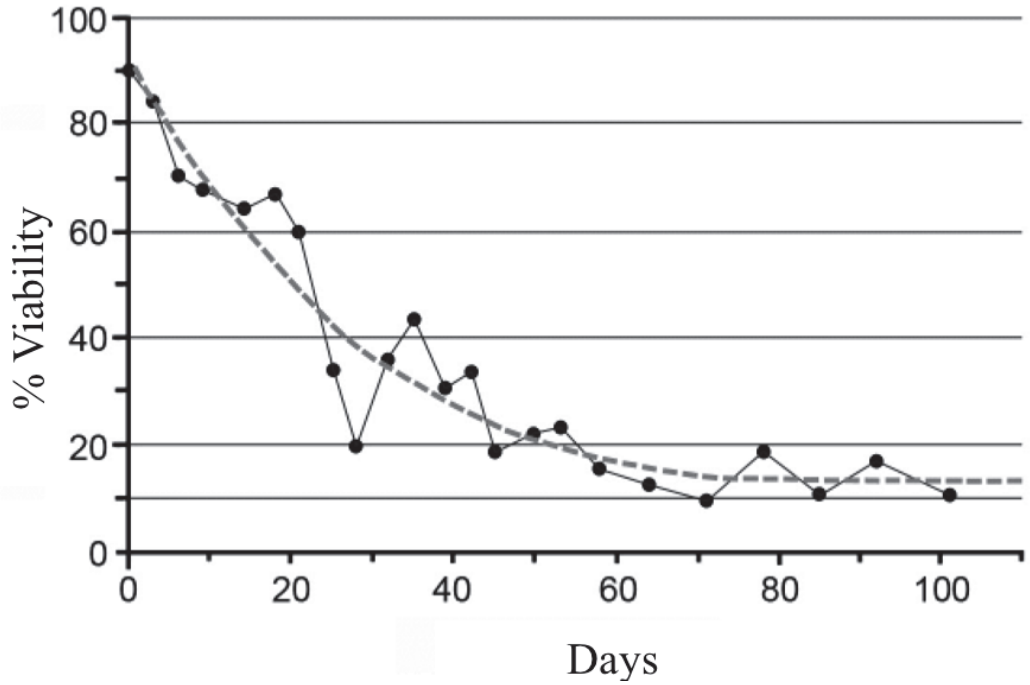


Fig. 3.2. The viability trendline of VBNC bacteria reaches an asymptote. Viability of SPF-VBNCCs in tap water at 45°C was followed for up to 101 days (56 days after SPFs entered the VBNC state) and graphed as % viability vs time. A trendline was drawn (broken gray line) that becomes asymptotic at ~13% viability. Dots show the mean of three independent measures, with error bars omitted for clarity.

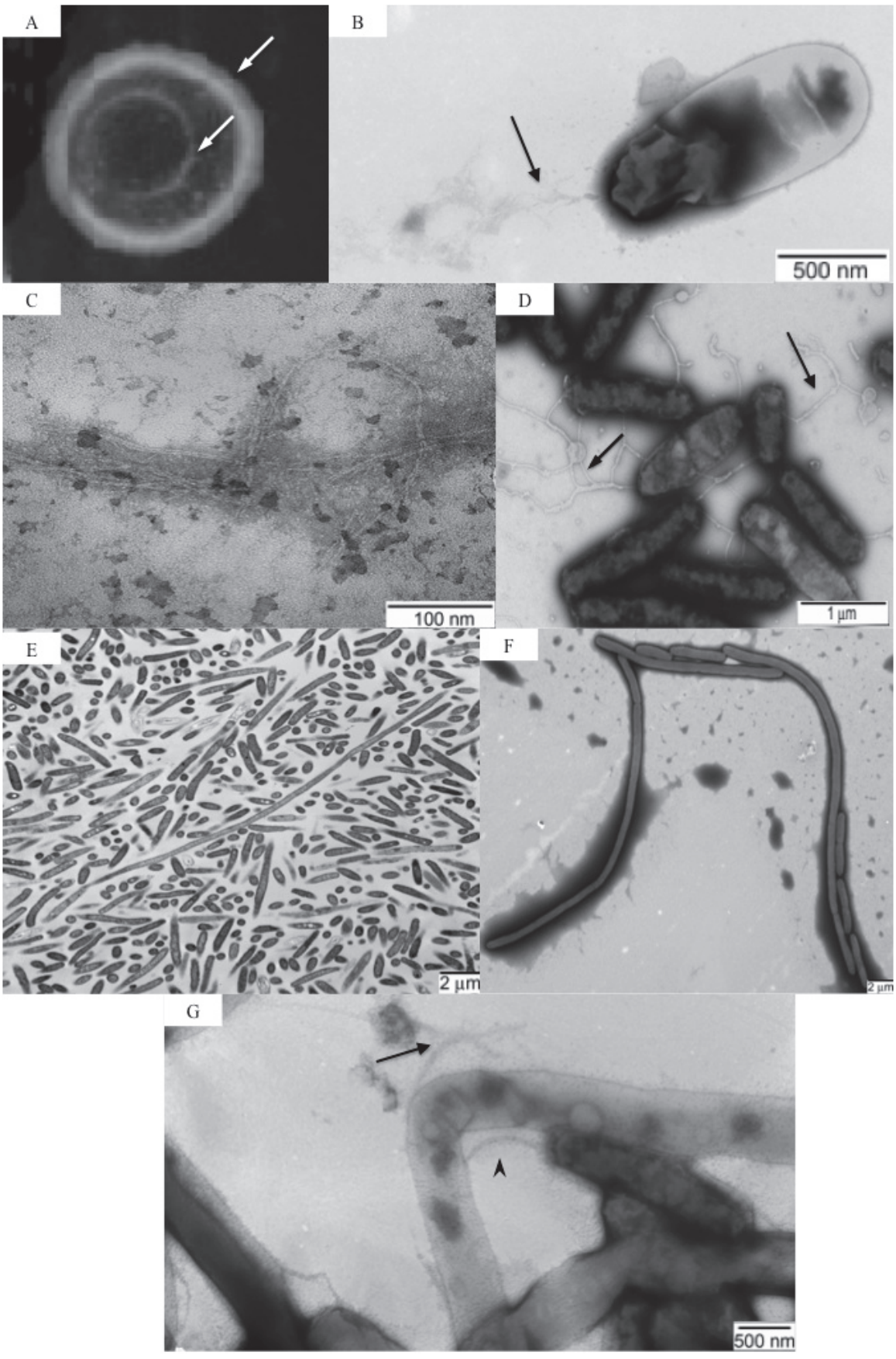

A
B
500 nm
C
100 nm
D
1 µm
E
2 µm
F
2 µm
G
500 nm

Fig. 3.3. Before entering the VBNC state, SPFs express pili and show surface motility on BCYE agar. Panel (A) Photograph showing a representative lawn produced after spotting on BCYE agar a dilution of SPFs. These lawns were produced by SPFs starved in tap water at 45°C at day 25-32. The lawn shown is about 1.7 cm in diameter. White arrows point at the often seen concentric circular zones of higher cell density. Panel (B) Electron micrograph of a SPF in negative stain, from a sample taken directly from a glass bottle containing the suspension of SPFs in tap water at 45°C, at day 31. Arrow points at a bundle of pili. Panel (C) Close-up of a bundle of flexible pili in negative stain TEM, from a sample taken from theouter ring of a lawn produced by SPFs starved in tap water at 45°C for 31 days. Panel (D) Electron micrograph of SPFs in negative stain, grown on BCYE agar. Arrows point at threads of dried hyaline extruded material. Panel (E and F) Filamentous cells in TEM (E) and negative stain TEM (F), from a sample taken from the outer ring of a lawn produced by SPFs starved in tap water at 45°C for 31 days. Panel (G) Negatively stained TEM specimen, showing a mixture of filaments and rods, prepared from a sample taken from the inner ring of a lawn produced by SPFs starved in tap water at 45°C for 31 days. Arrow points at a bundle of pili, and the arrowhead to a fragment of a flagellum.

2003; White *et al*., 2006). Moreover, flagella were uncommon, suggesting that the acquired surface motility was most likely mediated by pili, which have also been associated to the survival of *Salmonella* in low-nutrient environments (White *et al*., 2006). However, the possibility that surfactant-mediated sliding motility (Stewart *et al*., 2009; Stewart *et al*., 2011) was induced in SPFs starved in tap water cannot be ruled out, particularly because strings of an extruded clear material were observed around bacterial cells grown on BCYE agar (Fig. 3.3D). The outer ring growth zone on the BCYE lawns was enriched in filamentous *L. pneumophila* cells (Fig. 3.3E and F), the inner ring zone consisted of a mixture of rods and filaments expressing pili (Fig. 3.3G), and the central lawn mostly contained rods. Thus, it was concluded that before completely losing culturability, SPFs in tap water at 45°C become motile and are prone to form filaments. This phenomenon was not observed in starved MIFs.

3.2. Morphological Features of *L. pneumophila* VBNC Cells Determined by TEM

3.2.1. Morphology of SPF-VBNCCs from dd-Water

In agreement with the low viability values of SPF-VBNCCs in dd-water, the majority (91.1%) of these cells looked empty, with a ghost-like appearance. The few cells that appeared surrounded by a continuous cell membrane and still contained cytoplasmic material (Fig. 3.4 A and B) were assumed to be the *L. pneumophila* VBNC cells. In fact, the proportion of morphologically preserved cells in transmission EM specimens (8.9%) correlated well with the proportion of viable cells (5 ± 0.5%, n=3) determined by fluorescence microscopy with the BacLight vital stain. These SPF-VBNCCs from dd-water exhibited a rod-like shape, a typical Gram-negative envelope, an electron-translucent cytoplasm poor in ribosomes, and cytoplasmic inclusions (some of which were large in size). Unlike SPFs, SPF-VBNCCs showed obvious outer membrane vesiculation (Fig. 3.4B) and the presence of intraperiplasmic vesicles (Fig. 3.4C).

3.2.2. Morphology of SPF-VBNCCs from Tap Water

The proportion of cells that appeared morphologically preserved (18.7%) by transmission EM (assumed to be VBNCCs, as above) showed a good correlation with the proportion of viable cells (20.1 ± 4.3%, n=3) determined by the BacLight vital stain. These

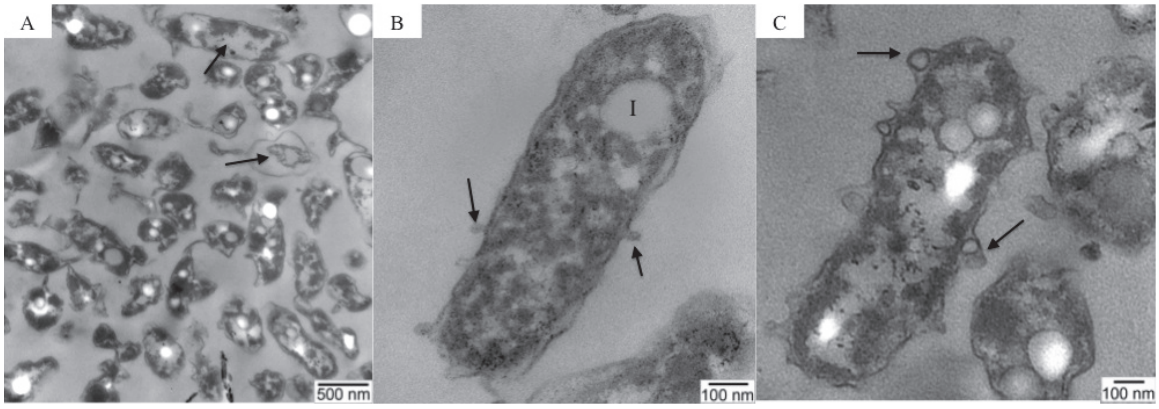


Fig.3.4. Analysis of TEM ultrathin sections of *L. pneumophila* SPF-VBNCCs from dd-water. Panel (A) Low magnification electron micrograph showing a representative field of cells. Two ghost-like cells are indicated by the black arrows. Panel (B) Close up of a VBNCC showing a continuous, apparently intact envelope, an electron-translucent cytoplasmic material, and an obvious inclusion (I). The outer membrane appears to be producing vesicles (black arrows). Panel (C) Example of an SPF-VBNCC with a wavy outer membrane and obvious intraperiplasmic vesicles (arrows).

VBNCCs displayed a typical Gram-negative envelope with clearly defined outer and inner membranes, and a periplasmic space, but also appeared very thin (Fig.3.5A). The outer membrane was rather straight and showed a thickened electron-dense inner leaflet, but no vesiculation. Interestingly, these SPF-VBNCCs had a pear-like shape in cross section, as if the cell envelope had collapsed on itself, producing a prominent pointed protrusion on one side of the cell that appeared to be associated with a spacious periplasmic space (Fig.3.5B, arrow). In sharp contrast with SPF-VBNCCs from dd-water, the cytoplasm of SPF-VBNCCs from tap water was denser and was devoid of cytoplasmic inclusions. Finally, dark spots were reproducibly evident in the cytoplasm of ~42.8% of the intact bacterial sections (Fig.3.5B, arrowhead). The molecular nature of these structures remains undetermined, but they were also prominently displayed by SPFs prior to the complete loss of culturability (Fig.3.5C).

3.2.3. Morphology of MIF-VBNCCs from dd-Water and Tap Water

The unique ultrastructural features of MIFs, previously reported by Faulkner and Garduno (2002), were well preserved in MIF-VBNCCs. Briefly, these cells had a straight outer membrane with a thickened electron-dense inner leaflet, a difficult-to-resolve inner membrane, an electron-dense cytoplasmic material, and an inconspicuous periplasmic space. However, as reported above for SPF-VBNCCs from tap water, MIF-VBNCCs also exhibited a lateral pointed protrusion in cross section (Fig. 3.6A and 3.6B). MIF-VBNCCs from tap water were very similar to MIF-VBNCCs from dd-water, with the only differences being that many were thin and cytoplasmic inclusions were absent (Fig.3.6C).

3.3. Effect of pH and Salts on The Culturability and Viability of *L. pneumophila*

The pH of dd-water was consistently measured as 4.4, whereas our tap water had a pH of 6.6. To determine whether the differences in VBNCCs' culturability and viability observed between dd- and tap water were solely due to pH, SPFs were suspended in dd-water buffered to pH 6.6 or tap water adjusted to pH 4.4. No differences were observed between untreated dd-water or dd-water buffered to pH 6.6 at 45°C with respect to the time required by SPFs to lose culturability and the final viability of the SPF-VBNCCs. Similarly, no major differences were observed in the viability of VBNCCs obtained from untreated tap water (pH

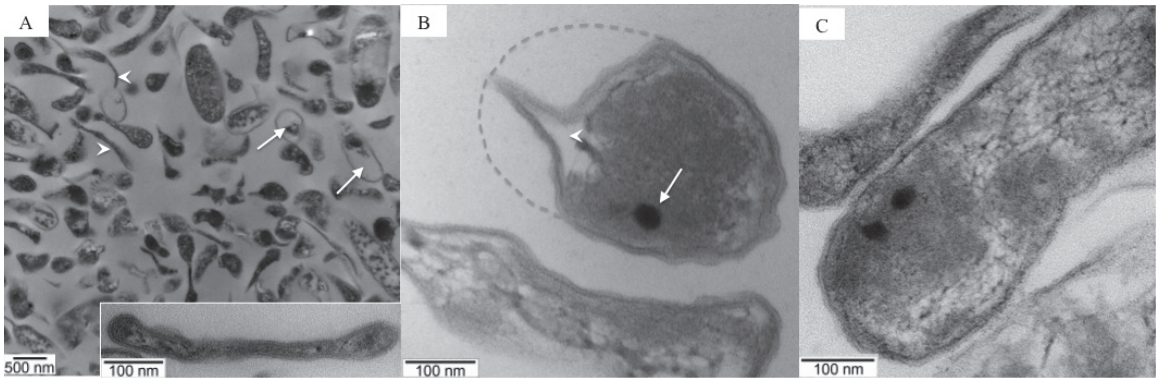


Fig.3.5. Analysis of TEM ultrathin sections of *L. pneumophila* SPF-VBNCCs produced in tap water. Panel (A) Low magnification electron micrograph of SPF-VBNCCs from tap water in which ghost-like cells are indicated (white arrows), as well as examples of thin bacterial cells (white arrowheads). Notice the lack of inclusions in these VBNC cells. Inset: Close-up of a thin VBNC cell depicting a continuous outer membrane and an inconspicuous periplasmic space and inner membrane. Panel (B) Close-up of a VBNC cell with a pointy end, speculated to be the result of a collapsed, structurally weakened cell envelope. The dotted line marks the hypothetical position of the outer membrane before the proposed collapse. There is evidence of a periplasmic space (marked by the white arrowhead) under the pointy end. The white arrow points at the electron-dense spot often seen in SPF-VBNCCs from tap water, and in SPFs suspended in tap water before completely losing culturability (panel C).

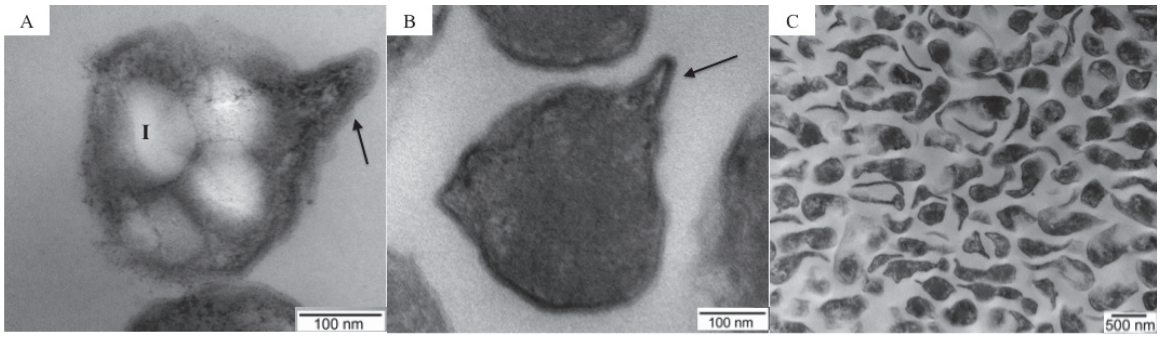


Fig. 3.6. Analysis of TEM ultrathin sections of *L. pneumophila* MIF-VBNCCs produced in dd-water and tap water. Panel (A) Electron micrograph of a MIF-VBNCC from dd-water depicting prominent inclusions (I) and a pointy end (black arrow). Panel (B) Close-up of a MIF-VBNCC from tap water also showing a pointy end (black arrow), but lacking inclusions. Panel (C) The absence of inclusions is best appreciated in this electron micrograph of MIF-VBNCCs from tap water, which also shows that several MIF-VBNCCs are thin.

6.6) versus tap water adjusted to pH 4.4 (data not shown) and incubated at 45°C. However, culturability of SPFs was lost earlier in tap water adjusted to pH 4.4 (Fig. 3.7A). Together, these data indicated that pH only played a minor role in the SPF-VBNCCs' differences observed between dd- and tap water.

States *et al*. (1985) previously reported that metal ions are important factors in the survival and growth of *L. pneumophila* in tap water (States *et al*., 1985). Therefore, after ruling out a major role for pH, the differences in SPF-VBNCCs' culturability and viability observed between dd- and tap water were hypothesized to be due to the presence or absence of mineral ions and salts. In fact, the culturability and viability of SPFs improved in buffered dd-water supplemented with individual salts (Fig.3.7B) added to a concentration that matched that of the corresponding ions found in our tap water. The best results were achieved by the addition of NaF, which extended the culturability and viability of SPFs to levels comparable to those observed in tap water. The addition of $FeSO_4$ helped in preserving viability (with a final value of 22%), but culturability was lost earlier (at day 28) than in tap water. A final viability of 39%(higher than that observed for SPF-VBNCCs in tap water), was achieved by the addition of $Ca(NO_3)_2 \bullet 4H_2O$. These data indicate that specific ions play a role in enhancing the culturability and viability of *L. pneumophila* in water at 45°C.

3.4. Resistance of VBNC *L. pneumophila* to Digestion in *Tetrahymena* Spp. Food Vacuoles

As a measure of environmental fitness, we examined whether VBNCCs were vulnerable to digestion inside the ciliate *Tetrahymena*. SPF-VBNCCs produced in dd-water were readily digested inside food vacuoles and expelled as membranous pellets that mostly contained outer membrane whorls (Fig. 3.8A and B). Interestingly, the few SPF-VBNCCs that appeared to have resisted digestion (observed both in food vacuoles and membranous pellets) exhibited MIF-like characteristics such as an irregular shape, a dense cytoplasm, a straight outer membrane, and a difficult-to-resolve inner membrane and periplasm (Fig. 3.8C). These traits were very different from those described above for SPF-VBNCCs produced in dd-water, suggesting that ciliates did act as a biological pressure to select for VBNC cells derived from SPFs that had differentiated into MIFs.

VBNC cells derived from SPF-VBNCCs produced in tap water or from MIFs in dd- and tap water were resistant to digestion by the ciliates and appeared intact inside food

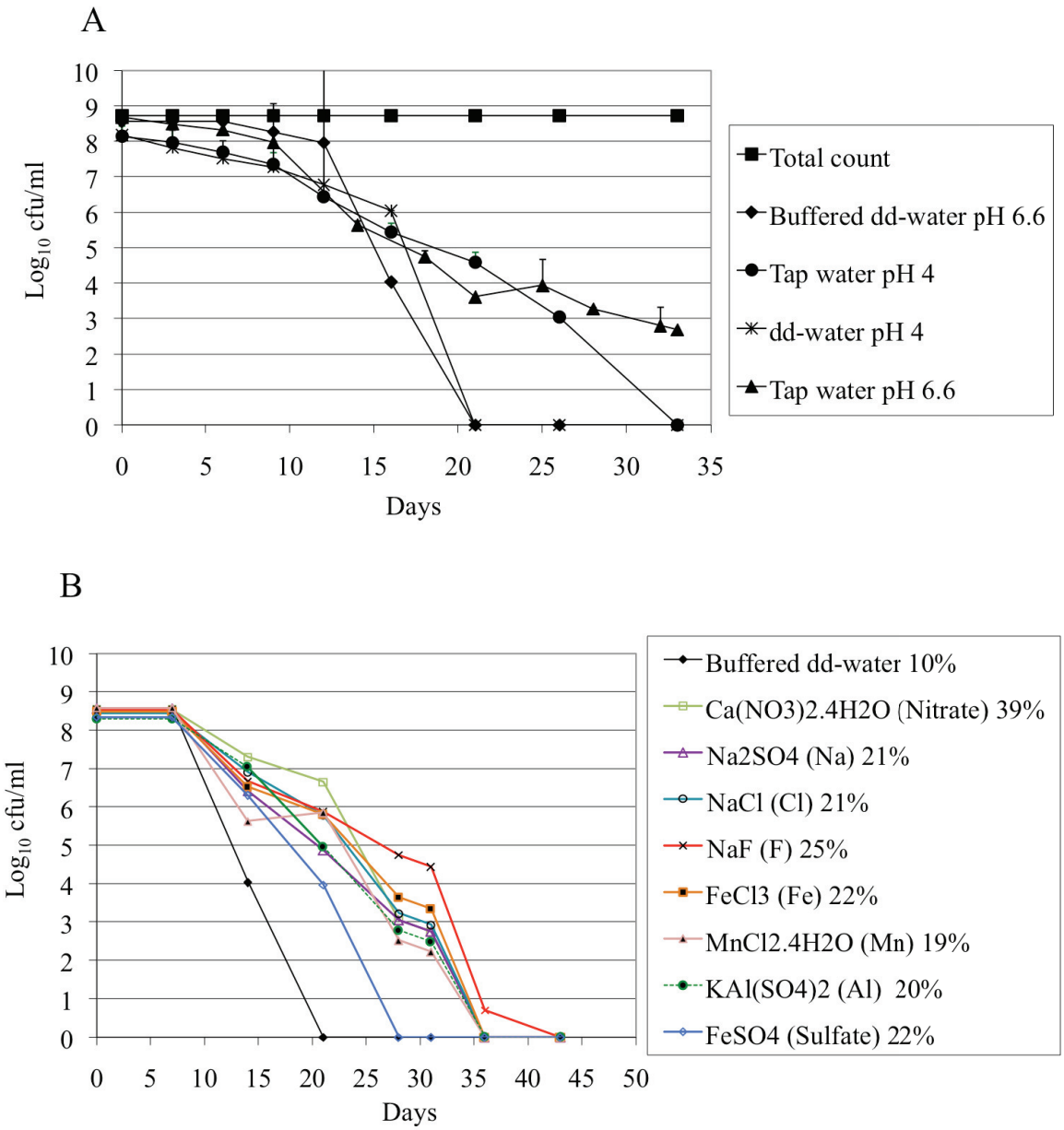


Fig. 3.7. Water pH plays a minor role in the kinetics of SPF-VBNCCs formation, whereas the addition of salts clearly enhances the preservation of culturability. Panel (A) Graph showing the loss of culturability of SPFs of *L. pneumophila* strain JR32 in dd-water and tap water at two different pHs. These experiments were done in duplicate. Panel (B) Graph showing the loss of culturability of SPFs of *L. pneumophila* strain JR32 suspended in dd-water buffered to pH 6.6 and with added salts. The ions whose effect was studied by adding the corresponding salts (as per Table 2.2) are indicated in the parentheses. The viability values observed at the time of entrance into the VBNC state (as determined by the fluorescent vital stain BacLight) are indicated as percent values. Results are means ± SD. These experiments were done in duplicate.

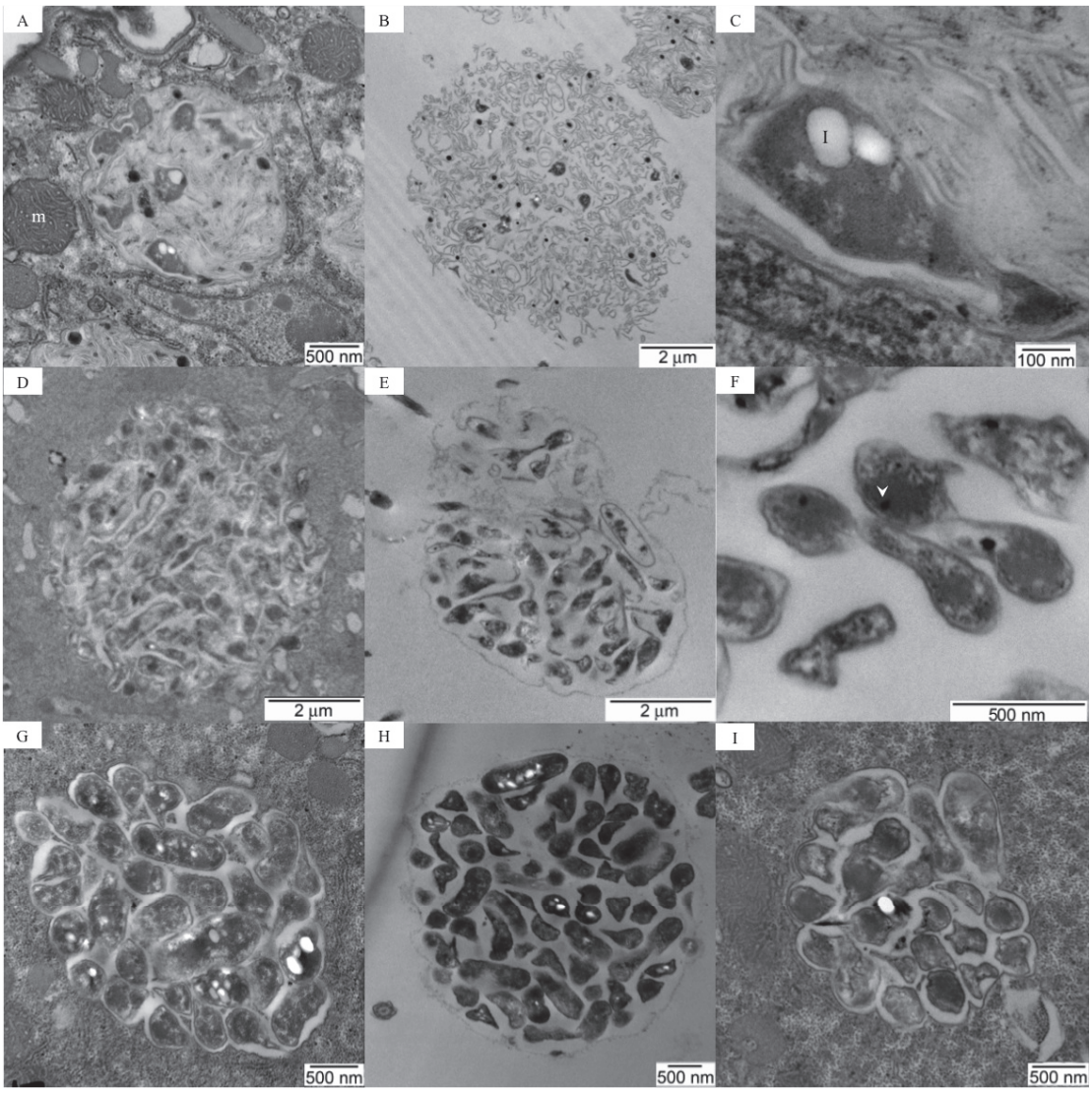


Fig. 3.8. VBNC *L. pneumophila* resist digestion inside the ciliate *Tetrahymena* and are packaged into pellets. Similar results were obtained in *T. tropicalis* and *T. thermophila*. Left column, panels A, D and G, shows micrographs of food vacuoles, and the central column, panels B, E and H, shows micrographs of pellets expelled by the ciliates. Panel (A-C) Most SPF-VBNCCs produced in dd-water and ingested by *Tetrahymena* are degraded into outer membrane whorls. The few surviving VBNC cells keep their inclusions ("I"). Panel (C) is a close up of one of the surviving cells in the food vacuole shown in panel A. Panel (D-F) Most SPF-VBNCCs produced in tap water are not degraded after ingestion by *Tetrahymena*. A large number of apparently intact VBNC cells are observed tightly packed in food vacuoles (D) and pellets (E). These bacterial cells had consumed their inclusions and displayed the dark spots typical of SPF-VBNCCs (white arrowhead in panel F). Panel (G and H) MIF-VBNCCs produced in dd-water and ingested by *Tetrahymena* showed a very robust ultrastructural preservation in food vacuoles (G) and expelled pellets (H). These VBNC cells kept some inclusions. (I) Micrograph of MIF-VBNCCs produced in tap water and ingested by *Tetrahymena* into a food vacuole. These VBNC cells also maintained a robust ultrastructure, but did not show inclusions. m = mitochondria.

vacuoles and expelled pellets. In general, the morphology of these VBNC cells was similar to that displayed before ingestion (Fig. 3.8D-F and 5G-I), except perhaps for MIF-VBNCCs from dd-water, in which the cytoplasmic inclusions were less evident (Fig. 3.8G and 5H), suggesting that after internalization in *Tetrahymena* MIF-VBNCCs can degrade inclusions.

3.5. Resistance of VBNC *L. pneumophila* to Detergent-Mediated Lysis

Detergents are used in the maintenance of cooling towers, so it is reasonable to surmise that *L. pneumophila* would be frequently exposed to them. In addition, biological surfactants might be naturally encountered in the freshwater environment. In fact, *L. pneumophila* is capable of secreting a surfactant that is inhibitory to other *Legionella* spp. (Stewart *et al*., 2011). Therefore, resistance of VBNC cells to a strongly ionic detergent was used as a second test for their environmental fitness. MIFs have been previously shown to be stable in a number of detergents (Garduno *et al*., 2002a) and MIF-VBNCCs preserved this trait showing limited lysis in 1% SDS. In contrast, SPFs and SPF-VBNCCs produced in dd-water rapidly lysed in the presence of detergents (Garduno *et al*., 2002a and Fig. 3.9A). However, SPF-VBNCCs from tap water were resistant to lysis in 1% SDS (Fig. 3.9A). Obviously, a change in cell envelope properties took place in SPFs as they entered the VBNC state in tap water. Additional support for a change in the envelope properties of SPFs came from the Giménez staining of SPF-VBNCCs (free or inside *Tetrahymena*), which were strongly Giménez positive with a bright red color (Fig. 3.9B). Since SPFs are typically stained with a dull shade of red (Garduno *et al*., 2002a), the bright red staining suggested changes in the molecular composition and surface properties of the SPF-VBNCC's envelope.

3.6. Resuscitation in *A. castellanii* and Quantitative Assessment of Resuscitated VBNC Cells

In agreement with previous reports (Steinert *et al*., 1997; Ohno *et al*., 2003), I was able to resuscitate SPF-VBNCCs produced in tap water in *A. castellanii*. However, all the other VBNCCs did not regain culturability. Examination of the resuscitated bacteria by negative stain TEM revealed the presence of flagella, as would be expected in MIFs (Fig. 3.10). Besides amoeba, I tried to resuscitate VBNC cells in ciliates and in different mammalian cell lines and culture media *in vitro*. None of the VBNC cells tested could be

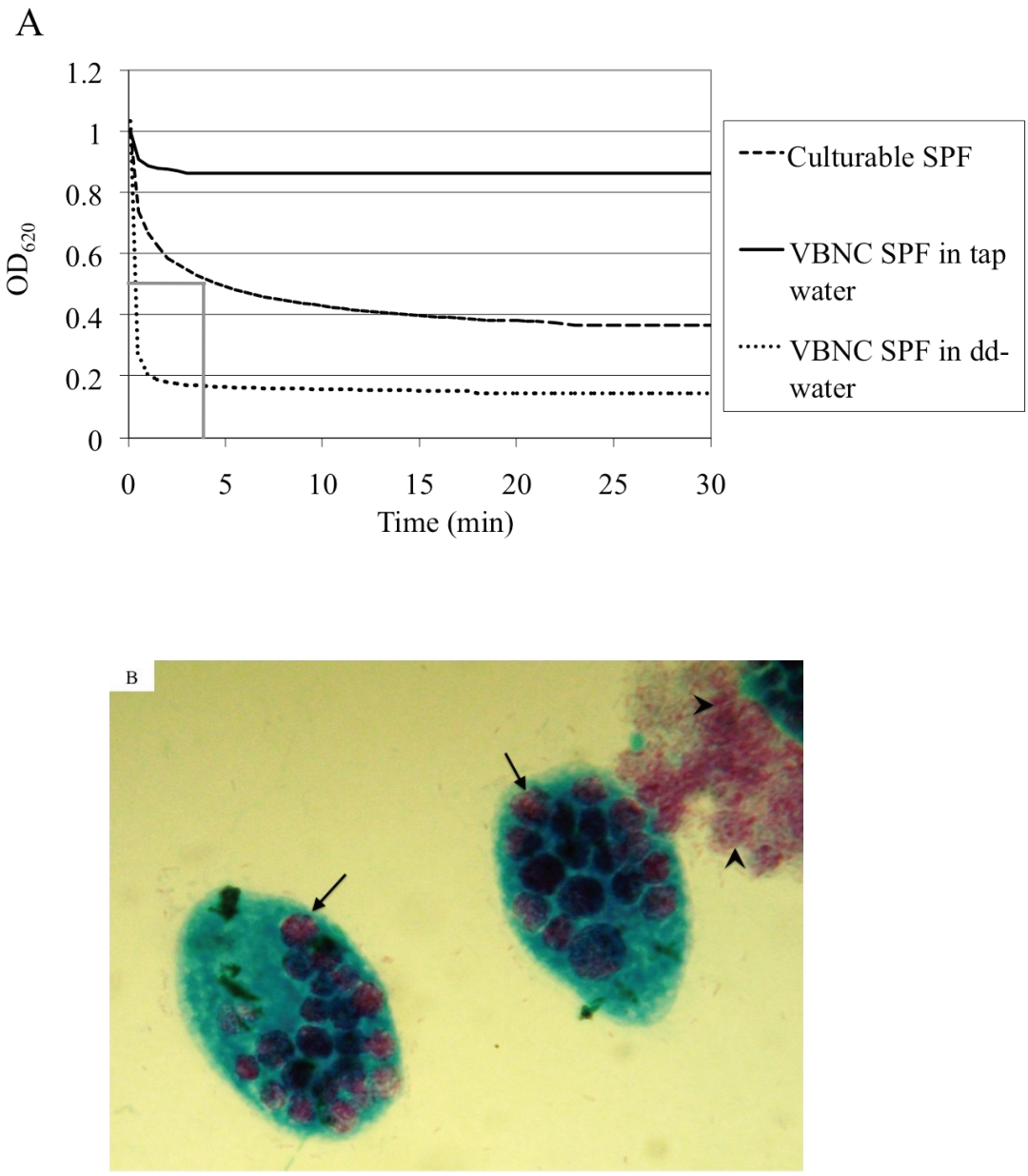


Fig. 3.9. Experimental evidence to support structural-compositional changes in the cell envelope of SPF-VBNCCs. Panel (A) SPF-VBNCCs become resistant to the strongly ionic detergent SDS. The graph shows $OD_{620\ nm}$ (an indicator of cell lysis) as a function of time, for SPFs, SPF-VBNCCs produced in dd-water and SPF-VBNCCs produced in tap water. The lysis index is defined as the time taken to reduce the optical density to 50% of the initial value. For culturable SPFs it is shown that the lysis index is 3.8 min. The estimated lysis index for SPF-VBNCCs in dd-water is ~20 s, and for SPF-VBNCCs in tap water is infinite. Results are means ± SD (n=3). Panel (B) Bright field light micrograph showing the bright red Giménez staining of SPF-VBNCCs inside *Tetrahymena* food vacuoles (arrows) and in expelled pellets (arrowheads).

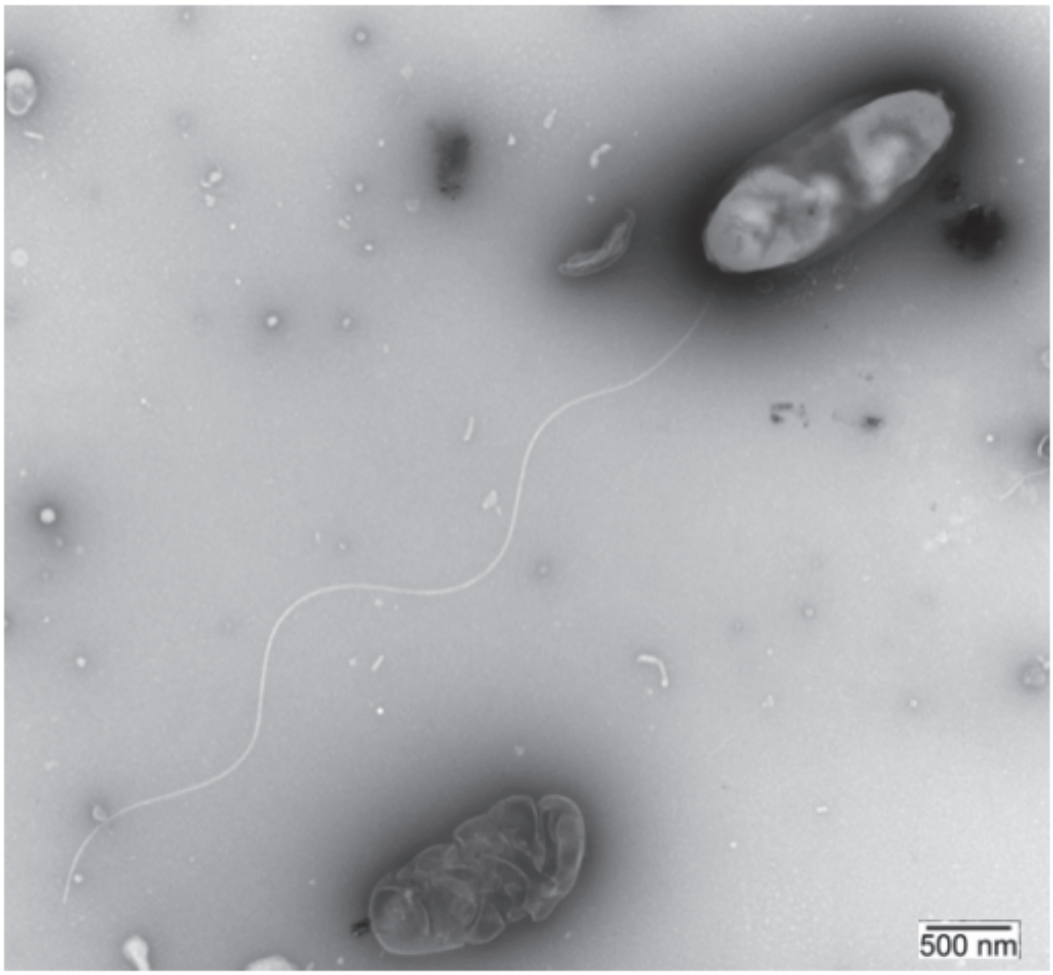


Fig. 3.10. Negative stain TEM analysis of resuscitated SPF-VBNCC in *A. castellanii* displaying flagella.

resuscitated inside *Tetrahymena tropicalis* or *T. thermophila*, U937-derived human macrophages, L929 murine fibroblasts, or human epithelial HeLa cells. Moreover, VBNC cells suspended in fresh BYE, spent BYE in which *L. pneumophila* was cultured overnight, or spent Modified Neff's in which *A. castellanii* was infected with *L.pneumophila*, did not resuscitate. Concentrated pellets expelled by ciliates that have fed on VBNC cells did not grow on BCYE plates and were unable to initiate infections in amoeba. These results indicate that the ability to regain culturability in amoeba depends on the form of *Legionella* and the water microcosm used to produce VBNC cells. In addition, it is clear that the resuscitation of *L. pneumophila* VBNC cells is not straightforward.

To quantify the number of SPF-VBNCCs from tap water that resuscitated in amoeba, I developed a new approach that involved the plating directly on BCYE plates of mixtures of amoeba cysts and serial dilutions of a suspension of VBNC cells. Table 3.1 shows that only 1-3 VBNC cells out of every 100,000 viable cells were able to resuscitate in amoeba. This is the first quantitative study of *L. pneumophila* resuscitation.

Results in this chapter showed that VBNCCs produced from two developmental forms of *L. pneumophila* (SPFs and MIFs) in two water microcosms (dd- and tap water) have distinct morphology. Resuscitation in amoeba was possible for VBNCCs produced from SPFs in tap water. Addition of salts to dd-water prolonged *L. pneumophila* viability to tap water levels. VBNCCs are environmently fit, as was evident from their resistance to detergent lysis as well as digestion in the ciliate *Tetrahymena. L. pneumophila* VBNCCs thus show distinct traits according to its originating developmental form and the surrounding water microcosm.

Table 3.1. Quantitation of tap water SPF-VBNCCs that resuscitated in *Acanthamoeba castellani*. Values show the number of colonies formed after plating VBNCC samples (in the $10^1 – 10^6$ range) on BCYE, in the presence of amoeba cysts suspended in Neff's medium.

	Colony Forming Units[a]							
	Concentrated	Undiluted	Diluted					
Sample	10^1 ($2x10^8$)[b]	10^0 ($2x10^7$)	10^{-1} ($2x10^6$)	10^{-2} ($2x10^5$)	10^{-3} ($2x10^4$)	10^{-4} (2000)	10^{-5} (200)	10^{-6} (20)
Controls[c]	0	0	0	0	0	0	0	0
S1	722	98	13	1	0	0	0	0
S2	TNTC[d]	TNTC	51	3	0	0	0	0
S3	TNTC	353	44	1	0	0	0	0

a Plating was done with either 10 ml samples taken from the corresponding glass bottle and concentrated 10 X to a final volume of 1 mL (10^1), undiluted 1 ml samples straight from the corresponding glass bottle (10^0), or 1 ml samples diluted in a ten-fold series (10^{-1} to 10^{-6}).
b The values provided in parentheses are VBNC cells equivalents calculated from the total direct microscopy count of the undiluted sample, multiplied by the concentration or dilution factor, and the average percent viability of SPF-VBNCCs in tap water (20%) determined by the viable fluorescent stain BacLight.
c Controls (negative) consisted of plated amoeba cysts and Neff's medium in the absence of any VBNC cell sample.
d TNTC = colonies were too numerous to count.
S1, S2, and S3 = the experiment was run in triplicate.

CHAPTER 4. RESULTS-IDENTIFICATION OF GENES AND GENE PRODUCTS INVOLVED IN VBNC CELL FORMATION

After characterizing the morphology and physiology of VBNC *L. pneumophila* cells produced in water and comparing the effect of dd- and tap-water on the production of VBNCCs, the next step was to identify genes and gene products involved in VBNC formation using transposon mutagenesis and proteomic analysis.

4.1. Screening of *L. pneumophila* Mini-Tn*10* Insertion Library

The mini-Tn*l0* insertion mutagenesis system in *L. pneumophila* (Pope *et al*., 1994) offers an effective approach for isolating random mutants that can be screened for various bacterial defects (Pope *et al*., 1994; Wagner *et al*., 2007; Viswanathan *et al*., 2000). This approach was adopted here in order to search for genes that might have a role in VBNC state formation in *L. pneumophila*. Results obtained in Chapter 3 showed that VBNC cells derived from MIFs gave the highest viability in both dd-water and tap water. Therefore, mini-Tn*10* insertion *L. pneumophila* mutants that were able to infect amoeba and produce MIFs were monitored for their ability to enter the VBNC state in water.

A library of 1743 mini-Tn*10* insertion clones was obtained. The transposon mutants were screened for the duration of culturability. Two mutant strains were selected from this library for further analysis. One of the mutants (N-E10) lost culturability earlier (2 days after incubation in water bath) than the parent strain (3 days after incubation), while the other mutant (C-E10) showed longer culturability (7 days after incubation). The two mutants were further analyzed using the method of producing VBNC cells from MIFs in 500 ml dd-water bottles that was described for the parent strain. Unlike the results obtained in the initial screening in 96-well plates, the time taken for both mutants to lose culturability in 500-ml dd-water bottles was similar to that of the parent strain (30 days after incubation) (Fig.4.1A). The viability of both mutants was also similar to that of the parent strain.

PCR using primers specific for mini-Tn*10* (Tn*10* up/Tn*10* down) was performed to confirm the insertion of mini-Tn*10* into the *L. pneumophila* genome of these two mutants. Transposon insertion was confirmed only in the N-E10 mutant (Fig.4.1B).

These results demonstrate the difficulty of screening transposon-insertion mutants for

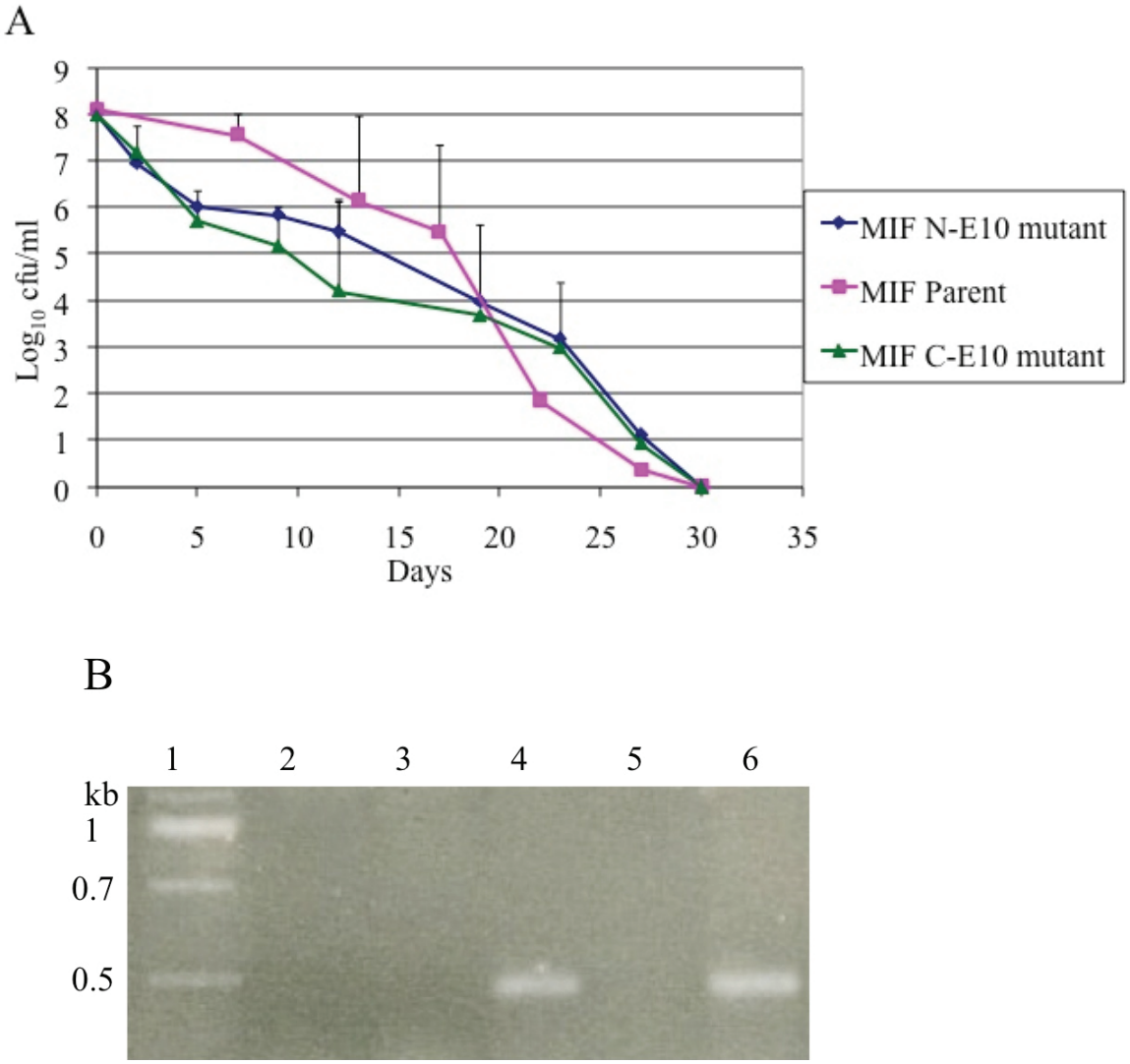


Fig. 4.1. Mini Tn*10* insertion library did not produce meaningful results. Panel (A) Graph showing the loss of culturability (measured as log cfu/ml) against time for two transposon mutants. The parent strain was used as reference. MIFs from two expected mini-Tn*10* insertion mutants (N-E10 and C-E10) in dd-water lost culturability at day 30 similar to parent strain. Results are means ± SD (n=3). Panel (B) Agarose gel electrophoresis of PCR analysis of mini-Tn*10* insertion in N-E10 and C-E10 strains revealed that N-E10 strain only carries the mini-Tn*10*. Lane (1) 1 kb ladder, lanes (2 and 3) no bands appeared when DNA from parent or C-E10 mini-Tn*10* insertion strains were used as the template, lanes (4) a band of 0.5 kb appeared when DNA from N-E10 strain was use as template, lane (5) no DNA template as control, and lane (6) a band of 0.5 kb appeared when DNA from pCDP05 plasmid was use as template.

their ability to produce VBNC cells. One explanation could be that the genes responsible for VBNC formation are not target for the mini-Tn*10*. However, screening for more mutanys could be needed to find a mutant for VBNC formation.

Thus, a different approach was adopted for identifying specific gene products that may play a role in the production of VBNC cells: shotgun proteomics and mass spectrometric analysis.

4.2. Shotgun Proteomic Analysis

Shotgun proteomic screening was performed to detect differences in proteins levels between MIF-VBNCs and culturable MIFs. From the screening, a list of 1904 proteins was obtained. This list was filtered to include only proteins with at least three matched peptides in MS/MS. This filtering reduced the list to 485 proteins. At least a 2-fold change in protein level in the VBNC state compared to the culturable state was used as a criterion for selecting proteins with altered expression levels. Based on this criterion, 18.1% of the 485 proteins showed increased levels in the VBNC state while, 46.6% showed decreased levels, and 35.3% had unchanged levels in the VBNC state. Putative uncharacterized proteins represented 20.7% of the total proteins; among those, 7.4% showed an increased level, 8.8% showed a decreased level, and 2.7% were unchanged in the VBNC state (Appendix I, II, and III).

The Gene Ontology (GO) database is one of the databases used by Proteome Discoverer to annotate gene products. The GO database has three structured, controlled vocabularies (ontologies) that describe gene products in a species-independent manner. These ontologies help classify gene products into those involved in biological processes in general, gene products that are cellular components, and gene products responsible for particular molecular functions. Thus, proteins within each group (increased, decreased, and unchanged protein levels) were further categorized according to these three ontologies (Fig.4.2). Among proteins that showed increased levels, the most common molecular function was catalytic activity (37.5%), followed by nucleotide binding and protein binding (each 9%), metal ion binding (6.8%), and enzyme regulator activity (2.3%). Each of RNA binding, transporter activity, signal transducer activity, and structural molecule activity represented 1.1%. The remaining 42% did not have an annotated molecular function, suggesting that nearly half

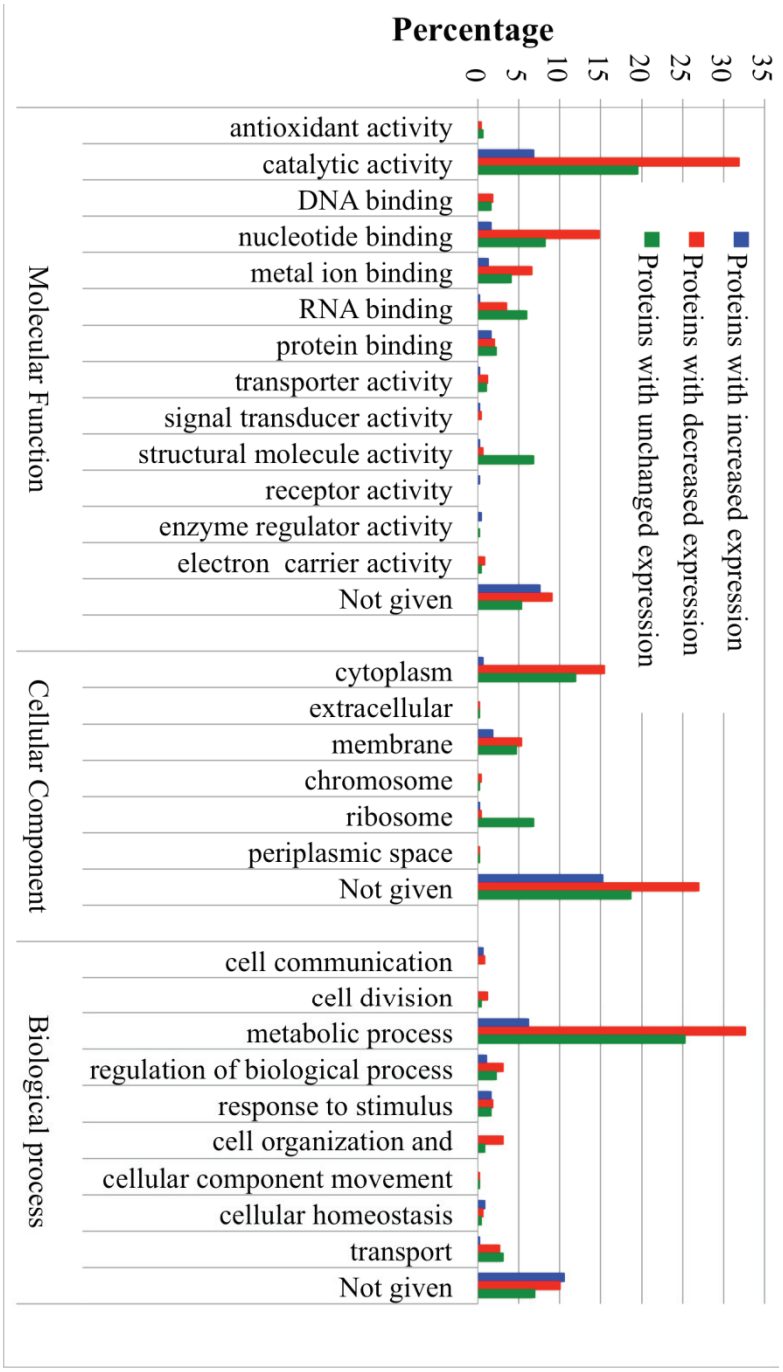


Fig.4.2. Bar graph showing the distribution of proteins identified by shotgun mass spectrometry present in MIF-VBNCCs in dd-water according to their molecular function, biological processes and cellular components. Data are presented as percentage of the total number of proteins identified.

of the proteins with increased levels are hypothetical proteins.

Among proteins with decreased levels, the most common molecular function was also catalytic activity (68.6%), followed by nucleotide binding (31.9%), metal ion binding (14%), RNA binding (7.5%), protein binding (4.4%), DNA binding (4%), transporter activity (2.7%), electron carrier activity (1.8%), structural molecule activity (1.3%), and signal transducer activity and antioxidant activity (0.9% for each). The remaining proteins (19.5%) were without known molecular function.

Categorization of proteins according to their cellular location revealed that the highest percentage of the proteins with increased levels was in the membrane (10.2%), followed by cytoplasmic and ribosomal proteins (3.4% and 1.1 %, respectively). However, the cellular location of the majority of the proteins (84%) with increased levels was not given. On the other hand, the highest percentage of the proteins with decreased levels was in the cytoplasm (33.2%), followed by the membrane (11.5%), ribosome and the chromosome (0.9% for each), and extracellular and periplasmic space (0.4% for each). As above, the location of the majority of the proteins (58%) with decreased levels was not known.

Categorization based on biological processes revealed that, among the proteins with an increased level, the percentage of proteins involved in metabolic processes was the highest (34%), followed by response to stimulus (9%), regulation of biological process (5.7%), cellular homeostasis (4.5%), cell-cell communication (3.4%), and transport (1.1%). The remaining proteins (58%) were not annotated with any known biological processes. Among those with decreased levels, proteins involved in metabolic processes also had the highest percentage (70.4%), followed by regulation of biological process and cell organization and biogenesis (6.6% for each), transport (5.8%), response to stimulus (4%), cell division (2.7%), cell communication (1.8%), and cellular homeostasis (1.3%). The remaining proteins (21.7%) were not annotated with any known biological processes.

Among the proteins with increased levels in the MIF-VBNCCs, 3-hydroxybutyrate dehydrogenase (BdhA) showed 2.4 folds increase in its level. Since TEM examination of MIF-VBNCCs produced in tap water showed the disappearance of PHB inclusions, therefore, BdhA was chosen for further analysis.

4.3. Validation of The Data from Shotgun Proteomic Analysis by Multiple Reaction Monitoring (MRM) Analysis

To validate the results of shotgun proteomic analysis, 12 proteins were chosen for further analysis using a quantitative MRM method. The 12 proteins consisted of seven from the increased protein level group, three from the decreased protein level group, and two from the unchanged protein level group. Table 4.1 compares the magnitude of change in the levels of these 12 proteins between the shotgun proteomics screen and the MRM proteomics methods.

Shotgun proteomic analysis and MRM analysis found comparable protein level changes for each of BdhA, acetyl CoA synthetase, RelA, and pyruvate dehydrogenase (Table 4.1). However, other proteins showed large differences with respect to protein level fold changes between the shotgun proteomics and MRM methods. For example, shotgun proteomics analysis showed that the level of small HspC2 (G8UYH4) in MIF-VBNC increased 87 fold over the culturable MIF; however, the MRM data showed that this increase was only 30.7 fold. Other examples were EnhA (6.3 in shotgun proteomics analysis vs 1.8 in MRM analysis), GGDEF/EAL (15.5 vs 2.4), and small HspC2 protein G8UX67 (11.2 vs 2). The differences in protein level fold changes between shotgun proteomics and MRM methods was also seen in protein with decreased level in the VBNC state. For example, the D-alanyl-D-alanyl carboxypeptidase protein level in MIF-VBNC decreased 0.01 fold over the culturable MIF; however, the MRM data showed this decrease as being 0.4 fold. Similar differences were evident with D-alanyl-D-alanyl ligase (0.07 vs 0.5) and acetyl CoA reductase (0.01 vs 0.35).

In spite of the discrepancies in the magnitude of fold changes, the results from the two methods are in agreement in defining proteins in the increase, decrease, or unchanged groups.

4.4. SDS-PAGE and Mass Spectrometry Analysis

To further investigate the changes in gene products, the protein profiles of *L. pneumophila* VBNCCs (SPFs-VBNCCs in tap water and MIFs-VBNCCs in dd- and tap water) were examined by SDS-PAGE and mass spectrometry. The results showed that the samples from SPF-VBNCCs from tap water contained a 21 kDa band at a much higher level

Table 4.1. Protein levels between culturable MIFs and MIF-VBNCCs in dd-water obtained by shotgun proteomic and MRM methods.

Accession #	Protein name	Description	Fold change MIF-VBNCCs/ culturable MIFs	
			MRM data	Shotgun proteomic analysis
G8UTX4	BdhA	Oxidize 3-Hydroxybutarate into acetoacetate	1.9	2.4
G8UY94	Acetoacetyl CoA synthetase	Catalyzes Acetyl CoA into Acetoacetyl CoA	2.1	2.3
A5IBU8	RelA	Synthesize ppGpp and pppGpp	1.8	2
A5IAT5	EnhA	Suggested in entry of *L. pneumophila* into monocytes	1.8	6.3
G8UX67	Small HspC2	Chaperones protect cellular proteins	2	11.2
A5IG24	GGDEF/EAL	Synthesis and degradation of c-di-GMP	2.4	15.5
G8UYH4	Small HspC2	Chaperone protect cellular proteins	30.7	87
D5TDA5	D-alanyl-D-alanyl carboxypeptidase	Remove terminal D-alanine from muramyl pentapeptide	0.4	0.01
I7HM34	D-alanyl-D-alanyl ligase	Catalyzes ligation of 2 D-alanine to D-alanyl-D-alanine	0.5	0.07
A5IF19	Acetyl CoA reductase	Reduce acetoacetyl-CoA to 3-hydroxybutyryl-CoA	0.35	0.01
I7I593	Pyruvate dehydrogenase	Contributes to transforming pyruvate into acetyl-CoA	1	1
G8UY88	Acyl CoA acetyltransferase	Catalysis of the transfer of an acyl group	1.3	1.1

than that from culturable SPF (Fig. 4.3). Mass spectrometric analysis of this 21 kDa band revealed the presence of a number of proteins (Table 4.2). Among the proteins in the list, hypothetical protein lpg0634, OmpA-like transmembrane domain containing protein, and the Trp repressor binding protein were only found in the VBNC samples, while the small heat shock protein HspC2 was more abundant in VBNC cell samples compared to the culturable SPF samples.

In the MIF samples, a protein band of *ca*. 24.5 kDa in MIF-VBNCCs from dd- water was more apparent compared to the corresponding area of the lanes for the culturable MIFs and MIF-VBNCCs from tap-water (Fig. 4.4). Mass spectrometry analysis of the gel slice from MIF-VBNCCs in dd-water showed the presense of two proteins, 3-hydroxybutyrate dehydrogenase (BdhA) and the hypothetical protein Lpg1960. These two proteins were absent in the samples from culturable MIFs. The same area for MIF-VBNCCs from-tap water samples did not show any stained band at this location.

4.4.1. RT-PCR Analysis of Genes Encoding Proteins Identified by SDS PAGE and Mass Spectrometric Analysis

RT-PCR was performed to examine the transcription of some of the genes encoding the upregulated proteins identified by SDS-PAGE analysis. These include 3-hydroxybutyrate dehydrogenase (BdhA), small heat shock protein HspC2, and hypothetical protein Lpg0634. Transcripts of *bdhA* (Fig. 4.5), *hspC2,* and *lpg0634* (data not shown) genes could not be detected in the VBNC state suggesting that the transcripts were degraded whereas the proteins persisted in the VBNC bacteria.

This chapter's results showed that VBNCCs produced from two developmental forms of *L. pneumophila* (SPFs and MIFs) have distinct protein profiles. A number of proteins present at a higher level in VBNC were identified. Shotgun proteomics was a successful technique to identify the VBNC proteins profile, and its findings were supported by the SDS-PAGE and MRM.

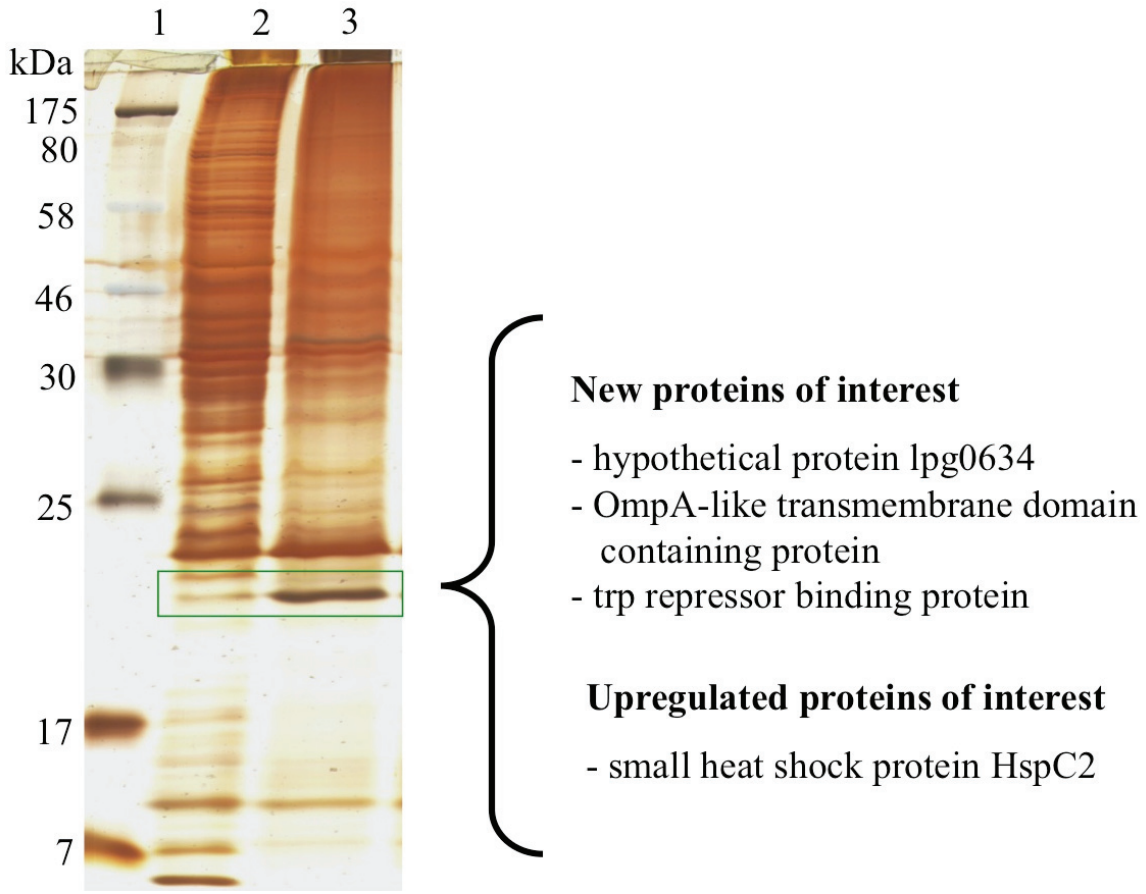


Fig. 4.3. A silver stained SDS-PAGE gradient (9-17%) gel of proteins from culturable SPFs and of SPF-VBNCCs from tap water. Lane (1) protein markers, lane (2) proteins from culturable SPFs, and lane (3) protein from SPF-VBNCCs in tap water. The green box indicates the 21 kDa band. The text on the right of the gel shows the newly found and upregulated proteins in the SPF-VBNCCs but not in culturable SPFs according to MS/MS analysis.

Table 4.2. Mass spectrometric analysis of the 21 kDa bands obtained from the SDS-PAGE gel from the culturable SPF and SPF-VBNCCs in tap water. The proteins are listed according to their abundance.

Order	Culturable SPF	SPF-VBNCCs in tap water
1	F0F1 ATP synthase subunit delta	Small heat shock protein HspC2
2	Small heat shock protein HspC2	Major outer membrane protein precursor
3	50S ribosomal protein L5	Major outer membrane protein
4	Hypothetical protein lpg1526	50S ribosomal protein L5
5	Peptide deformylase	F0F1 ATP synthase subunit delta
6	NADPH-dependent FMN reductase domain-containing protein	MompS
7	Short chain dehydrogenase	NADPH-dependent FMN reductase domain-containing protein
8	Major outer membrane protein precursor	Short chain dehydrogenase
9	Glutathione S-transferase	Hypothetical protein lpg0634
10	D-ribulose-5-phosphate-3-epimerase	Small HspC2 heat shock protein
11	Hypoxanthine-guanine phosphoribosyltransferase	Trp repressor binding protein
12	Deoxycytidine triphosphate deaminase	Elongation factor Tu
13		Glutathione S-transferase
14		OmpA-like transmembrane domain containing protein

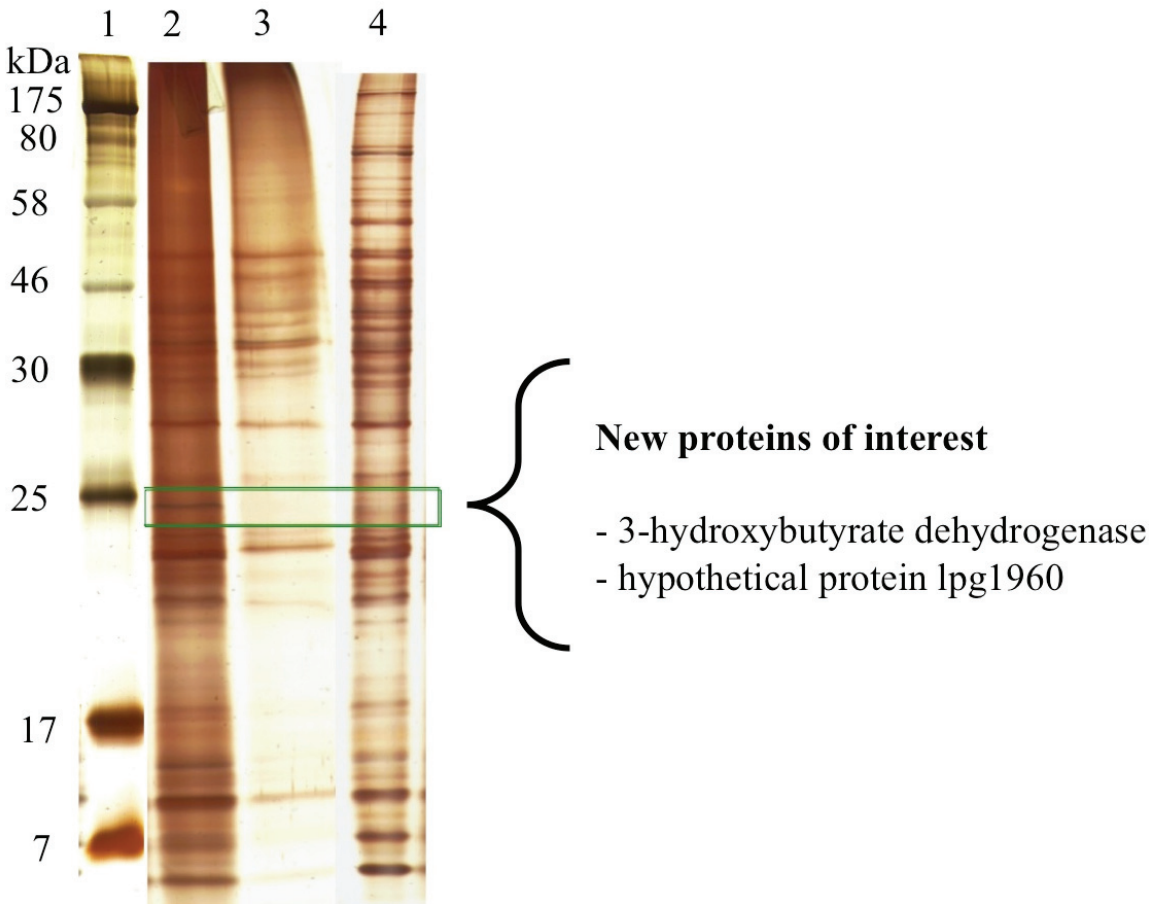


Fig. 4.4. A silver stained SDS-PAGE gradient (9-17%) gel of proteins from culturable MIFs and of MIF-VBNCCs from dd- and tap water. Lane (1) protein markers, lane (2) proteins from MIF-VBNCCs in dd-water, lane (3) proteins from MIF-VBNCCs in tap water, and lane (4) proteins from MIFs. The green box indicates the 24.5 kDa band. The text on the right of the gel shows the newly found proteins in the MIF-VBNCCs from dd-water but not from culturable MIFs and MIF-VBNCCs from tap-water according to MS/MS analysis

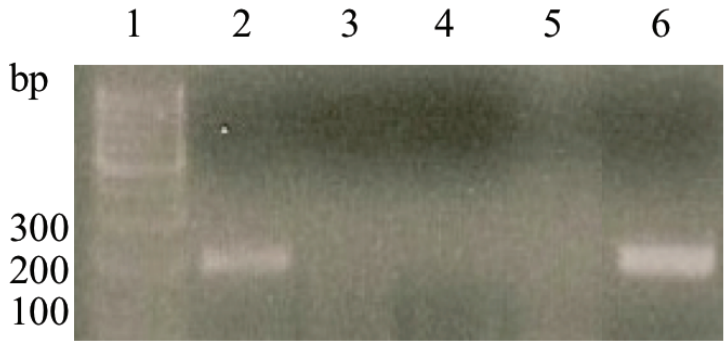


Fig.4.5. Agarose gel electrophoresis showing the absence of *bdhA* transcript by RT-PCR. Lane (1) 100 bp ladder, lanes (2-6) PCR amplification of a 200 bp internal piece in the *bdhA* gene using 3hbdF/3hbdR primer pair. cDNA from the culturable MIF was used as template in lane 2. cDNA from the MIF-VBNC from dd-water was used as template in lane 3. cDNA from the MIF-VBNC from tap water was used as template in lane 4. Lane (5) no DNA template as control. Genomic DNA from the culturable MIF was used as template in lane 6.

CHAPTER 5. RESULTS- ROLE OF 3-HYDROXYBUTYRATE DEHYDROGENASE ON VBNC CELLS FORMATION IN *L. PNEUMOPHILA*

5.1. Construction of a *bdhA* Mutant

As described in Chapter 4, shotgun proteomics analysis of MIF-VBNCCs in dd-water showed a 1.8 fold increase in the level of BdhA compared to culturable MIFs. BdhA was also identified by SDS-PAGE and mass spectrometry analysis from the 24.5 kDa protein band of MIF-VBNCCs from dd-water. Furthermore, TEM examination of both SPF-VBNCCs and MIF-VBNCCs produced in tap water showed the disappearance of PHB inclusions. With these results in mind, a mutant defective in BdhA was constructed in order to study the role of BdhA in *L. pneumophila* VBNC formation in water.

Initially, I tried to mutate the *bdhA* gene using the erythromycin resistance cassette. A pBluescript *bdhA*::*ermAM* plasmid was constructed, as described in the Materials and Methods section. Restriction of pBluescript *bdhA*::*ermAM* using BamHI showed a single band of 5.2 kb, the expected size of the plasmid (Fig. 5.1A). PCR analysis using the pBluescript *bdhA*::*ermAM* fragment as a template yielded the expected size of 2.2 kb for the *bdhA*::*ermAM* construct (Fig. 5.1B). When the PCR-amplified *bdhA*::*ermAM* construct was restricted with either EcoRV or BamHI, two bands with the expected sizes 1.5 kb and 0.7 kb appeared (Fig. 5.1C). The above results indicate that the *bdhA*::*ermAM* construct has been successfully created.

L .pneumophila JR32 was electroporated with the *bdhA*::*ermAM* construct, and transformants were selected on BCYE containing erythromycin. Fifty erythromycin resistant colonies were screened by PCR amplification of *ermAM* using the SL609/SL729 primer pair. An erythromycin resistant colony, which showed the 0.8 kb *ermAM* amplicon, was chosen for RT-PCR analysis. RT-PCR results showed that *bdhA* was still expressed in this erythromycin resistant colony (Figure 5.2). Furthermore, 80 erythromycin resistance colonies were screened by PCR amplification of a 130 bp internal region in the *bdhA* gene using the 3hbd-Q F/3hbd-Q R primer pair. All of the colonies showed the *bdhA* amplicon (data not shown). These results indicate that the parent copy of the *bdhA* gene still existed in the transformants.

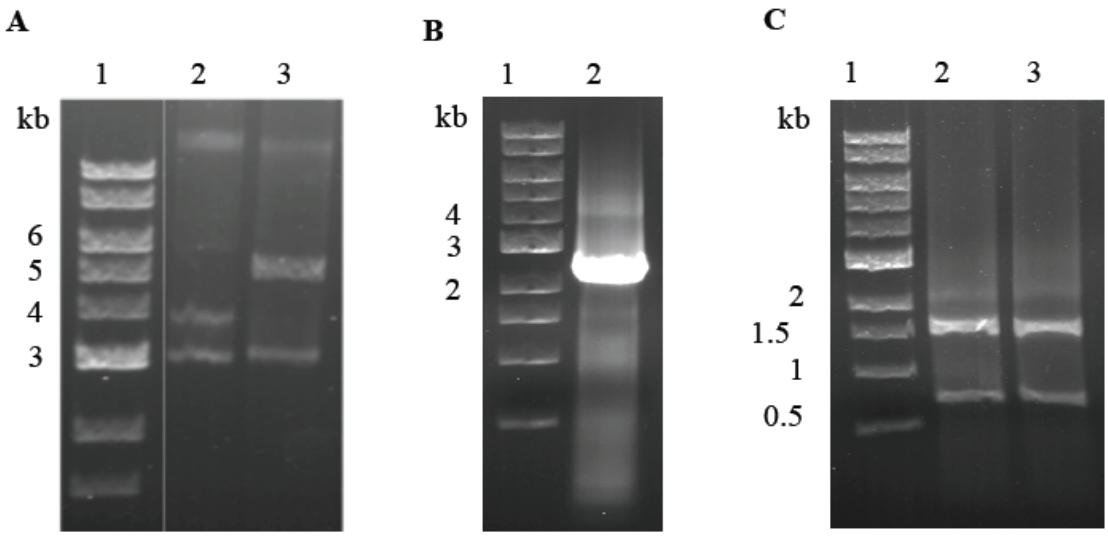


Fig.5.1. Agarose gel electrophoresis of the *bdhA::ermAM* construct. Panel (A) Restriction analysis of pBluescript *bdhA*::*ermAM* by BamHI showed the expected size of 5.2 kb. Lane (1) 1 kb ladder, lane (2) undigested pBluescript *bdhA*::*ermAM* as control, and lane (3) BamHI digested plasmid. Panel (B) PCR amplification of *bdhA* upstream-*ermAM*-*bdhA* downstream fragment from pBluescript *bdhA::ermAM* plasmid using SL960/SL963 primer pair. Lane (1) 1 kb ladder and lane (2) PCR product showed the expected size of 2.2 kb. Panel (C) Restriction analysis of *bdhA* upstream-*ermAM*-*bdhA* downstream fragment resulted in 2 bands 1.5 kb and 0.7 kb which indicate the expected size of the fragment. Lane (1) 1 kb ladder, lane (2) EcoRV digestion of the fragment, and lane (3) BamHI digestion of the fragment.

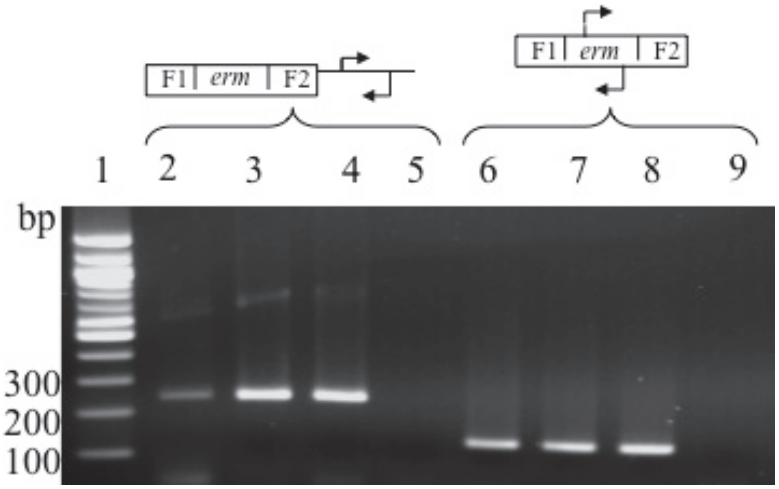


Fig.5.2. Agarose gel of RT-PCR revealed that *bdhA* gene was still expressed in the erythromycin resistant. Lane (1) 100 bp ladder. Lanes (2-5) PCR amplification of 240 bp region in the second gene of the *bdhA* operon using SL9721/SL972 primer pair. Lanes (6-9) PCR amplification of 130 bp internal region in *bdhA* using 3hbd-Q F/3hbd-Q R primer pair. cDNA from the transformant was used as template in lane 2 and 6. Genomic DNA from the suspected mutant was used as template in lane 4 and 8. DNA from the parent strain was used as template in lane 3 and 7. Lane 5 and 9: no DNA template as control.

Given the inability to obtain a *bdhA* mutant using the erythromycin resistance cassette, I decided to use the kanamycin resistance cassette to construct the *bdhA* mutant. A pBluescript *bdhA*::*aphA3* plasmid was constructed. Restriction of pBluescript *bdhA*::*aphA3* using BamHI showed a single band of 5.4 kb, the expected size of the plasmid (data not shown). PCR analysis using the pBluescript *bdhA*::*aphA3* as template and the SL960/SL963 primer pair yielded the expected 2.4 kb band for the *bdhA*::*aphA3* construct (Fig. 5.3). The above results indicate the successful construction of the *bdhA*::*aphA3* construct.

L. pneumophila JR32 was electroporated with the *bdhA::aphA3* fragment and transformants were selected on BCYE containing kanamycin. Fifty-four kanamycin resistant colonies were screened by colony PCR amplification of the 130 bp internal region in the *bdhA* gene using the 3hbd-Q F/3hbd-Q R primer pair. Results revealed a colony lacking this region (Fig 5.4). This colony was considered as a candidate *bdhA* mutant and was chosen for further analysis. Amplification of the kanamycin cassette using the SL801/SL823 primer pair yielded an expected 1 kb DNA band (Fig. 5.5A). When the *bdhA* mutant was further analyzed for a larger (280 bp) *bdhA* internal region using the SL982/SL983 primer pair, no PCR product was obtained (Fig. 5.5B). This internal piece was easily amplified from the parent.

To further analyze the *bdhA* mutant, additional PCR reactions were performed, as shown in Figure 5.6A and B. When DNA from the mutant was used as template to PCR amplify the *bdhA* upstream-*aphA3*-*bdhA* downstream fragment using the SL960/SL963 primer pair, a single band with the expected size of 2.4 kb resulted (Fig. 5.6A). Similarly, the expected 1.7 kb *aphA3*-*bdhA* downstream fragment, 1.7 kb *bdhA* upstream-*aphA3* fragment, and 2 kb *bdhA::aphA3*-*bdhA* downstream fragment plus region in the downstream gene were amplified from the mutant using the primer pairs SL801/SL963, SL960/SL823, and SL801/SL972, respectively (Fig. 5.6A).

When PCR was performed on the mutant using 3hbd-Q F/SL972 and SL960/ 3hbd-Q R primer pairs, no product was obtained (Fig. 5.6B). The expected products, 1.2 kb and 1.1 kb respectively, were easily obtained from the parent.

To further confirm the inactivation of *bdhA*, RT-PCR was performed. First, efficient DNase treatment of RNA from the *bdhA* mutant was confirmed by PCR amplification of the 2.3 kb region in *rpoS* gene using the SL956/SL959 primer pair. As shown in Figure 5.7A, a

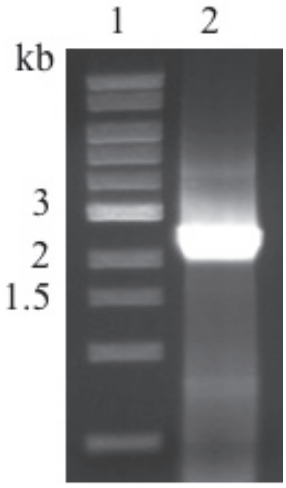


Fig.5.3. Agarose gel electrophoresis of *bdhA* upstream-*aphA3*-*bdhA* downstream fragment amplified from pBluescript *bdhA::aphA3* plasmid using SL960/SL963 primer pair. Lane (1) 1 kb ladder and lane (2) PCR product showed the expected size of 2.4 kb.

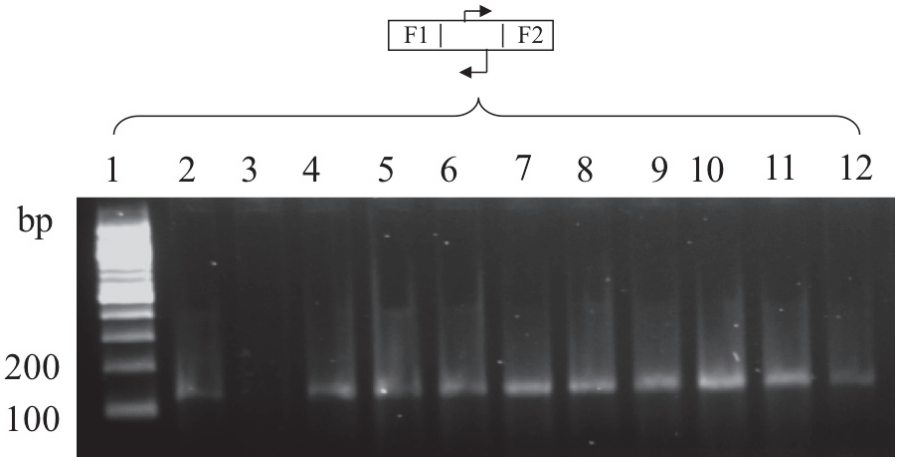


Fig.5.4. PCR screening of kanamycin-resistant *Legionalla* transformants. Lane (1) 100 bp ladder. Lane (3) no PCR band was shown for the candidate *bdhA* mutant. Lane (2 and 4-12) other transformants showing a PCR product of 130 bp.

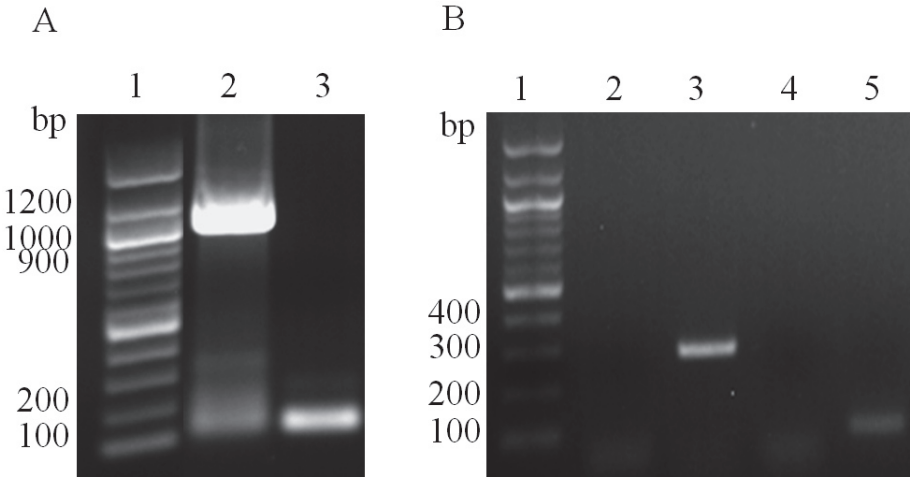


Fig.5.5. PCR analysis of the *Legionella bdhA* mutant. Panel (A) PCR amplification of the kanamycin resistance cassette. Lane (1) 100 bp ladder, lane (2) PCR product (1kb) of the *aphA3* gene appeared, and lane (3) internal region in *rpoS* gene (90 bp) was amplified as control using DNA from *bdhA* mutant and RpoS-Q F/ RpoS-Q R primer pair. Panel (B) PCR amplification of the internal region of *bdhA* gene (280 bp). Lane (1) 100 bp ladder, lane (2) No PCR product amplified when *bdhA* DNA from the mutant was used as the template, lane (3) PCR using parent DNA as template showed band with the expected size (280 bp), lane (4) no DNA template as control, and lane (5) the internal region in *rpoS* gene (90 bp) was amplified from the *bdhA* mutant DNA using the RpoS-Q F/ RpoS-Q R primer pair.

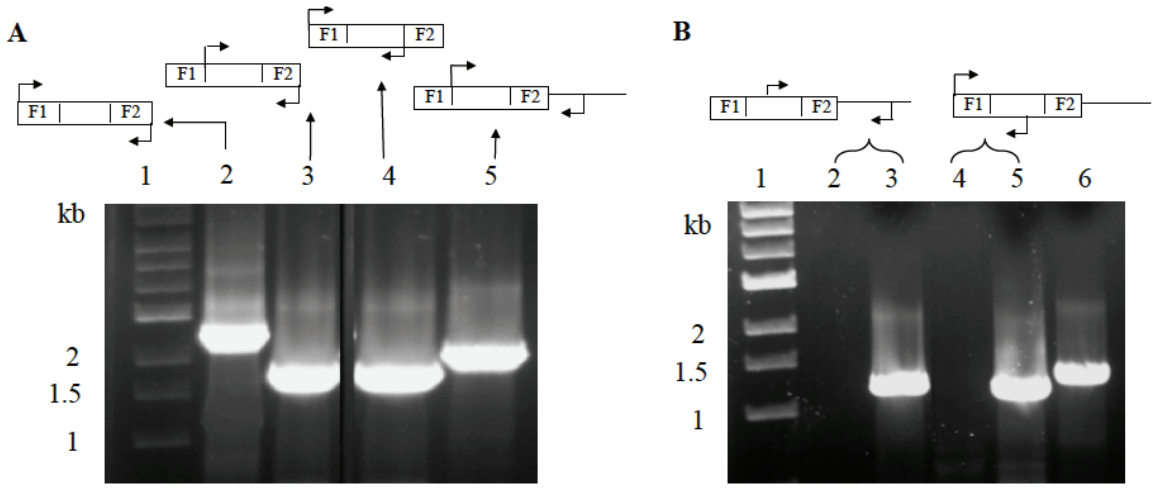


Fig.5.6. Further analysis of the *Legionella bdhA* mutant. Panel (A) PCR amplification of different regions using DNA from the *bdhA* mutant as template. Lane (1) 1 kb ladder, lane (2) amplification of *bdhA* upstream-*bdhA*::*aphA3*-*bdhA* downstream fragment, lane (3) amplification of *bdhA*::*aphA3*-*bdhA* downstream fragment, lane (4) amplification of *bdhA* upstream- *bdhA*::*aphA3* fragment, and lane (5) amplification of *bdhA*::*aphA3*-*bdhA* downstream fragment with region in the second gene of the operon. Panel (B) PCR amplification of different regions using DNA from the parent and *bdhA* mutant strains as the templates. Lane (1) 1 kb ladder, lanes (2 and 4) no PCR products appeared when DNA from the *bdhA* mutant was used, lanes (3 and 5) PCR products appeared when DNA from the parent was used, and lane (6) 1.3 kb region in *rpoS* gene was amplified as control using DNA from the *bdhA* mutant as template and SL956/RpoS-Q R primer pair.

single band of the expected size 2.3 kb was absent in the DNase-treated RNA from the *bdhA* mutant. Second, cDNA synthesis from the *bdhA* mutant RNA was confirmed by PCR amplification of the 0.7 kb region in *rpoS* gene using the SL956/SL957 primer pair. As shown in Figure 5.7B, a single band of the expected size (0.7 kb) appeared in the cDNA from the *bdhA* mutant. Third, RT-PCR was run to amplify a 370 bp region in *bdhA* gene using the SL982 /3hbd-Q R primer pair. Results showed a single band of the expected size (370 bp) from the cDNA sample of the parent but not from the *bdhA* mutant (Fig. 5.7C). Moreover, RT-PCR results also showed that *bdhA* deletion has no effect on the expression of the downstream gene. When the SL971/972 primer pair was used to amplify 240 bp in the downstream gene, a band with the expected size of 240 bp was detected from the *bdhA* mutant and parent cDNA samples (Fig. 5.7C).

5.2. Complementation of The *bdhA* Mutant

Complementation of the *bdhA* mutation was pursued by introducing the parent *bdhA* gene into the mutant. First, the upstream region of the *bdhA* gene was analysed for the existence of promoters by the Neural Network Promoter Prediction software. Results showed four hypothetical promoters (Figure 5.8). As explained in the Materials and Methods, the *bdhA* gene with one, three, and four upstream promoters was PCR amplified and cloned into pMMB207C plasmid to yield pBdhA1, pBdhA3, and pBdhA4 recombinant plasmids, respectivly. *L. pneumophila* JR32 *bdhA* mutant was electroporated with the pBdhA1, pBdhA3, and pBdhA4 plasmids. Thus, Comp 1P represents *bdhA* mutant complemented with pBdhA1, Comp 3P represents *bdhA* mutant complemented with pBdhA3, and Comp 4P represents *bdhA* mutant complemented with pBdhA4. Transformants were selected on BCYE containing kanamycin, streptomycin, and chloramphenicol. Twenty transformants obtained from each recombinant plasmid were analysed by restriction analysis and PCR. PCR amplification of *bdhA* with one promoter from pBdhA1, using the SL1019/3hbd-Q R primer pair, showed a single band with the expected size of 0.9 kb (Fig. 5.9A). Restriction of the cloned fragment (*bdhA* with one promoter) by double digestion of pBdhA1 with BamHI and PstI showed a single band with the expected band size (0.9 kb) (Fig. 5.9B). Restriction analysis of pBdhA1 by EcoRV resulted in two bands with the expected sizes of 6.4 kb and 3.6 kb (Fig. 5.9C). Similar analysis of pBdhA4 revealed that PCR amplification of *bdhA* with

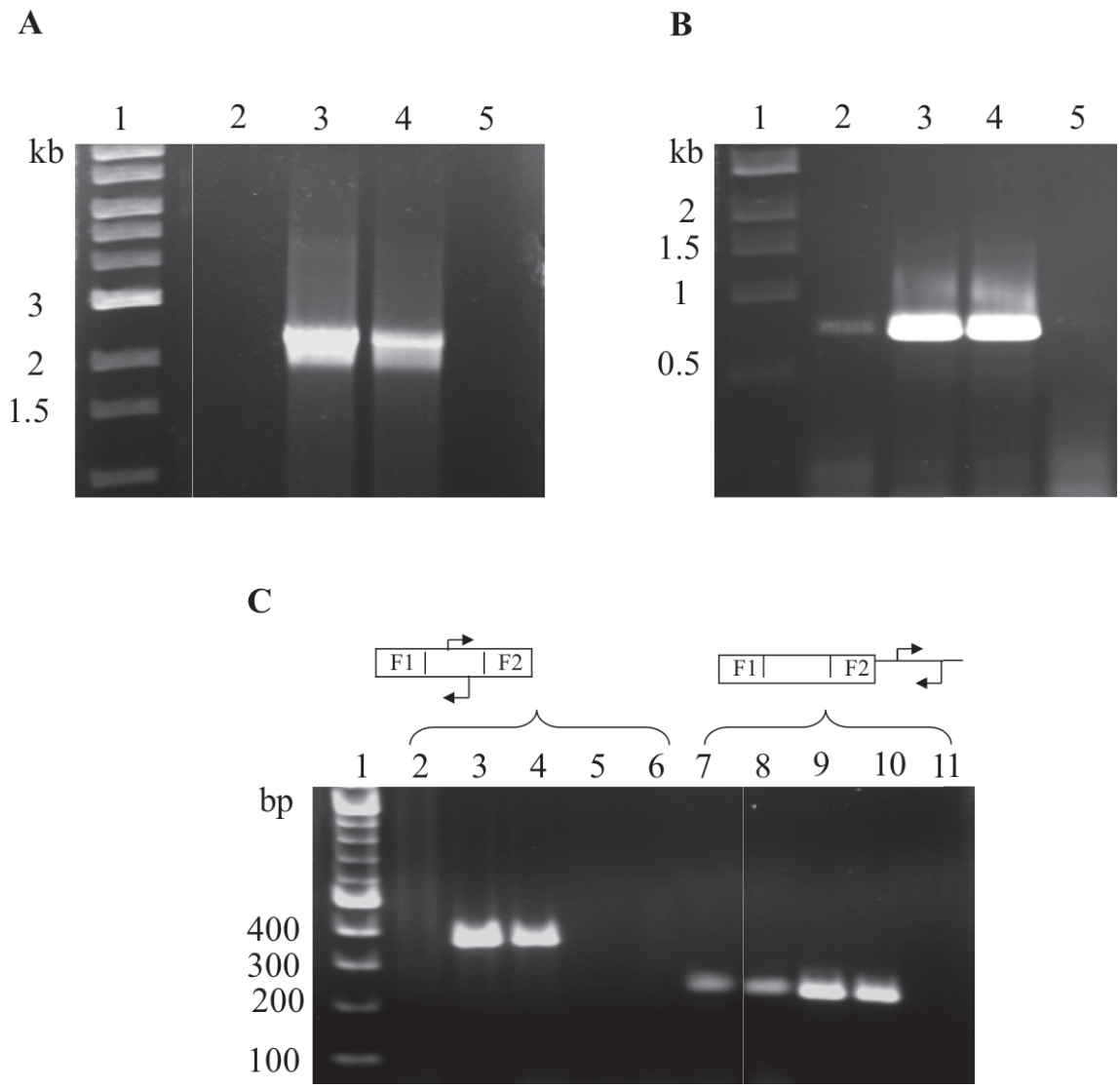


Fig.5.7. Agarose gel electrophoresis showing confirmation of the *bdhA* mutation by RT-PCR. Panel (A) A 2.3 kb region of the *rpoS* gene was amplified to check for the efficiency of DNase treatment of the *bdhA* mutant RNA. Lane (1) 1 kb ladder. DNase treated RNA from the *bdhA* mutant (lane 2), DNA from the parent (lane 3), DNA from the *bdhA* mutant (lane 4) were used as template. Lane (5) no DNA template as control. Panel (B) A 0.7 kb region of the *rpoS* gene was amplified to check for the efficiency of cDNA synthesis from the *bdhA* mutant. Lane (1) 1 kb ladder. cDNA from the *bdhA* mutant (lane 2), DNA from the parent (lane 3), DNA from the *bdhA* mutant (lane 4) were used as the templates. Lane (5) no DNA template as control. Panel (C) RT-PCR analysis of the *bdhA* gene and the downstream gene expressions in the *bdhA* mutant. Lane (1) 100 bp ladder, lanes (2-6) PCR amplification of a 370 bp internal piece in the *bdhA* gene, lanes (7-11) PCR amplification of 240 bp internal piece in the downstream gene. cDNA from the *bdhA* mutant was used as template in lane 2 and 7. Genomic DNA from the *bdhA* mutant was used as template in lane 5 and 10. cDNA from the parent strain was used as template in lane 4 and 8. DNA from the parent strain was used as template in lane 3 and 9. Lanes (6 and 11) no DNA template as control.

Upstream gene: encode hypothetical protein

bdhA gene: encode 3-hydroxybutyrate dehydrogenase

Downstream gene: encode transmembrane protein

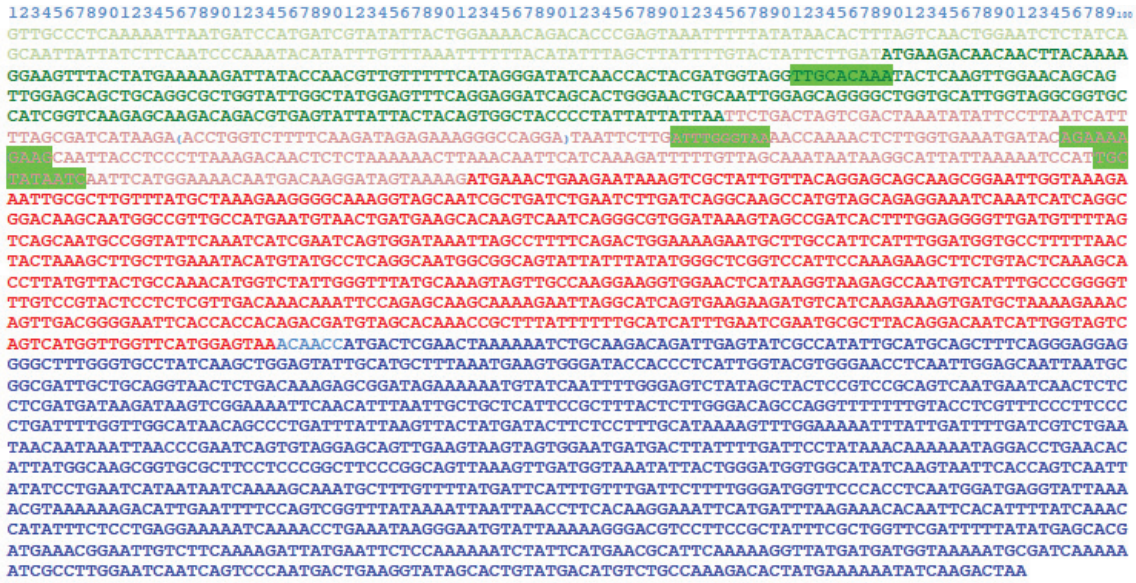


Fig.5.8. Analysis of upstream region of the *bdhA* gene by the Neural Network Promoter Prediction software revealed 4 hypothetical promoters (green-highlighted sequences). The upstream hypothetical gene is given in green. The *bdhA* gene is given in red. The downstream gene in the *bdhA* operon, encoding transmembrane protein, is given in blue.

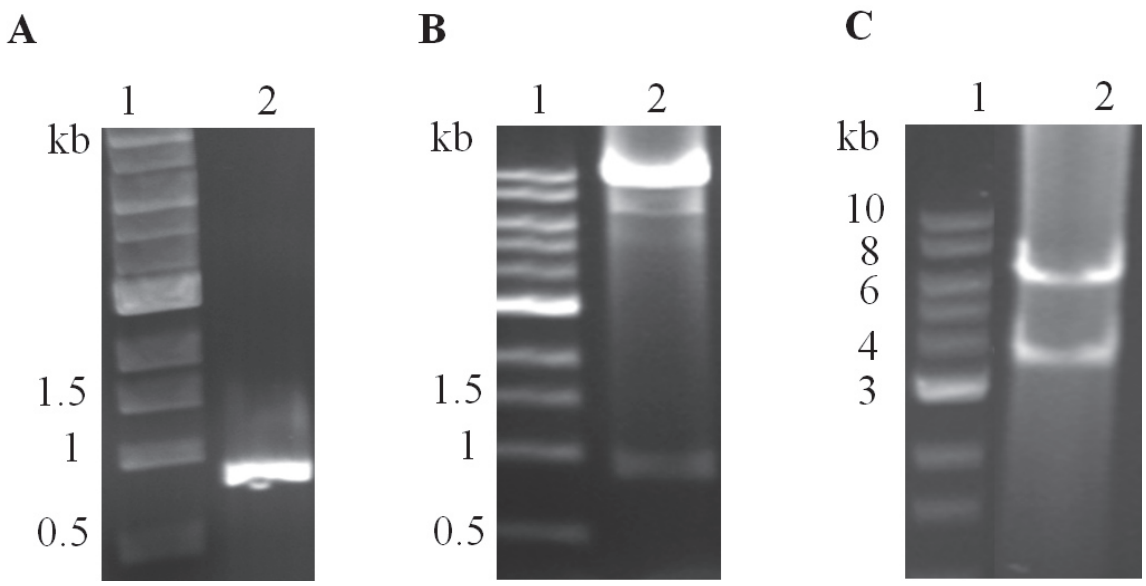


Fig.5.9. Agarose gel electrophoresis of pBdhA1. Panel (A) PCR amplification of *bdhA* gene with one promoter from the pBdhA1. Lane (1) 1 kb ladder, and lane (2) PCR product showed the expected size of the cloned fragment (0.9 kb). Panel (B) Double digestion of pBdhA1 by BamHI and PstI results in the excision of the cloned fragment. Lane (1) 1 kb ladder and lane (2) the expected size (0.9 kb) of the cloned fragment (*bdhA* and one promoter). Panel (C) Restriction analysis of pBdhA1 by EcoRV yielded in two fragments. Lane (1) 1 kb ladder and lane (2) two bands 6.4 kb and 3.6 kb were expected from EcoRV digestion.

four promoters fragment, using the SL1018/3hbd-Q R primer pair, showed a single band of the expected size (1.1 kb) (Fig. 5.10A). Restriction of the cloned fragment (*bdhA* with four promoter) by double digestion of pBdhA4 with BamHI and PstI showed a single band with the expected band size of 1.1 kb (Fig. 5.10B). Restriction analysis of pBdhA4 by EcoRV resulted in two bands with the expected sizes 6.6 kb and 3.6 kb (Fig. 5.10C). Data for pBdhA3 analysis is not shown because Comp 3P did not restore the parent strain BdhA's enzyme activity.

To control for any effect that the empty pMMB207C plasmid might have had on the complemented mutant, the parent and *bdhA* mutant were transformed with the empty pMMB207C plasmid to yield parent207C and *bdhA207C* strains, respectively. To confirm the expected size of the empty pMMB207C plasmid from the parent207C and *bdhA*207C, restriction analysis of pMMB207C was conducted. EcoRV digestion resulted in two bands of the expected size (5.5 kb and 3.6 kb) (Fig. 5.11A), while restriction analysis of pMMB207C by PstI, which has a single cut site in this plasmid, linearized the pMMB207C into a single band of the expected size (9 kb) (Fig. 5.11B).

5.3. BdhA Enzyme Activity

BdhA enzyme activity was tested to further confirm the mutation in the *bdhA* gene, as well as the regaining of activity in the complemented mutant. The results showed that the parent strain exhibited enzyme activity reaching maximal (0.469 unit/mg) after 31 minutes. The *bdhA* mutant showed no enzymatic activity and the heat-inactivated protein samples showed a background value of 0.126 unit/mg (Fig. 5.12). The Comp 1P and Comp 4P strains showed enzyme activity reaching maximal (0.489 unit/mg) after 6 minutes. Thus, these two complemented mutants exhibited higher enzyme activity compared to the parent strain. Interestingly, the Comp 3P strain showed no enzymatic activity, indicating that the sequence upstream of the *bdhA* gene could play a role in its expression—probably by providing binding sites for some of the regulatory DNA binding proteins which were predicted using the Virtual Footprint Promoter Matches website (http://prodoric.tu-bs.de/vfp/index2.php). Finally, the parent207C strain exhibited enzyme activity similar to the parent strain while the *bdhA207C* strain showed no enzymatic activity, similar to the *bdhA* mutant, indicating that the enzyme activity observed in Comp 1P and Comp 4P was due to the cloned *bdhA* gene.

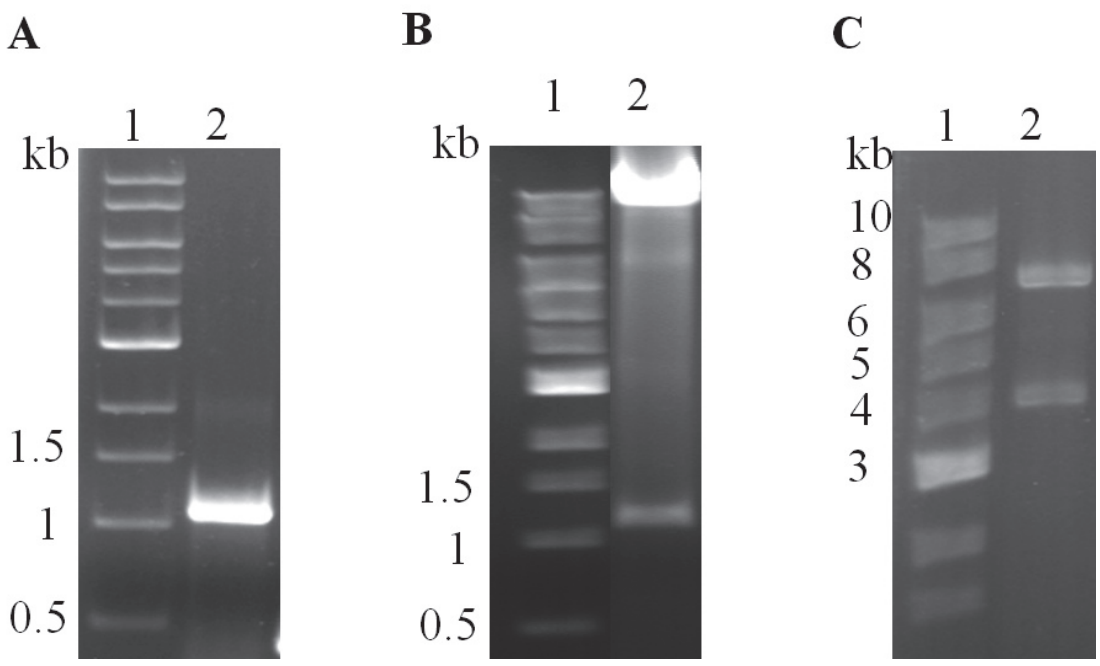


Fig.5.10. Agarose gel electrophoresis of pBdhA4. Panel (A) PCR amplification of *bdhA* gene with 4 promoters from the pBdhA4. Lanel (1) 1 kb ladder, and lane (2) PCR product showed the expected size of the cloned fragment (1.1 kb). Panel (B) Double digestion of pBdhA4 by BamHI and PstI results in the excision of the cloned fragment. Lane (1) 1 kb ladder and lane (2) the expected size (1.1 kb) of the cloned fragment (*bdhA* and 4 promoters). Panel (C) Restriction analysis of pBdhA4 by EcoRV yielded in two fragments. Lane (1) 1kb ladder and lane (2) two bands 6.6 kb and 3.6 kb were expected from EcoRV digestion.

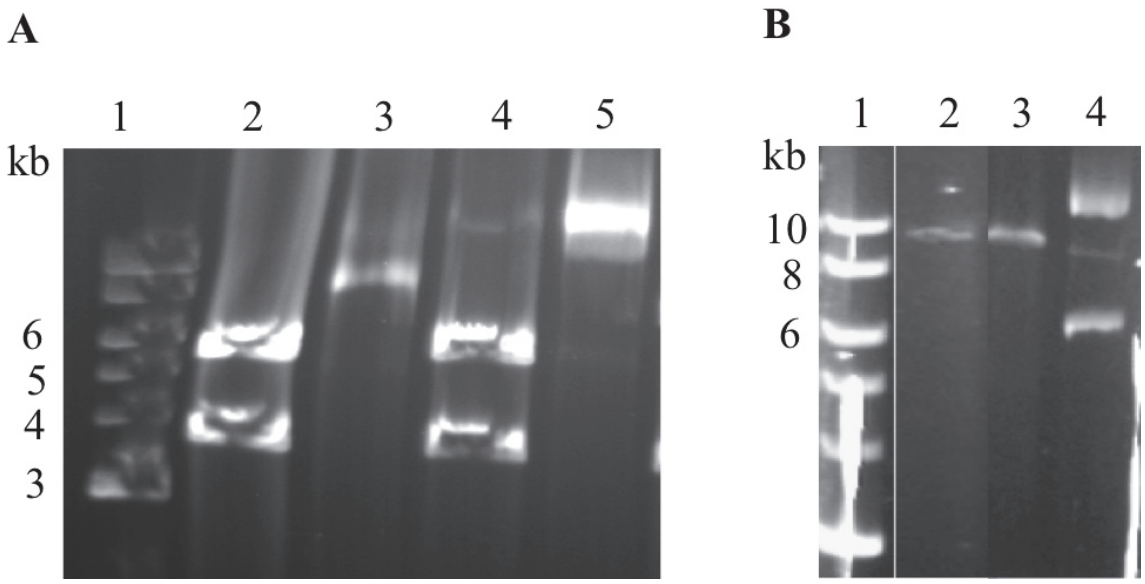


Fig.5.11. Agarose gel electrophoresis of pMMB207C isolated from the parent207C and *bdhA207C* strains. Panel (A) Restriction analysis of pMMB207C by EcoRV results in two fragments of 5.5 kb and 3.6 kb. Lanel (1) 1 kb ladder. pMMB207C from the parent207C (lane 2 digested and lane 3 undigested). pMMB207C from the *bdhA207C* (lane 4 digested and lane 5 undigested). Panel (B) Restriction analysis of pMMB207C by PstI (single cut) showed 9 kb band. Lanel (1) 1 kb ladder, lane (2) digested pMMB207C from the *bdhA207C*, lane (3) digested pMMB207C from the parent207C, and lane (4) undigested pMMB207C.

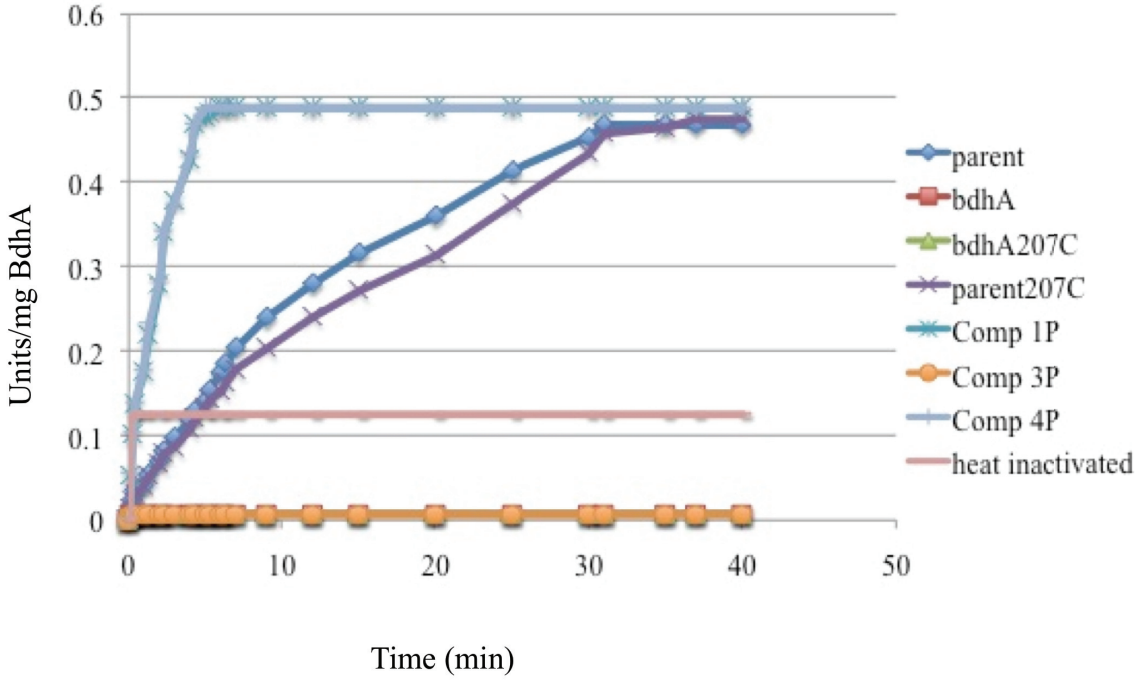


Fig.5.12. BdhA enzyme activity of protein extracts from *L. pneumophila* strains. The graph represents enzyme activity (units/mg BdhA) versus time, in which the amount of NADH produced is monitored. All assays were performed with crude whole cell lysates with a total content of 138 mg of protein per sample. BdhA enzyme activity was detected from protein extracts from the parent, parent207C, Comp 1P, and Comp 4P but not that from the *bdhA* mutant, *bdhA207C*, and Comp 3P. Protein extracts from heat inactivated *L. pneumophila* samples showed a background value of 0.126 unit/mg. Results are means ± SD (n=3). Results were from independent experiments. The error bars were not shown for clearity.

5.4. Growth Kinetics of Culturable *L. pneumophila* Strains

The *L. pneumophila bdhA* mutant, *bdhA*207C, parent207C, and the complemented strains displayed similar growth kinetics to the parent strain JR32 in BYE broth. Thus, knocking out *bhdA* has no effect on growth (Fig. 5.13).

5.5. Morphological Features of Culturable *L. pneumophila bdhA* Mutant

SPFs produced from the *L. pneumophila bdhA* mutant displayed a morphology similar to that of the parent strain, as evident from TEM studies indicating that *bdhA* mutation has no effect on *L. pneumophila* differentiation into SPF (Fig.5.14). However, the number of inclusions (I) in the *bdhA* mutant was significantly higher (0.5 I/cell, n=1000) ($P < 0{\cdot}0001$) compared to the parent strain (0.35 I/cell, n=1000).

5.6. Differentiation of *L. pneumophila bdhA* Mutant SPF into MIF

Similar to the parent strain, *L. pneumophila bdhA* mutant SPFs successfully infected the *A. castellanii* monolayer and efficiently produced MIFs, which displayed morphology comparable to MIFs produced from the parent strain (Fig.5.15A). Also similar to the parent strain, the *bdhA* mutant SPF could infect *Tetrahymena* spp., and the infection resulted in differentiation of SPF into MIF (Fig.5.15B-E). Thus, *bdhA* mutation does not seem to have a role in *L. pneumophila* differentiation into MIFs. Giménez stain reaction of the culturable *bdhA* mutant SPFs and MIFs bacteria were also comparable to the parent strain, with both displaying a Gimenez+ reaction. (Fig.5.15F-H).

5.7. Production of VBNC Cells from *L. pneumophila bdhA* Mutant in dd- and Tap Water

Next, I examined the ability of the *bdhA* mutant to form VBNC. When inoculated in dd-water, the *bdhA* mutant SPF lost culturability in 15 days while the parent strain's culturability lasted 18 days (Fig. 5.16A). The difference was statistically significant ($P = 0{\cdot}05$). In another experiment (after a long time from the first one) the *bdhA* mutant SPF lost culturability in 9 days while the parent strain's culturability lasted 15 days (Fig. 5.16B). The complemented mutants Comp 1P and Comp 4P showed similar culturability to the parent (Fig. 5.16B). The parent strain and the strain carrying the empty plasmid (parent207C)

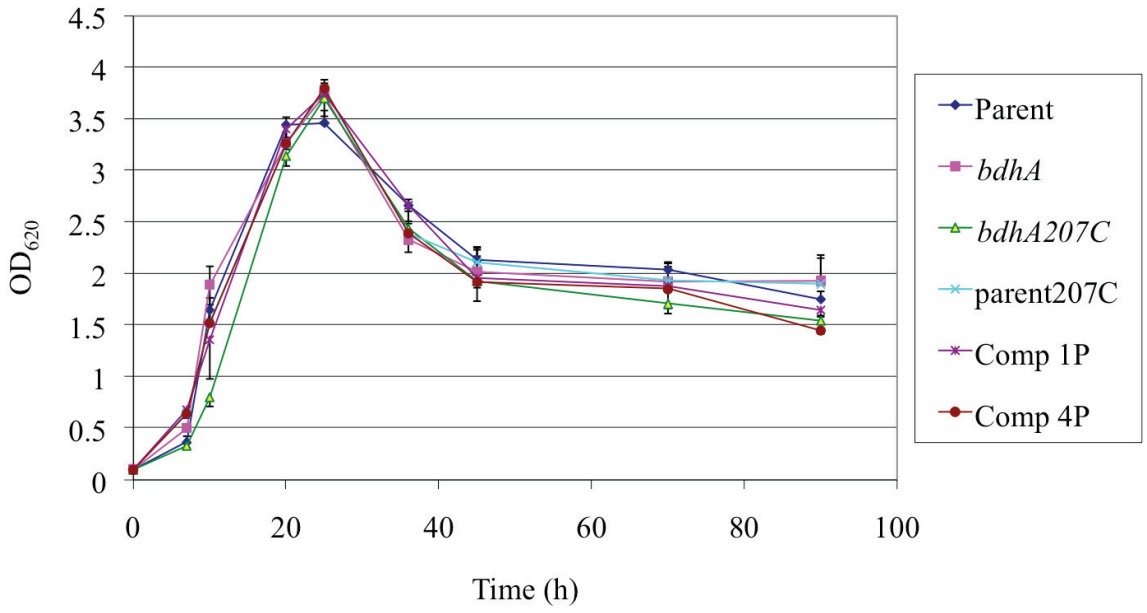


Fig.5.13. Growth kinetics of *L. pneumophila* parent, *bdhA* mutant, *bdhA*207C, parent207C, Comp 1P and Comp 4P strains. Following inoculation of BYE broth with 0.1 OD_{620} of *L. pneumophila* cells, all the strains used entered stationary phase of growth at ~ 20 h.

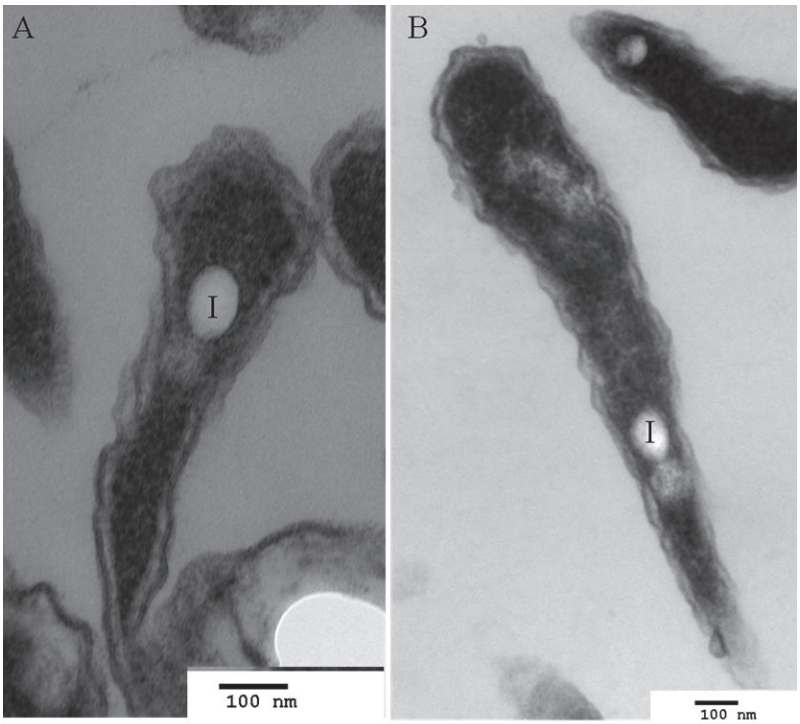
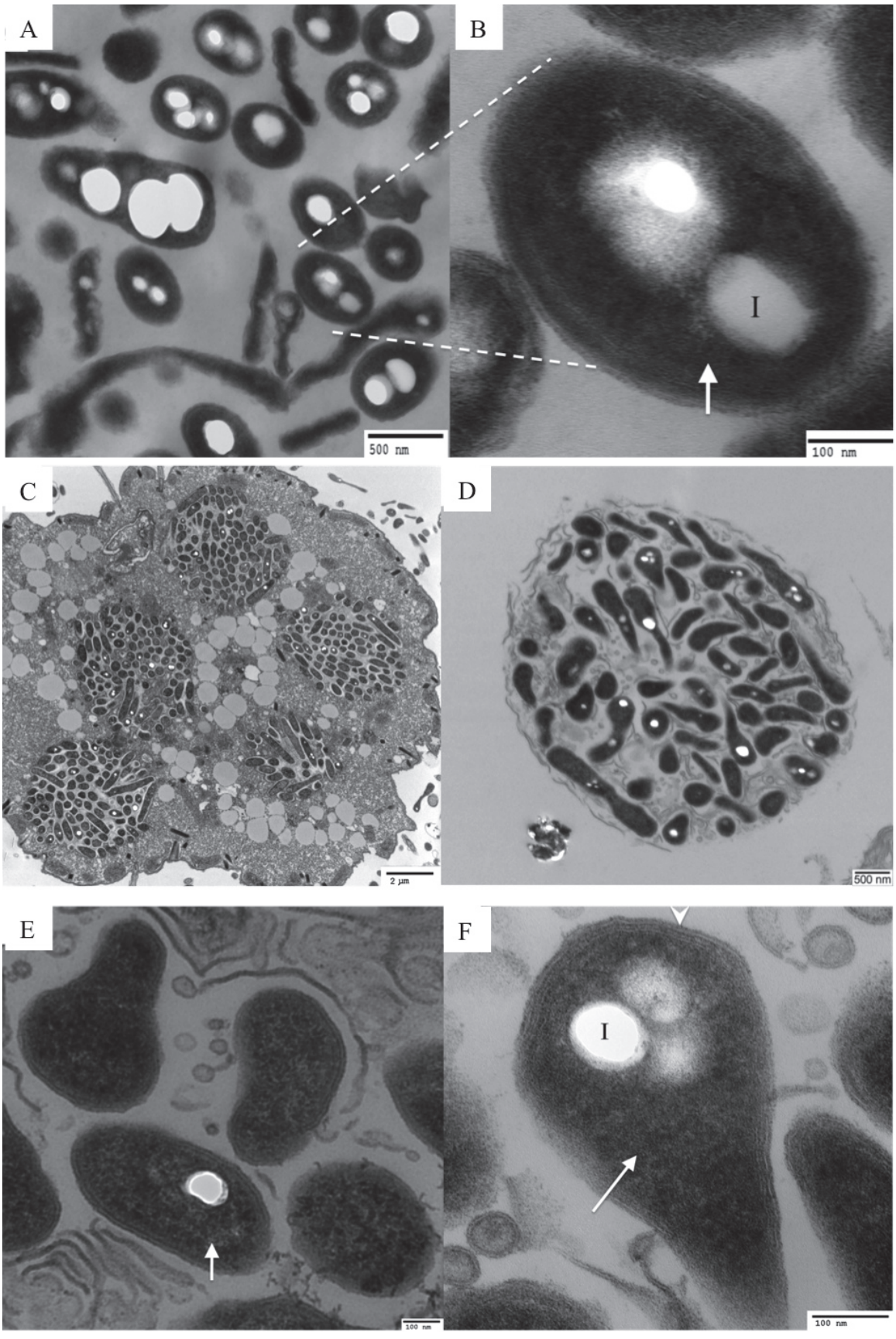


Fig.5.14. TEM micrographs of culturable SPF of *L. pneumophila bdhA* mutant (A) showing morphology similar to the parent strain (B). Bacteria appear as rods displaying wavy outer membranes, well-defined inner membranes, obvious periplasmic spaces, and a uniformly dense cytoplasm with presence of inclusions ("I").


A
500 nm
B
I
100 nm
C
2 µm
D
500 nm
E
100 nm
F
I
100 nm

Continuation

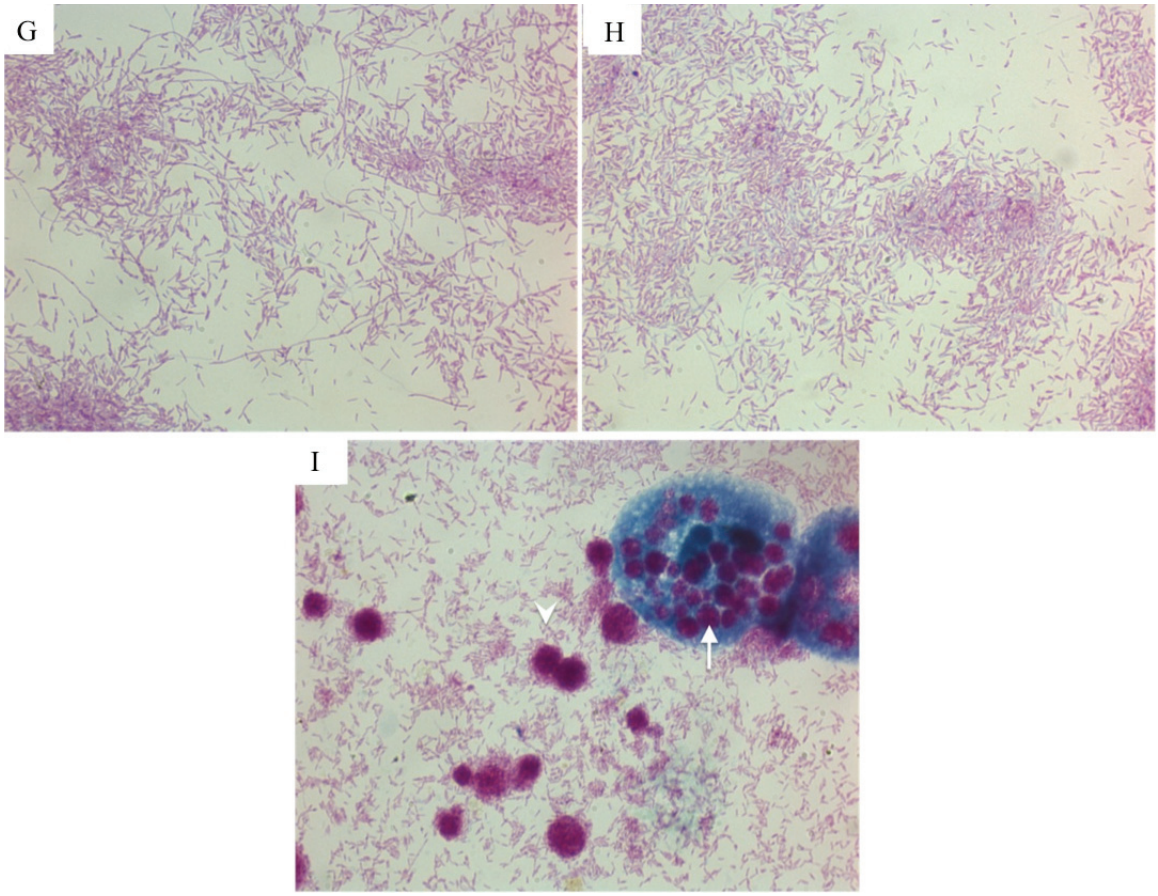


Fig.5.15. The *bdhA* mutant SPFs differentiate into MIFs. Panel (A-F) TEM of MIFs produced from *bdhA* mutant SPFs. (A) MIFs produced from *bdhA* mutant SPFs after infecting *A. castellanii*. (B) Magification of one of the *bdhA* mutant MIFs showen in (A). Panel (C-F) MIFs produced from *bdhA* mutant SPFs after infecting *Tetrahymena* spp. MIFs produced from *bdhA* mutant SPFs resist digestion inside *Tetrahymena* food vacuoles (C) and are expelled in pellets (D). MIFs produced by infection of either amoeba or *Tetrahymena* spp. by *bdhA* mutant SPFs displayed morphology similar to MIFs produced by parent strain. Note the presence of inclusions "I" (B and F), straight outer membranes, thick inner leaflet of outer membranes, absence of periplasmic space (arrow head) (F), and dense cytoplasm (arrow) (B, E and F). Panel (G-I) Gimenez stain reaction of *L. pneumophila bdhA* mutant SPFs and MIFs. *L. pneumophila bdhA* mutant SPFs (G) displayed dull red Giménez+ reaction comparable to parent strain (H). (I) *L. pneumophila bdhA* mutant MIFs showed bright red Giménez+ reaction inside *Tetrahymena* food vacuoles (arrow) as well as in the expelled pellets (arrow head).

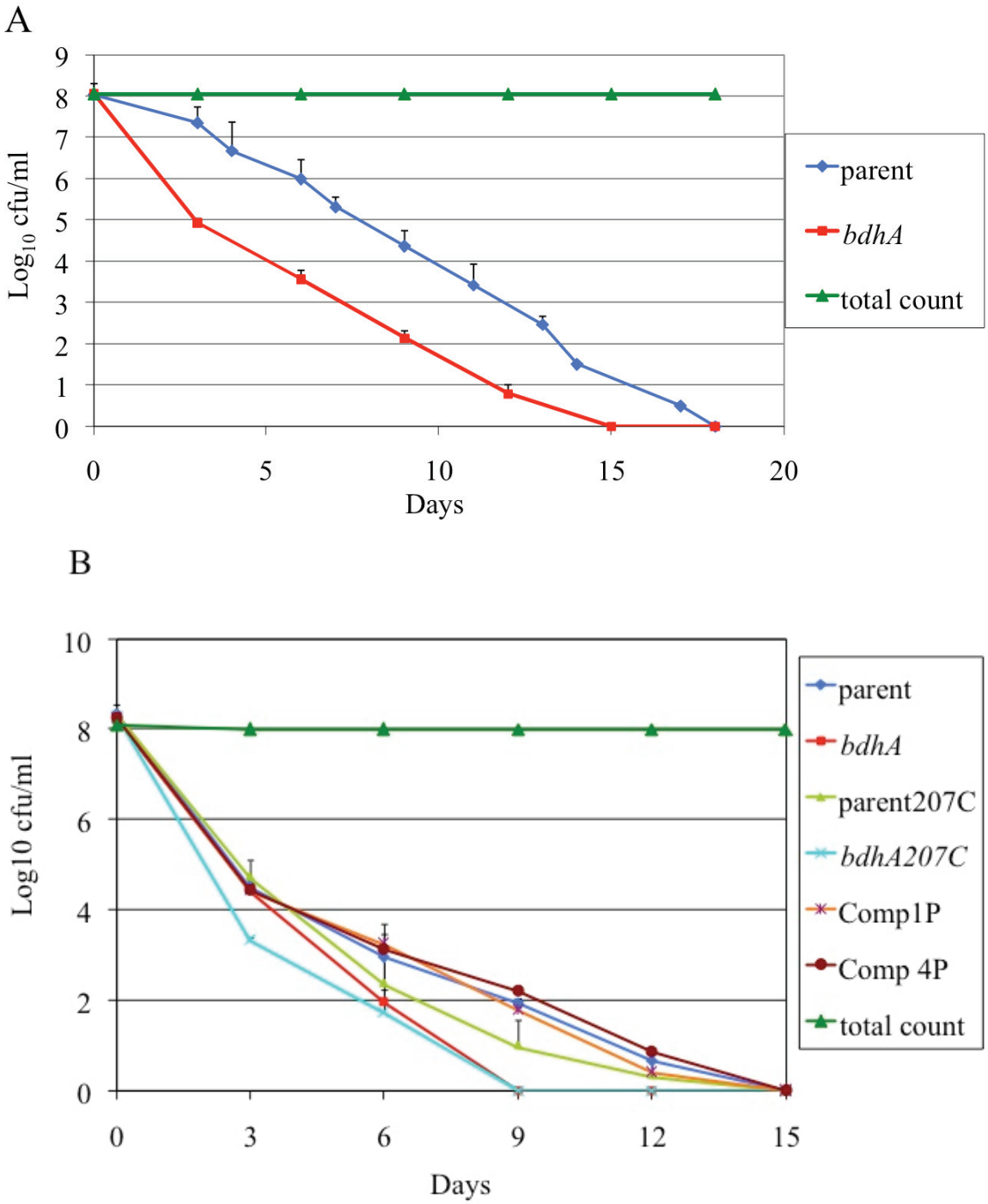


Fig.5.16. BdhA is important for the culturability of *L. pneumophila* SPFs in dd-water. Panel (A) *bdhA* mutant lost culturability earlier (15 days) than the parent strain (18 days). Panel (B) *bdhA* and *bdhA207C* mutant lost culturability earlier (9 days) than the parent, parent207C, Comp 1P and Comp 4P strains (15 days). The experiment was repeated once with similar results. Results are means ± SD (n=3).

showed similar culturability. This is also the case for the *bdhA* mutant with and without the empty plasmid. The difference in culturability between the parent, parent207C, Comp 1P, and Comp 4P SPFs in dd-water compared to that of the *bdhA* mutant and *bdhA207C* SPFs in dd-water was statistically significant ($P = 0{\cdot}05$).

The *bdhA* mutant was not viable, for the same two sets of experiments mentioned above, when bacteria in the dd-water samples lost culturability (Fig.5.17A and B). However, the parent strain viability was 6.2 ± 0.9% (n=3) in the first experiment (Fig.5.17A) and 5.9 ± 0.7% (n=3) in the second experiment. The complemented mutants Comp 1P and Comp 4P showed viability of 6.3 ± 1.3% and 6.8 ± 1.8%, respectively (Fig.5.17B). Similar to the *bdhA* mutant, *bdhA207C* was not viable when bacteria in the dd-water samples lost culturability, whereas parent207C was viable (4.5 ± 1.6%).

The *bdhA* mutant MIF in dd-water entered the VBNC state at day 17 with a final mean viability of 76.6 ± 4.1% (n=2), whereas the parent strain MIF culturability lasted 30 days with a final viability of 87.4 ± 5.7% (n=2). The difference in culturability between the parent and the *bdhA* mutant MIFs in dd-water was statistically significant ($P = 0{\cdot}05$). Interestingly, a dramatic decrease in viability of the mutant was observed beyond the point of complete loss of culturability. At day 90, the final mean percentage of VBNCCs was 11.45 ± 0.35% (n=2) for the mutant strain; however, it was 73.2 ± 1.69% (n=2) for the parent strain (Fig.5.18). Collectively, the above results indicate that BdhA is important for the survival of *L. pneumophila* in dd-water.

In tap-water, the *bdhA* mutant SPF entered the VBNC state at day 38 with a final mean viability of 34.5 ± 2.1% (n=3), which is similar to the parent strain's viability 38.8 ± 1% (n=3) (Fig. 5.19). Both the MIFs from the parent and *bdhA* mutant strains in tap water entered the VBNC state at day 45 with a mean viability of 74.3 ± 4.2% (n=2) and 79±2.1% (n=2), respectively (Fig. 5.20).

Collectively, these results indicate that *bdhA* plays an important role in maintaining the culturability and viability of both SPF and MIF of *L. pneumophila* in dd-water, but not in tap water. Unfortunately, repeated attempts to generate MIFs from complemented mutants were unsuccessful, as the complemented mutants failed to infect amoeba.

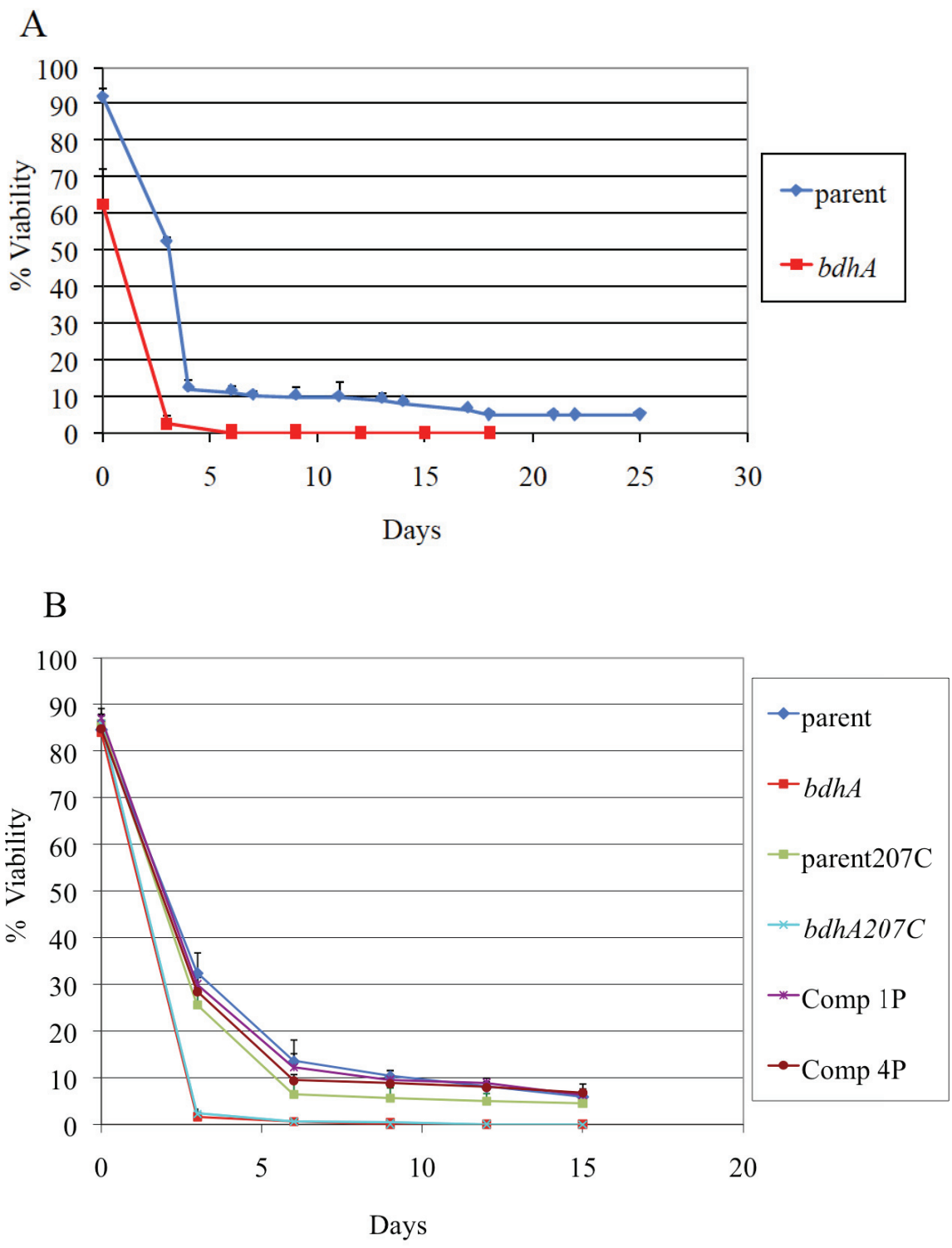


Fig.5.17. BdhA is important for the survival of *L. pneumophila* SPFs in dd-water. Panel (A) At day 18, *bdhA* mutant were not viabile while the parent strain still retained a viability of 6.2 ± 0.9%. Panel (B) At day 15, *bdhA* mutant and *bdhA207C* were not viable while the parent, parent207C, Comp 1P, and Comp 4P strains retained a viability of (5.9 ± 0.7%, 4.5 ± 1.6%, 6.3 ± 1.3%, and 6.8 ± 1.8%) respectively. Results are means ± SD (n=3).

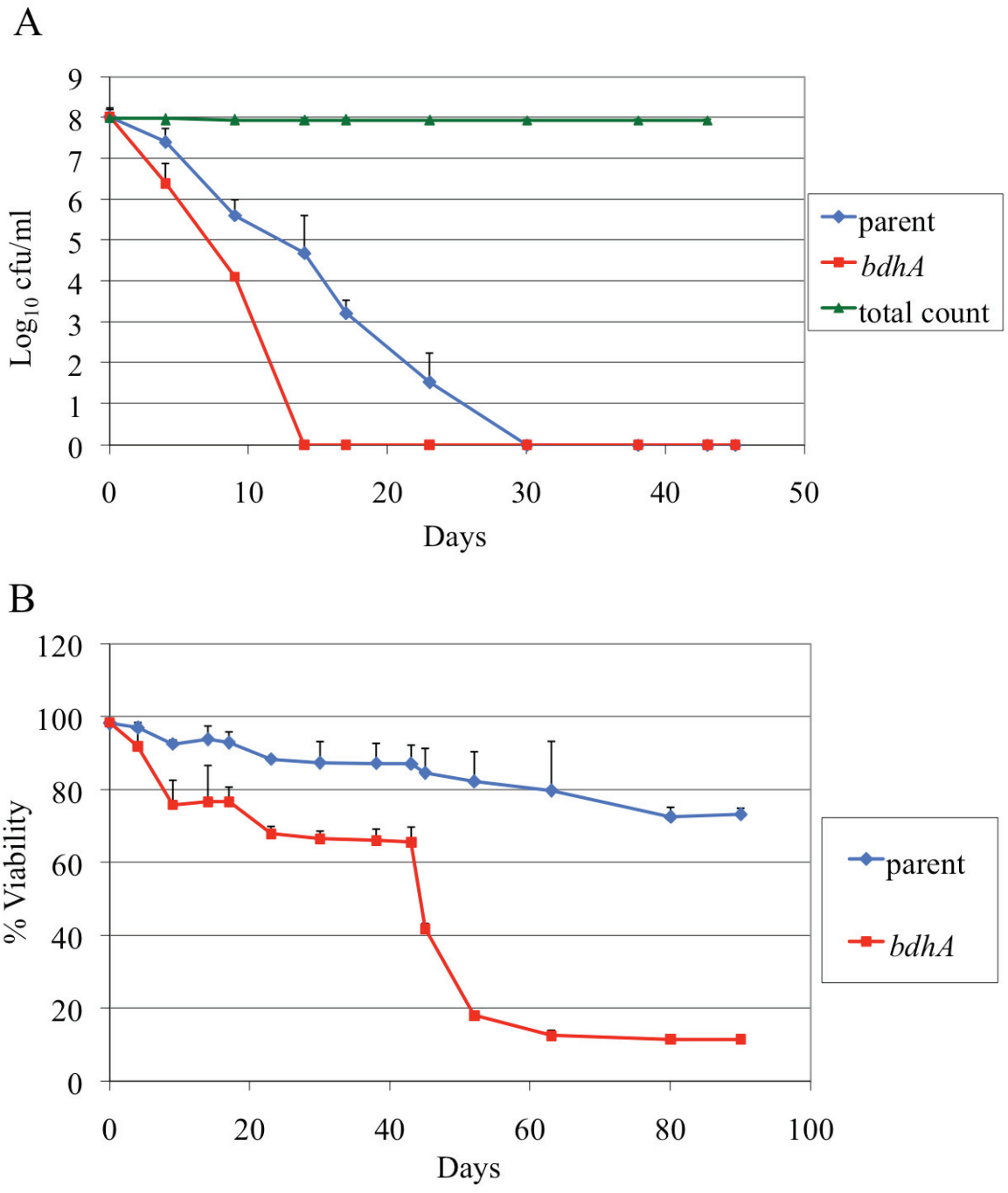


Fig.5.18. BdhA is important for the survival of *L. pneumophila* MIFs in dd-water. Panel (A) *L. pneumophila bdhA* mutant MIF in dd-water lost culturability earlier (day 17) than the parent strain (day 30)). Panel (B) A dramatic decrease in viability of the *bdhA* mutant MIF-VBNCCs (11.45 ± 0.35%) was observed at day 90 compared to the parent MIF-VBNCCs (73.2 ± 1.69% (n=2)). Results are means ± SD (n=2).

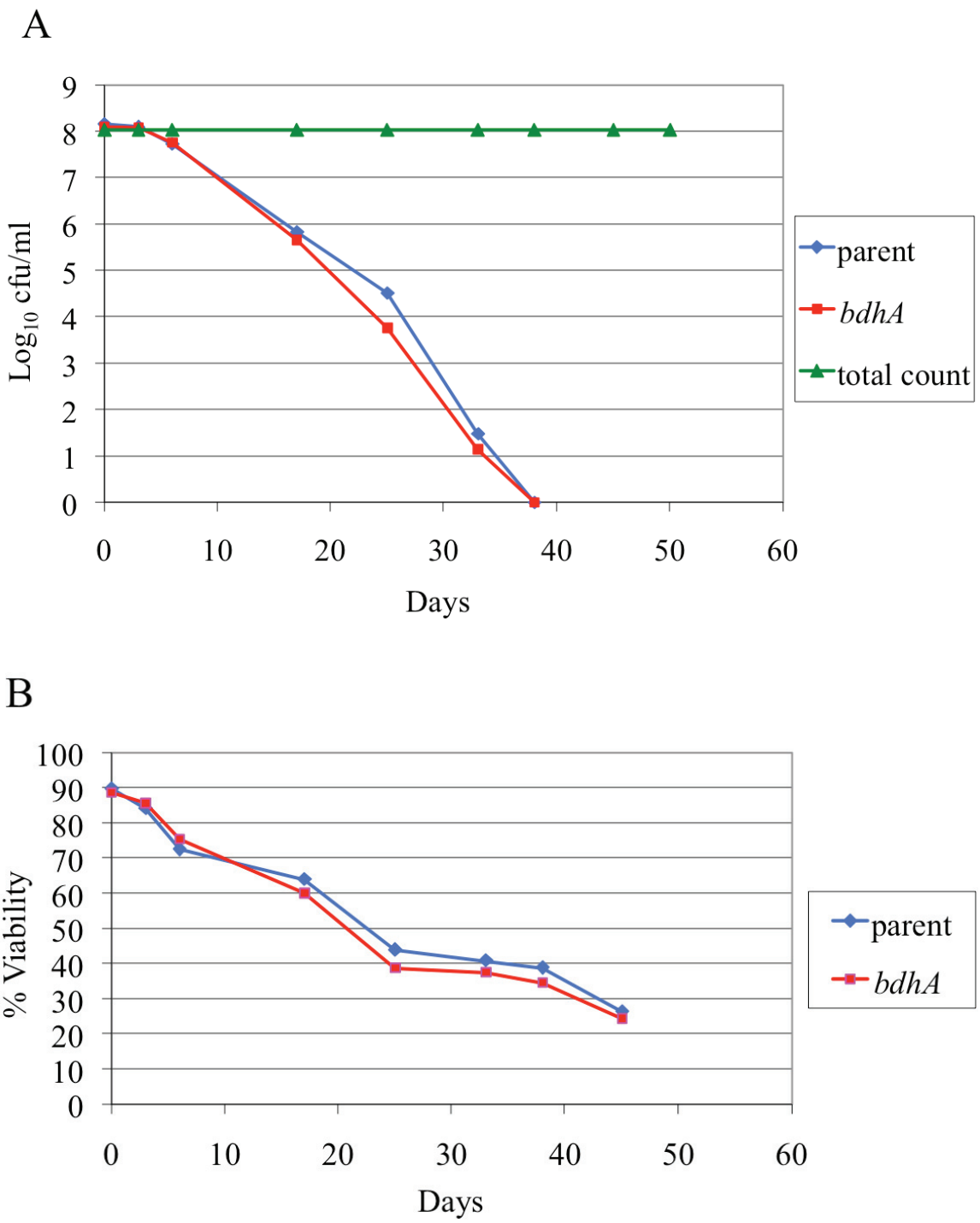


Fig.5.19. The *bdhA* mutant and parent strain SPFs in tap water entered the VBNC state on the same day. Panel (A) Both *bdhA* mutant and the parent strain loss culturability at day 38. Panel (B) *bdhA* mutant SP-VBNC viability was 34.5 ± 2.1% (n=3) similar to the parent strain SPF-VBNC viability (38.8 ± 1% (n=3)). Results are means ± SD (n=3).

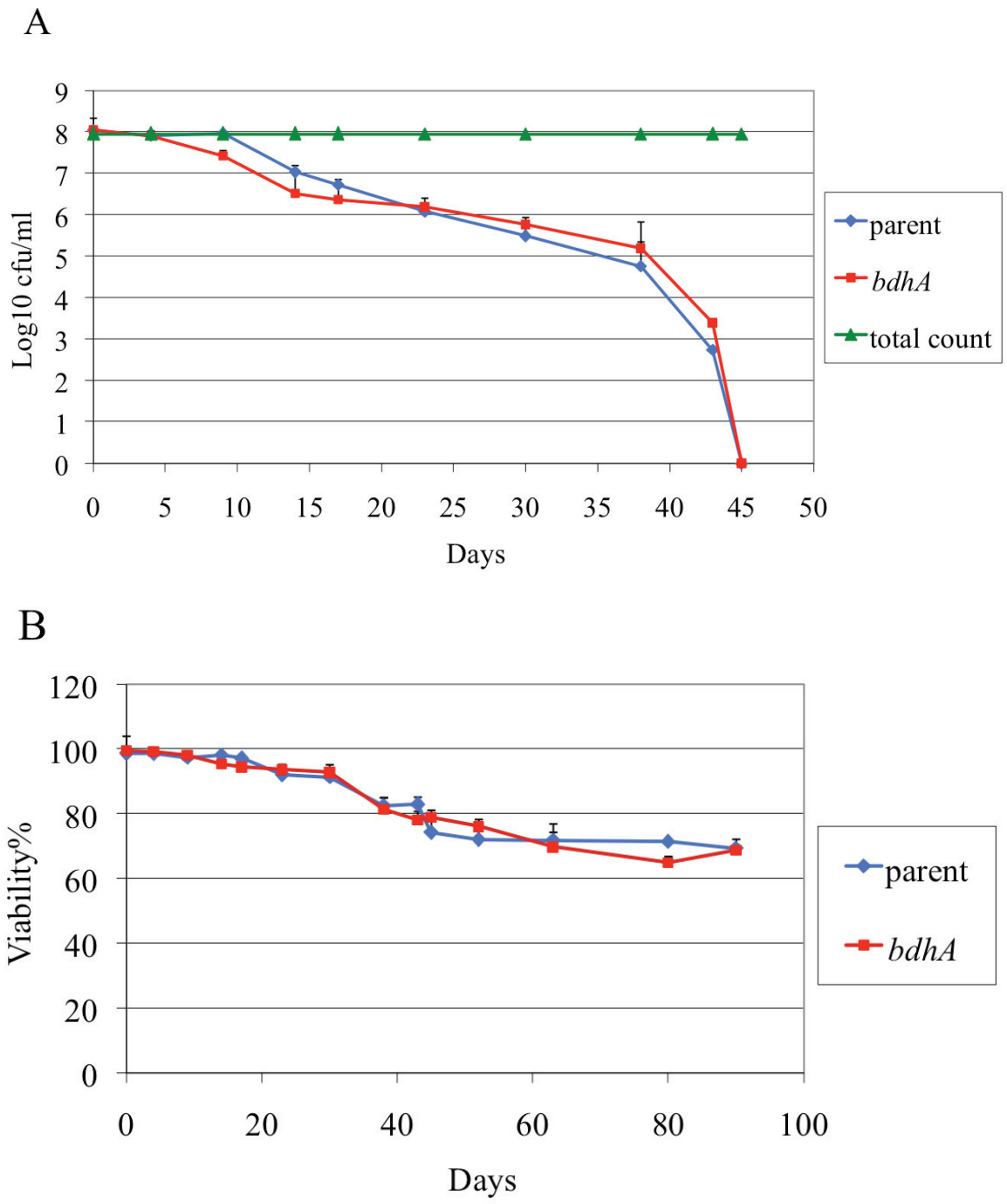


Fig.5.20. The *bdhA* mutant and parent strain MIFs in tap water entered the VBNC state on the same day. Panel (A) Both *bdhA* mutant and the parent strain loss culturability at day 45. Panel (B) *bdhA* mutant MIF-VBNC viability was 74.3 ± 4.2% similar to the parent strain MIF-VBNC viability 79±2.1%. Results are means ± SD (n=2).

5.8. Morphological Features of *L. pneumophila bdhA* Mutant VBNC Cells

5.8.1. Morphology of *L. pneumophila bdhA* Mutant SPFs in dd-Water after Complete Loss of Culturability

After complete loss of culturability, the majority of *bdhA* mutant SPF bacteria in dd-water showed cell lysis which makes it difficult to determine the proportion of morphologically intact cells. Only a few cells appeared morphologically intact, similar to what was observed in the parent cells (Fig. 3.4 B and C). These morphologically intact cells appeared to be surrounded by a continuous cell membrane and still contained cytoplasmic material. They exhibited a rod-like shape, a typical Gram-negative envelope, an electron-translucent cytoplasm poor in ribosomes, cytoplasmic inclusions, and numerous outer membrane vesicles (Fig. 5.21A and B).

5.8.2. Morphology of *bdhA* Mutant VBNCCs Produced from SPFs in Tap Water

Morphology of the SPF *bdhA* mutant after reaching the VBNC state was similar to that described for the parent SPF-VBNCCs (Fig.3.5 B and C). These VBNCCs showed a pear-like appearance (Fig. 5.22A and B). SPF-VBNCCs displayed a rather straight outer membrane with a thickened electron-dense inner leaflet (Fig. 5.22B), a dense cytoplasm (Fig.5.22A), but no vesiculation or inclusions. Finally, similar to the parent strain (Fig. 3.5B and C), dark spots were repeatedly observed in the cytoplasm of the mutant (Fig. 5.22A). These dark spots were also prominently observed before complete loss of culturability.

5.8.3. Morphological Features of *bdhA* Mutant MIF-VBNC Cells in dd- and Tap Water

In dd-water, MIF-VBNC *bdhA* mutant cells showed similar morphological characteristics to those described for the parent VBNC cells in dd-water (Fig. 3.6A) in terms of membrane features, cytoplasm density and the presence of inclusions. These VBNCCs displayed a straight outer membrane, a thickened electron-dense inner leaflet, and a dense cytoplasm with inclusions (Fig. 5.23).

Morphology of the MIF-VBNC *bdhA* mutant cells from tap-water was similar to that described for the parent MIF-VBNCCs (Fig. 3.6B). These MIF-VBNC *bdhA* mutant

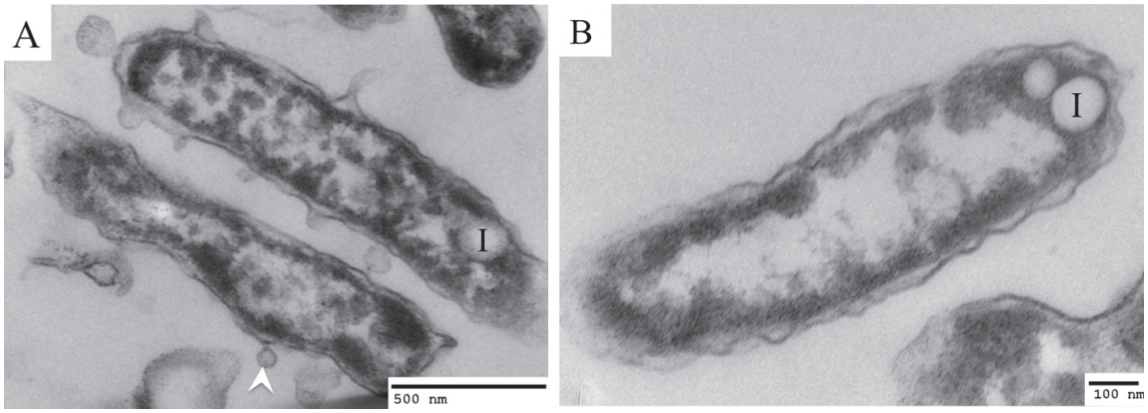


Fig.5.21. TEM of *bdhA* mutant SPFs in dd-water after complete loss of culturability. Panel (A) Numerous outer membrane vesicles (white arrow). Panel (B) Cell exhibiting low density cytoplasm and inclusions “I". See Fig. 3.4B and C TEM of the parent SPFs in dd-water showing low density cytoplasm, inclusions, and outer membrane vesicles.

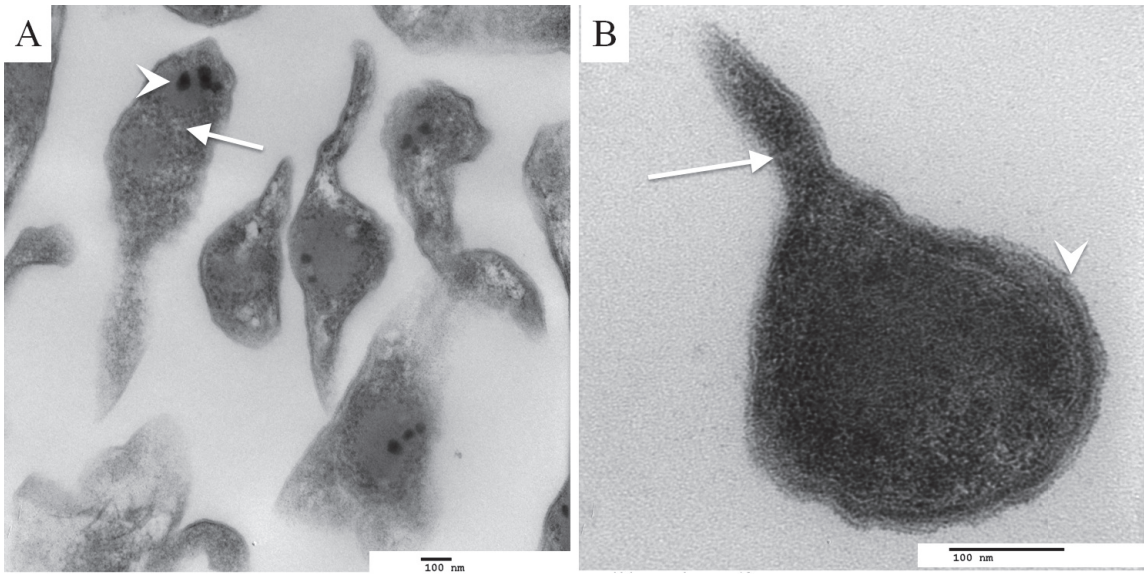


Fig.5.22. TEM of SPF-VBNCCs produced from *bdhA* mutant in tap water. Panel (A) SPF-VBNCCs of the *bdhA* mutant look intact cells with no inclusions. Dark spots were evident (arrow head). These VBNCCs showed electron-dense cytoplasmic material (arrow). Panel (B) SPF-VBNCCs of the *bdhA* mutant exhibited pear-like shape with a lateral pointed protrusion (arrow). These VBNCs displayed a straight outer membrane with a thickened electron-dense inner leaflet and a difficult-to-resolve inner membrane (arrow head). See Fig. 3.5A and B TEM of the parent SPFs in tap water showing pear-like shape cells, electron-dense cytoplasm containing dark spots, no inclusions, straight outer membrane, thickened electron-dense inner leaflet, and a difficult-to-resolve inner membrane.

displayed a straight outer membrane, a thickened electron-dense inner leaflet, and a dense cytoplasm with no inclusions (Fig. 5.24).

5.9. Resistance of VBNC Cells Produced from *L. pneumophila bdhA* Mutant to Detergent-Mediated Lysis

Resistance to SDS-mediated lysis in culturable and VBNC cells obtained from *bdhA* mutant SPFs and MIFs in dd- and tap water was comparable to those obtained from parent strains (Fig. 3.9A). As shown in Figure 5.25, SPF-VBNC *bdhA* mutant cells in tap water and MIF-VBNC *bdhA* mutant cells in dd- and tap water were resistant to SDS-mediated lysis, while SPF *bdhA* mutant cells in dd-water after complete loss of culturability were susceptible to lysis. These results indicate that BdhA has no role in mediating resistance to detergent-mediated lysis in VBNC cells.

5.10. Resistance of *L. pneumophila bdhA* Mutant VBNC Cells to Digestion Inside *Tetrahymena* Food Vacuoles

Deletion of the *bdhA* gene had no effect on the ability of *L. pneumophila* to resist digestion inside *Tetrahymena* food vacuoles since both culturable and VBNC *bdhA* mutant bacteria behaved similarly to the parent strain (Fig. 3.8). Except for *bdhA* mutant SPFs in dd-water, all VBNC cells tested were resistant to digestion inside *Tetrahymena* (Fig.5.26). Moreover, *bdhA* mutant VBNC cells showed Giménez stain reactions similar to those of parent VBNC cells (previously shown, see Figure 3.9). As shown in Figure 5.27, both *bdhA* mutant VBNC cells fed to *Tetrahymena* and *bdhA* mutant VBNC cells in the expelled pellets were Giménez +, displaying a bright red shade (Fig.5.27).

To conclude results from this chapter, BdhA seems to influence *L. pneumophila* survival and VBNC formation. However, the effect on survival was only demonstrated in dd-water, not in tap-water. The *bdhA* mutant showed similar morphological features to the parent. BdhA has no role in resistance of *Legionella* to digestion in *Tetrahymena* food vacuole or to SDS lysis.

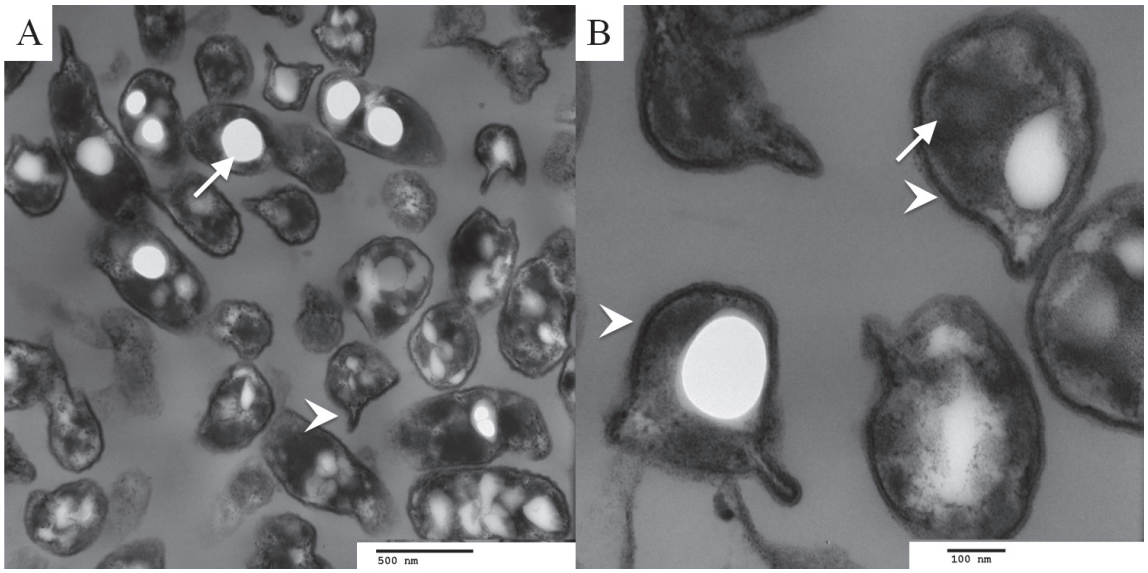


Fig.5.23. TEM of *bdhA* mutant MIF-VBNC cells in dd-water. Panel (A) *bdhA* mutant MIF-VBNCCs appeared as intact cells with inclusions (arrow). These MIF-VBNCCs exhibted pear-like shape with a lateral pointed protrusion (arrow head). Panel (B) *bdhA* mutant MIF-VBNCs displayed a straight outer membrane with a thickened electron-dense inner leaflet and a difficult-to-resolve inner membrane as well as an inconspicuous periplasmic space (arrow head). These MIF-VBNCCs showed electron-dense cytoplasmic material (arrow). See Fig.3.6A TEM of the parent MIF-VBNCs in dd-water exhibiting pear-like shape with a lateral pointed protrusion, electron-dense cytoplasm, inclusions, straight outer membrane, thickened electron-dense inner leaflet, and a difficult-to-resolve inner membrane.

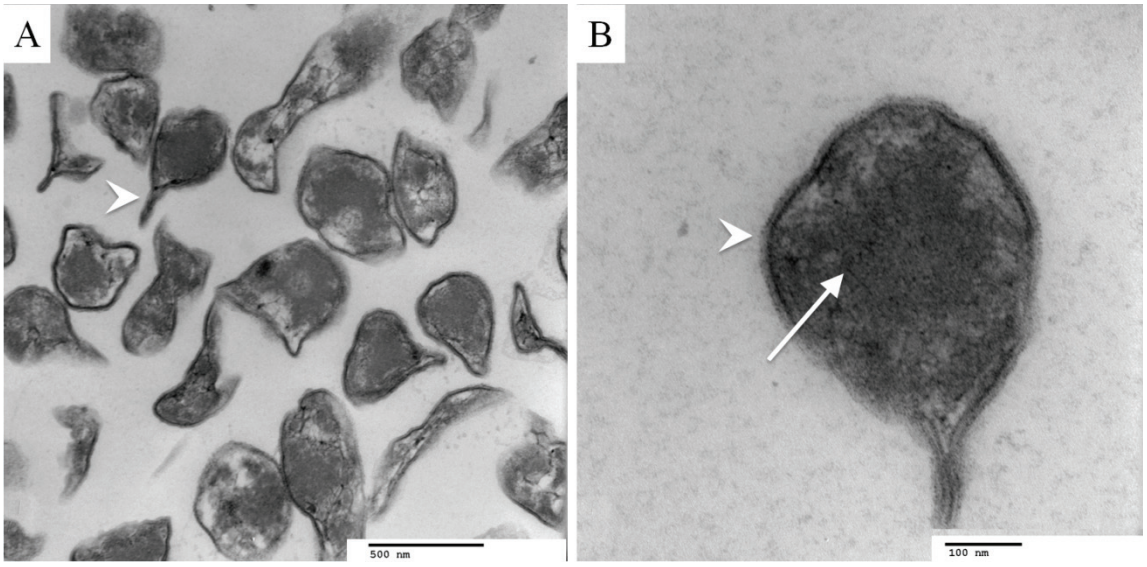


Fig.5.24. TEM of *bdhA* mutant MIF-VBNC cells in tap water. Panel (A) *bdhA* mutant MIF-VBNCCs appeared as intact cells with no inclusions. These MIF-VBNCCs exhibited pear-like shape with a lateral pointed protrusion (arrow head). Panel (B) *bdhA* mutant MIF-VBNCs displayed a straight outer membrane with a thickened electron-dense inner leaflet and a difficult-to-resolve inner membrane as well as an inconspicuous periplasmic space (arrow head). These MIF-VBNCCs showed electron-dense cytoplasmic material (arrow). See Fig.3.6B and C TEM of the parent MIF-VBNCs in tap water exhibiting pear-like shape with a lateral pointed protrusion, electron-dense cytoplasm, straight outer membrane, thickened electron-dense inner leaflet, and a difficult-to-resolve inner membrane.

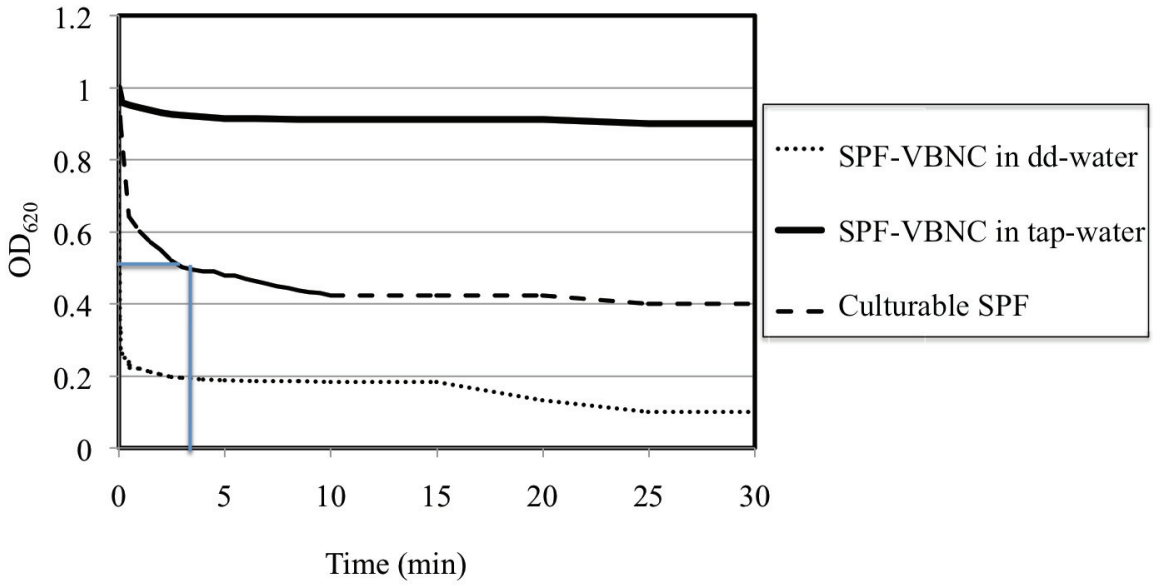
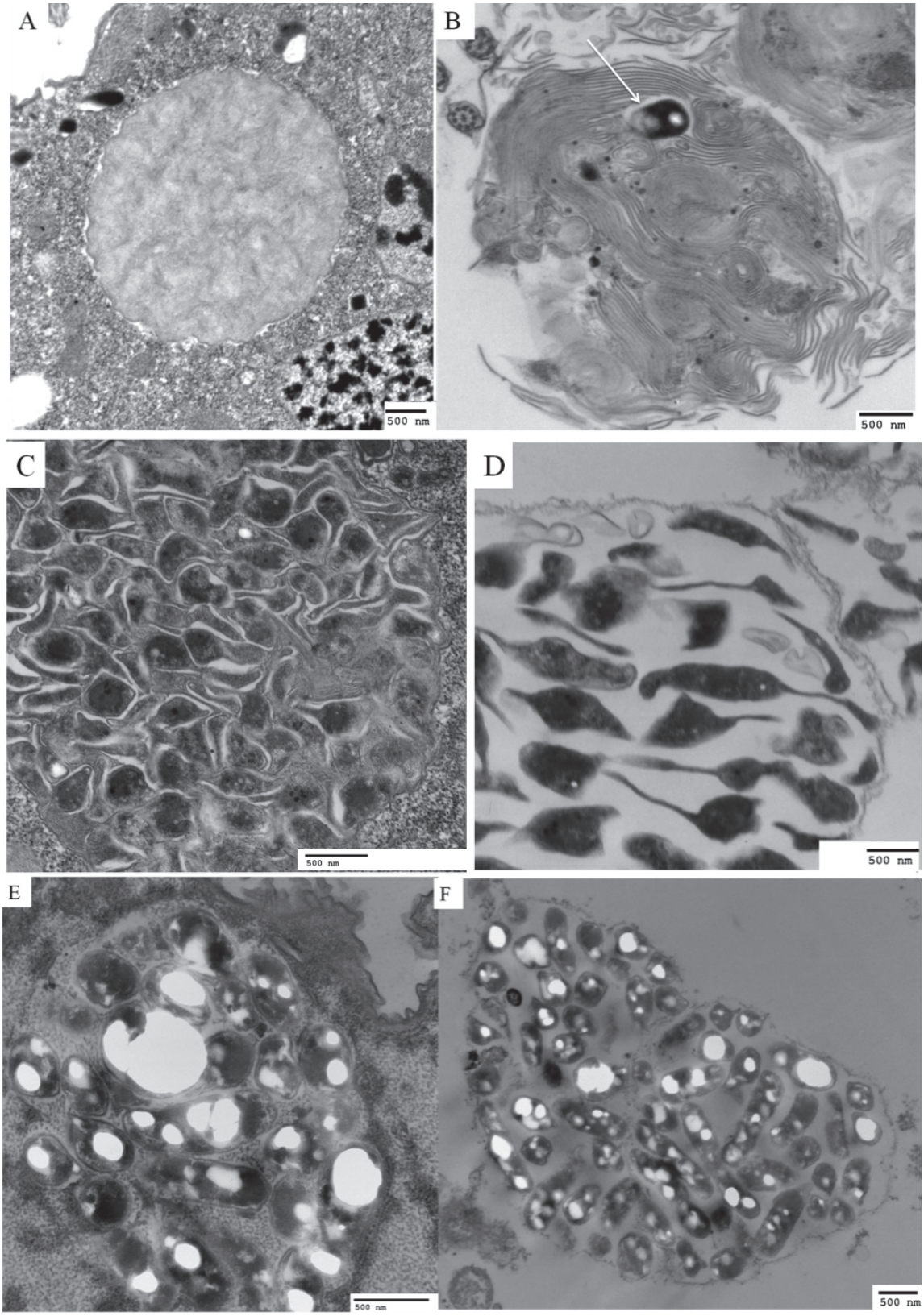


Fig.5.25. BdhA plays no role in detergent-mediated lysis of *Legionella*. The SPF-VBNCCs of the *bdhA* mutant was resistant to detergent SDS. The graph shows $OD_{620\ nm}$ (an indicator of cell lysis) as a function of time, for SPFs, SPF-VBNCCs produced in dd-water and SPF-VBNCCs produced in tap water. The lysis index is defined as the time taken to reduce the optical density to 50% of the initial value. For culturable SPFs, the lysis index was 3.5 min. The estimated lysis index for SPF-VBNCCs in dd-water was 3 s, and for SPF-VBNCCs in tap water was infinite. Results are means ± SD (n=3). See detergent-mediated lysis of the parent strain in Fig.3.9A in which the lysis index was 3.8 min, 20 s, and infinite for the culturable SPFs, SPF-VBNCCs in dd-water, and SPF-VBNCCs in tap water respectively.


A
500 nm
B
500 nm
C
500 nm
D
500 nm
E
500 nm
F
500 nm

Continuation

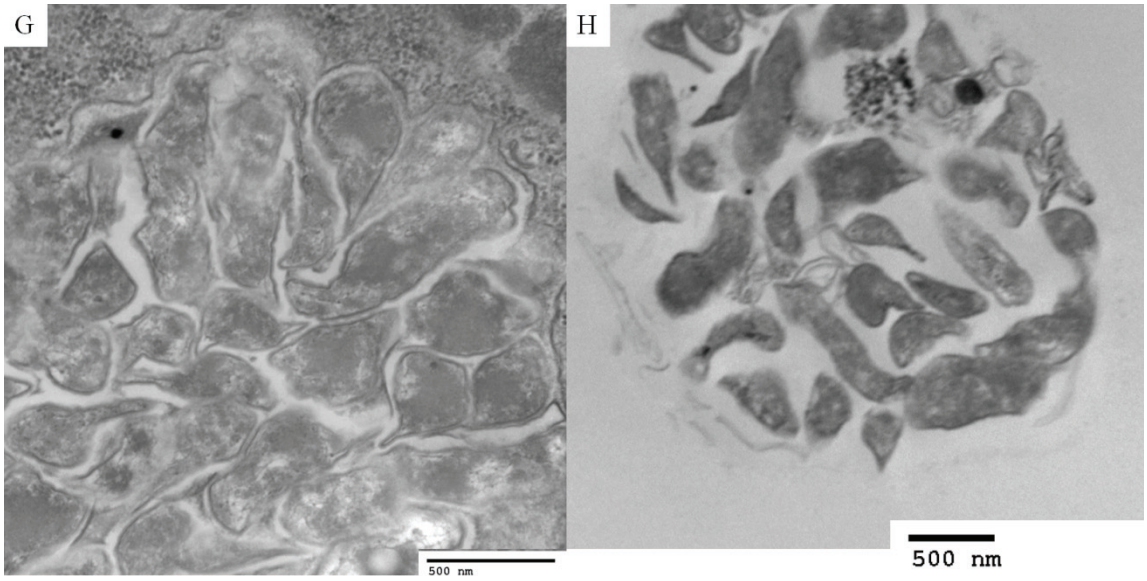


Fig.5.26. Resistance of *L. pneumophila bdhA* mutant VBNC cells to digestion inside *Tetrahymena* food vacuole. Panel (A and B) *L. pneumophila bdhA* mutant SPFs in dd-water after complete loss of culturability were digested inside *Tetrahymena* food vacuole (*T. thermophila*) (A) and in the expelled pellets (*T. tropicalis*) (B) and were manifested as membrane whorls; only very few bacteria inside the vacuoles and the pellets appeared intact and exhibited irregular shapes (arrow). Panel (C and D) *bdhA* mutant SPF-VBNCCs in tap water were resistant to digestion inside *Tetrahymena* as they could be seen inside *T. thermophila* food vacuole (C) and appeared intact in the expelled pellets (D). Panel (E and F) *bdhA* mutant MIF-VBNC produced in dd-water were resistant to digestion inside *Tetrahymena* as they could be seen inside *T. tropicalis* food vacuole (E) and appeared intact in the expelled pellets (F). Panel (G and H) *bdhA* mutant MIF-VBNC produced in tap water were resistant to digestion inside food vacuoles (*T. thermophila*) (G) and appeared intact in the expelled pellets (*T. tropicalis*) (H).

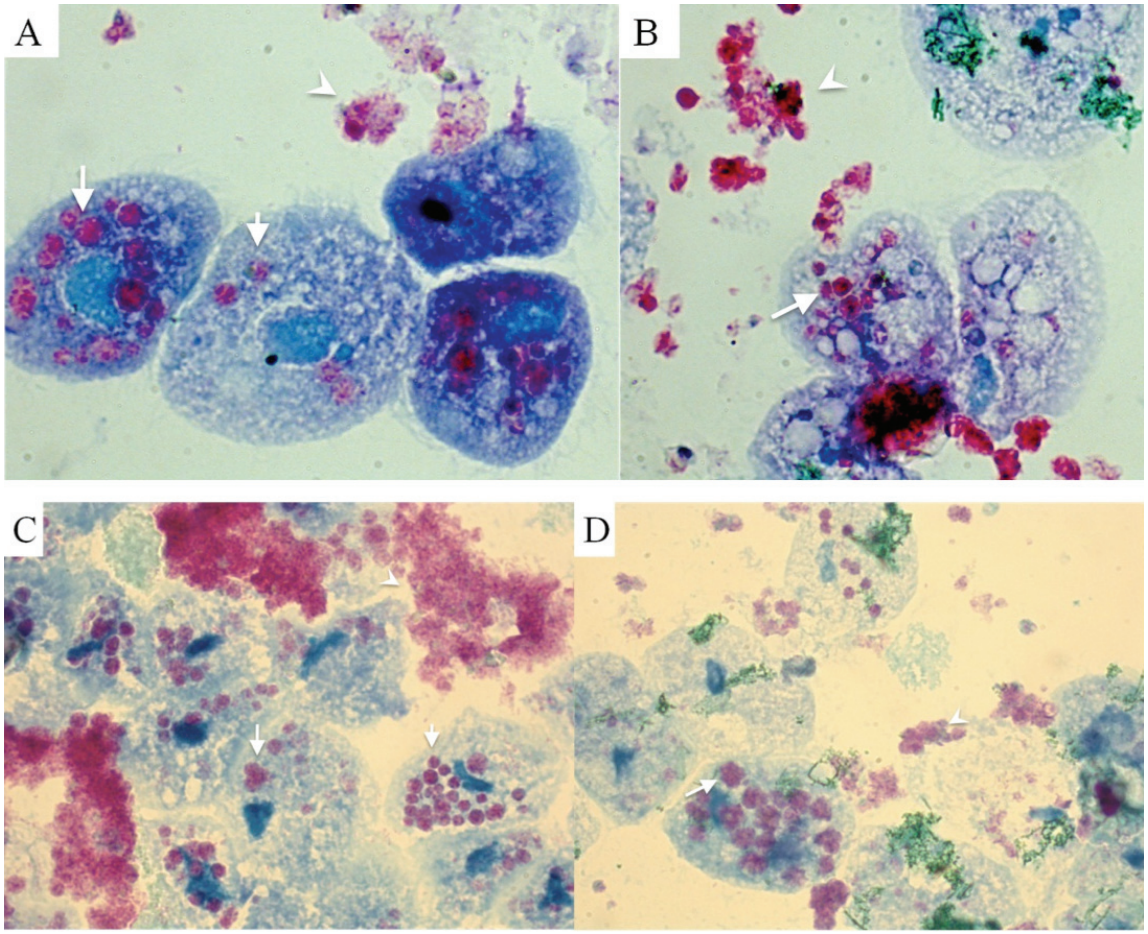


Fig.5.27. Gimenez staining of *bdhA* mutant MIF-VBNCCs from tap (panel A and B) and dd-water (panel C and D) fed to *T. theromphila* (panel A and C) and *T. tropicalis* (panel B and D). All showed Gimenez+ reaction inside food vacuoles (white arrows) and expelled pellets (white arrow heads).

CHAPTER 6. DISCUSSION

6.1. *L. pneumophila* SPFs and MIFs Produce Distinct VBNC Cells in dd- and Tap Water

As a survival strategy, many bacteria, including *L. pneumophila*, respond to various environmental stresses by entering into a different physiological state in which the cells remain viable but are no longer culturable on standard laboratory media. Upon resuscitation, these viable but non-culturable cells regain culturability and the ability to cause infection (reviewed in Oliver, 2010). The ability of *L. pneumophila* to enter into the VBNC state, and the implications of this ability for water disinfection efficiency and *L. pneumophila* detection, have been previously reported and discussed (England *et a*l., 1982; Hussong *et al*., 1987; Paszko-Kolva *et al*., 1992; Steinert et al., 1997; Ohno *et al*., 2003; Hwang *et al*., 2006; Alleron *et al*., 2008). Entry into the VBNC state may account for that *L. pneumophila* cannot be cultured from cooling towers and other water systems suspected to be the source of infectious bacteria (England *et al*.,1982; Hussong *et al*., 1987). However, *L. pneumophila* VBNC cells remain poorly understood.

In this study, *L. pneumophila* VBNC cells were produced using two different developmental forms, SPFs and MIFs. For the first time, these forms were shown to produce VBNC cells that display very different characteristics in terms of morphology, survival, and environmental fitness, which in turn depend on the surrounding water microcosm. These new findings clearly indicate that we cannot assume that all *L. pneumophila* VBNC cells are equal.

If the characteristics of VBNC cells depend on the developmental *L. pneumophila* form used to produce them, replicative forms should also produce unique (and perhaps fragile) VBNC cells. In support of this idea, Ohno *et al*. (2003) found that *L. pneumophila* cells harvested during the exponential growth phase in BYE broth and resuspended at a concentration of 10^6 cells/mL in distilled water at 42°C rapidly lost both culturability (at day 4) and viability (at day 10). In similar conditions in my current study, SPFs resuspended to 10^6 cells /mL in dd-water at 45°C were more resilient and lost culturability at day 9 while retaining 30-50% viability. When directly compared in tap water at 42°C and at a cell density of 10^8/ml, exponential phase *L. pneumophila* lost culturability at day 85, whereas SPFs did so

at day 145 (Ohno *et al*., 2003). These results suggest that VBNC cells produced by replicative forms of *L. pneumophila* are indeed different from those produced by SPFs, and indicate that the characteristics of *L. pneumophila* VBNC cells depend on the developmental form used to produce them.

The type of water in which the VBNC cells are produced also seems to influence *L. pneumophila* entry into this state. In dd-water, *L. pneumophila* SPF lost its culturability after 18 days and retained a low percentage of viable cells (5%). These viable cells can not be resuscitated in *Acanthamoeba*. In tap water, however, SPF culturability lasted longer (45 days) and retained a high viability (20%). These viable cells could be resuscitated in *Acanthamoeba*. *L. pneumophila* culturability in the experiments reported by Ohno *et al.* (2003) lasted longer (>140 days at 42^{o}C) than in my work, probably because of the slightly lower temperature and/or the source of tap water used in their study. In my work, there was a difference in the time to losing culturability between experiments of SPF in dd-water. The difference in time frame is likely due to the quality of dd-water obtained at different times over the course of my study.

The percentage of metabolically active cells in the Ohno *et al.* (2003) study with initial inoculum of 10^6 cells /mL was within -1 log of the initial value in all microcosm samples throughout the study period. It is noteworthy that once the VBNC state was reached, the viability appeared to remain constant, with an asymptotic trendline. This indicates that in tap water, *L. pneumophila* seems to enter a stable VBNC state and can survive for a long time in water systems that may impose health risks if the cells regain culturability after encountering amoeba.

L. pneumophila has the capacity to grow as a free bacterium in nutrient-rich media, either artificially prepared or naturally provided in association with microalgal and (or) bacterial communities (Tison *et al*., 1980; Pope *et al*., 1982; Wadowsky and Yee, 1983). However, it is generally accepted that in nature, extracellular growth is a minor contributor compared to intracellular growth in amoeba with respect to the total numbers of planktonic and biofilm-associated *L. pneumophila* (Fields, 1993; Murga *et al*., 2001; Kuiper et al., 2004). Therefore, it seems reasonable to surmise that the developmental form that emerges from wasted amoeba (i.e., MIFs) constitutes the predominant form of *L. pneumophila* that would be available in the freshwater environment to enter the VBNC state. This implies that

L. pneumophila VBNC cells produced naturally would be mostly derived from MIFs and, like the MIF-VBNCCs produced in this study, would be able to maintain an ultrastructural integrity and high levels of viability.

6.2. Resuscitation of The VBNC *L. pneumophila*

Resuscitation of SPF-VBNCCs produced in tap water was successful in amoeba but not in macrophages, suggesting that amoeba might be providing molecules essential to the resuscitation process. *L. pneumophila* relies on amino acids as the major sources of carbon and energy production through the TCA cycle. Cysteine and serine are converted by *Legionella* into pyruvate that feeds the TCA cycle. The amino acids essential for *Legionella* are also essential for the two evolutionarily distant hosts amoeba and macrophages, with the exception of cysteine, which is scarce in human cells (reviewed in Al-Quadan,T. *et al*., 2012). Thus, cysteine might be one of the factors found in amoeba that contributes to resuscitation of *L. pneumophila*.

Resuscitation of *L. pneumophila* in amoeba emphasizes the importance of eradication of amoeba from drinking water. In fact, in a 2008 study of domestic water in South Florida in USA, 19.4% of all tap water samples were positive for *Acanthamoeba*, *Hartmannella* and *Vahlkampfia* spp. (Shoff *et al*., 2008), all of which have been shown to harbor intracellular *Legionella* spp. (reviewed in Lau and Ashbolt, 2009). In this study, only a small percentage of the VBNCs could be resuscitated and regained culturability. This is consistent with statements by Ohno *et al.* (2003) that VBNC cells from both RF and SPF in tap water that underwent resuscitation represented only a minor portion of the VBNC population.

In my current study, resuscitation was not successful in media. When others were investigating resuscitation of *V. vulnificus*, it was observed that nutrients may be inhibitory to the resuscitation of VBNC cells (Whitesides and Oliver, 1997). The presence of nutrients would, in fact, be bacteriostatic or bacteriocidal to VBNC cells, and would explain their inability to develop into colonies on routine media. Koch (1996) proposed that, upon exposure to high nutrients, dormant cells might transport the nutrients so rapidly that they would experience a cell damaging internal nutrient concentration. Such an event, which might occur within seconds, would likely be due to inadequate feedback inhibition or

overflow metabolism occurring as the dormant cells transported the nutrient. Koch (1996) speculated that death could arise simply from the osmotic effects of the large internal nutrient concentration, or to the specific effects of one or more components of the nutrient. Others speculated that, upon exposure to high nutrient levels, growth-arrested cells would likely undergo an imbalance in metabolism leading to a near instantaneous production of super-oxide and other free radicals (Bloomfield *et al*., 1998).

Wong *et al.* (2004) found that the *V. parahaemolyticus* VBNC state induction period and the age at which the VBNC cells could be successfully resuscitated by temperature upshift were strain dependent (Wong *et al*., 2004). Thus, it is possible that other strains of *L. pneumophila* might have the ability to recover from the VBNC state in amoeba more efficiently.

The resuscitation of *L. pneumophila* VBNC cells in amoeba has never been quantified. For instance, in the studies by Steinert *et al*. (1997) and Ohno *et al*. (2003), the originally resuscitated VBNC cells were not distinguished from their progeny and, consequently, the number of cells that actually resuscitated could not be determined. The quantitative method that I used in this study indicated that only 1-3 cells in every 10^5 VBNC cells regained culturability in the presence of *A. castellanii*. This result could mean that the vast majority of VBNC cells were damaged beyond repair, or that exposure to *A. castellanii* is not an efficient way to resuscitate *L. pneumophila* VBNC cells. The second explanation is more likely, mainly because it is difficult to argue that MIF-VBNCCs, which maintained their ultrastructural integrity and high viability but could not regain culturability in amoeba because they were all irreversibly damaged. Moreover, others have reported that a number of VBNC *L. pneumophila* samples were actually non-resuscitable in amoeba (Ohno *et al*., 2003).

It seems that *L. pneumophila* VBNC cells might require a complex set of signals—perhaps a combination of environmental cues in addition to the presence of amoeba—to exit the VBNC state and regain culturability. That is, the fact that MIF-VBNCCs didn't resuscitate does not necessarily mean that they cannot be resuscitated; but that further investigation is needed to better understand their physiology. It's possible that using different species of amoeba or *Tetrahymena* might result in more efficient resuscitation.

6.3. The Role of Salts or Ions in Extending *L. pneumophila* Survival in Water

In the present study, I found that the viability of *L. pneumophila* SPF-VBNCCs in dd-water was particularly dependent on the presence or absence of salt ions. These results are in agreement with previous reports suggesting that minerals and salt ions are needed for the extended survival of *L. pneumophila* in water (States *et al*., 1985; Heller *et al*., 1998; Ohno *et al*., 2003; Hwang *et al*., 2006). Ohno *et al*. (2003) demonstrated that a lack of salts could result in the simultaneous loss of culturability and viability of *L. pneumophila* under extreme starvation conditions, such as in distilled water. Dilution of two hot spring water samples with sterilized distilled water decreased the survival ability of *L. pneumophila* depending on the dilution rate (Ohno *et al*., 2003).

States *et al*. (1985), on the other hand, showed that lower levels of certain ions, such as iron, zinc, and potassium, are important factors in the survival and growth of *L. pneumophila* in tap water. Hwang *et al.* (2006) suggested that *L. pneumophila* could maintain its viability for a long time by reducing metabolic activities and losing culturability as long as those salts or minerals that are involved in starvation survival were present. Heller *et al.* (1998) found that addition of small amounts of NaCl (0.1%-0.5%) enhanced survival of *Legionella*.

The specific role played by sodium and chloride ions in metabolism of *L. pneumophila* has not been examined, but sodium is well known to be involved in metabolic carrier systems and is also an important co-factor in enzymes (Heller *et al*., 1998). For chloride ions, a mild enhancement of *L. pneumophila* growth has been described (States *et al*., 1985). In my work, NaF, $FeSO_4$ and $Ca(NO_3)_2$ were found to be the most beneficial salts in extending the culturability of SPFs and improving the yield of SPF-VBNC cells. However, a comprehensive analysis of several metal fluorides, sulfates and nitrates would be required in order to identify the specific ions responsible. The role of ions in extending *L. pneumophila* survival in tap water is unknown; however, they might act as co-factors for some enzymes that participate in catalyzing important processes (e.g., utilization of energy sources).

6.4. Morphological Changes in The VBNC State

Morphological changes have been previously observed in bacteria entering the VBNC

state (Catrenich and Makin, 1991; Jiang and Chai, 1996; Chaiyanan *et al*., 2001; Signoretto *et al*., 2002). Rod-shaped Gram-negative bacteria, when in the VBNC state, become cocci-like and smaller than vegetatively growing cells (Catrenich and Makin, 1991; Jiang and Chai, 1996). Similar to *Y. pestis* (Pawlowsk *et al*., 2011), *Edwardsiella* (Du *et al*., 2007), and *Vibrio* (Su e*t al*., 2013) VBNC cells, *Legionella* VBNC cells also demonstrated a morphological change. The *Legionella* VBNC cells became pear-like in shape, as shown by TEM sections.

EM of Gram-negative VBNC bacteria revealed alterations in envelope structure. In particular, micrographs of *V. cholerae* and *V. parahaemolyticus* showed that the coccoid VBNC state had a loosely bound cell envelope and a gap between the inner and outer membrane with the formation of blebs (Chaiyanan *et al*., 2001; Jiang and Chai, 1996). The periplasmic space of nonculturable *Y. pestis* cells was also greatly increased compared to laboratory grown cells and their cytoplasm was electron-translucent (Pawlowski *et al*., 2011). The formation of a spacious periplasmic space and the production of outer membrane blebs were also observed in *Legionella* SPFs-VBNCCs, which also displayed an electron-translucent cytoplasm.

The spacious periplasmic space may be formed during EM processing (e.g., dehydration and or embedment) of *L. pneumophila*, during which materials may be extracted from the spacious periplasm causing the collapse of a weakened cell envelope and the formation of pointy protrusions (e.g. Fig. 3E). Jiang and Chai (1996) hypothesized that outer membrane blebs could be formed by pieces of cell envelope. Because of cell size reduction, the excess cell envelope material may be formed and allow cell volume adjustment via bleb formation (Jiang and Chai, 1996). The formation of similar outer membrane vesicles during starvation has also been observed in *V. cholerae* (Marden *et al*., 1985).

Outer membrane vesicles (OMV) are shed from the outer membrane by *L. pneumophila* and most other Gram-negative bacteria. They are between 100 and 250 nm in diameter and consist of components from both the outer membrane, including LPS, and the periplasm. *L. pneumophila* OMVs contain a disproportionately high number of virulence-associated proteins and display lipolytic and proteolytic activities (Galka *et al*., 2008). In general, OMVs from other bacteria can mediate interbacterial contact and also contact with eukaryotic cells. Membrane vesicles are also thought to serve as a source of nutrients

(reviewed in Shevchuk *et al*., 2011). Quorum sensing functions have also been assigned to OMVs (Mashburn and Whiteley, 2005). In this way, OMVs might contribute to the communication of the *Lp* VBNC cells or to transporting nutrient from one VBNC cell to another. Thus, those VBNC cells that are producing OMVs might be showing an altruistic behavior to improve the survival of other VBNC cells.

The fact that SPF-VBNCCs showed MIF-like characteristics, becoming resistant to detergent-mediated lysis, displaying morphological changes, and changing their Giménez staining properties, suggests structural and/or chemical changes in the bacterial cell envelope. However, more work is needed to elucidate the nature of such changes.

An important change observed in VBNC *L. pneumophila* cells produced in tap water was the lack of PHB inclusions. It seems that *L. pneumophila* degrades PHB in order to maintain an active metabolism and extend its culturability during starvation. In fact, James *et al*. (1999) previously monitored the utilization of PHB in *L. pneumophila* and found that PHB reserves were utilized during starvation and that PHB depletion correlated with loss of culturability. Fluorescence microscopy provided visual evidence of PHB utilization, with a marked reduction in the number of Nile red-stained granules during starvation.

The presence of PHB in bacteria correlates with reduced degradation of RNA and proteins during nutrient starvation, and PHB is known to serve as a carbon and energy source during the encystment of *Azotobacter* (reviewed in Anderson and Dawes 1990). In my current study, utilization of PHB happens only in tap water since the inclusions were still seen in both SPF- and MIF-VBNCCs in dd-water. The difference might be related to ions or other tap water components playing a role in the utilization of PHB, but this remains uncertain. The utilization of PHB might enhance the ability of the VBNC cells to resuscitate, as was observed in SPF-VBNCCs from tap water. Further study is required to investigate this notion.

The changes in shape of VBNC cells in some bacteria were attributed to changes in peptidoglycan structures. Signoretto *et al.* (2002) reported that in *E. coli* VBNC cells, in comparison to the exponential phase, there was a high degree of cross-linking, a threefold increase in unusual DL-2,6-diaminopimelic acid interpeptide bonds (DAP-DAP) cross-linking, an increase in muropeptides bearing covalently bonded lipoprotein, and a shortening of the average length of glycan strands. Signoretto *et al*. (2002) further suggested that the

unusual DAP-DAP crosslinking seen in the VBNC state might occur in response to a shortage of the pentapeptide.

In their experiments with vancomycin, Del Mar Lleo *et al*. (2007) suggested a lack of synthesis of D-ala-D-ala by *E. faecalis* VBNC cells. According to the shotgun proteomic data in my work (Table 4.1), decreases in the protein levels of D-alanine-D-alanine ligase and D-alanyl-D-alanine carboxypeptidase were observed in VBNC protein samples. As peptidoglycan rearrangements have been observed in both Gram-positive and -negative cells as they enter the VBNC state (Costa *et al*., 1999; Signoretto *et al*., 2002), such changes may be hallmarks of the VBNC state (Oliver, 2010).

6.5. A Model for *L. pneumophila* VBNC within The Developmental Cycle

Differentiation mainly refers to distinct morphological, physiological, or biochemical characteristics expressed by an organism at some particular stage of development (Garduno *et al.*, 2008). During the development of living organisms, cells displaying specific sets of distinguishing characteristics are produced, which are referred to as 'forms'. Unicellular organisms usually follow a 'circular' sequence of differentiation steps that may define a developmental cycle. The alternating nature of this developmental process implies that the closing step of any given cycle constitutes the opening step of the next cycle. The development of any specialized form that withstands specific physiological challenges (like starvation), while keeping the ability to quickly resume replication in favourable conditions, is an excellent example of bacterial differentiation (Garduno *et al.*, 2008).

The synthesis of stage-specific proteins controlled by temporally integrated regulatory networks can lead to complex forms within markedly different phenotypes (Errington, 2003). Stage specific proteins may have direct regulatory or structural roles, and/or be involved in the synthesis of compounds necessary to carry out specific changes that will ultimately determine the differentiation stage of the organisms (Garduno *et al.*, 2008).

As explained in Chapter 3, VBNC *L. pneumophila* displayed unique morphology. In comparison to the culturable cells, VBNC *Legionella* underwent changes in cell shape, membrane, cytoplasm density, and inclusions. *Legionella* VBNC cells were environmentally fit since they resisted SDS-mediated lysis and digestion inside *Tetrahymena* food vacuoles. Moreover, these VBNC cells showed a distinct protein profile than that of the culturable

Legionella. Therefore, using the same criteria used by Garduno et al. (2002) to define the MIF as a developmental form of *L. pneumophila*, I postulate that the consistent morphological traits of VBNCCs, their fitness profile and their unique complement of proteins, support the notion that VBNCCs are separate developmental forms of *L. pneumophila*. Moreover, the resuscitation processes itself suggest that the VBNCC is a developmental form that keeps the genetic program to differentiate back to the culturable state. Thus, the above-mentioned findings suggest that the VBNC cell can be considered as a differentiated form and hence, VBNC *L. pneumophila* could be integrated into *L. pneumophila* developmental cycle as shown in Figure 6.1.

The absence of existence of protein related to the oxidative stress (stochastic detorioratio model) or Rel E-Rel B toxin-antitoxin proteins (programmed cell death model) in the shotgun prteomic data of the MIF-VBNCCs suggest that these two models are not involved in the VBNC cell diffrentiation. Also, the infinte level of the viability once the bacteria enter the VBNC state role out out any role for the stochastic detorioratio or programmed cell death models. On the contrast, proteins related to the adaptive response model, for example Rel A and GGDEF-EAL domain, were increased in their level in MIF-VBNCCs indicating that VBNC formation is differentiantion process in response to an stress.

The data from the current study widen our knowledge about VBNC *Legionella*. However, further studies are required to understand the pathway through which such differentiation proceed. These studies may include monitoring changes in protein level at different time points during *Legionella* entry into the VBNC state or monitoring *L. pneumophila* ability to enter the VBNC state after deleting (or expressing) genes that could be within the putative VBNC regulatory network.

6.6. Proteomic Analysis as A Tool to Study Gene Products Important for VBNC Cell Formation

The study of gene expression can be undertaken at the transcriptional level, with technologies such as cDNA and oligonucleotide microarrays facilitating the quantitative analysis of the expression of thousands of genes. However, the abundance and activity of the end product of genes (i.e., proteins) is not exclusively determined by regulatory events at the transcriptional level. Indeed, it is proteins that act as the cellular building blocks that

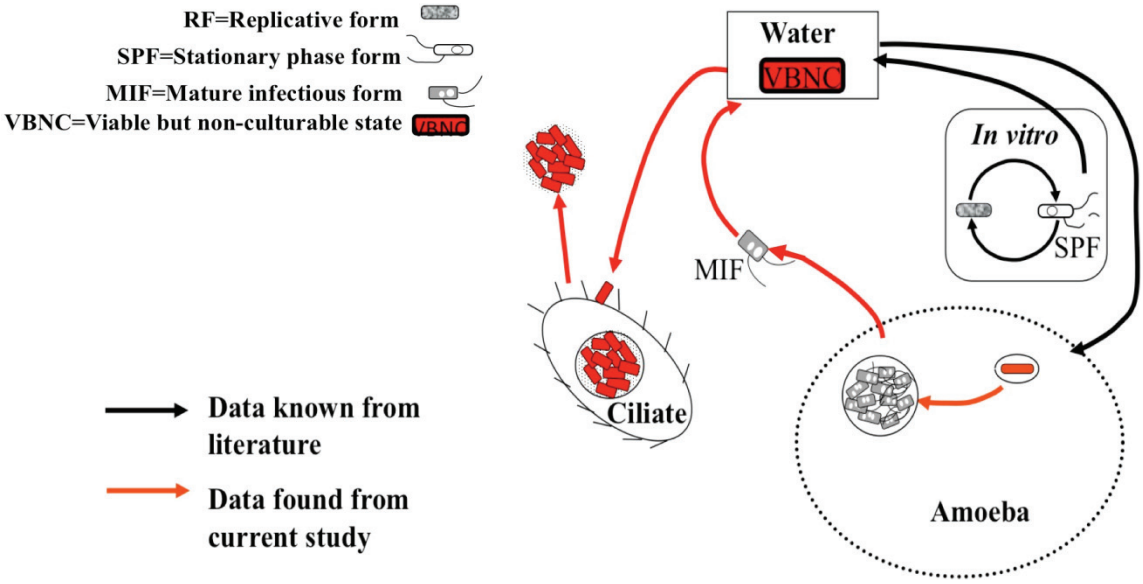


Fig.6.1. A model for integration of VBNC cells into the developmental cycle of *L. pneumophila*. Black arrows represent the known pathways from literature, red arrows represent the pathways found in the current study. *L. pneumophila* alternates *in vitro* between the RF and the SPF. MIFs are produced from infecting amoeba. In this model, VBNC cells are produced either from SPFs or MIFs in water. The VBNC cells are resuscitated in amoeba and are suspected to differentiate into MIFs. The VBNC cells are environmentally fit as they get packaged in *Tetrahymena* food vacuoles and expelled as pellets containing intact cells.

ultimately carry out the function of genes, including enzymatic catalysis, molecular signaling, and physical interactions. Analyses undertaken at the protein levels are necessary for the following reasons. First, there is poor correlation between mRNA abundance and corresponding protein levels, indicating that protein levels cannot simply be predicted from corresponding mRNA levels. Second, the translocation of each protein from its site of synthesis to its site of activity cannot always be deduced from sequence data. Third, proteins undergo post-translational modifications that have potentially enormous functional consequences but cannot always be predicted from gene sequences (reviewed in Pardanani *et al*., 2002).

Common post-translational modifications include phosphorylation, acetylation, glycosylation, ubiquitination, and hydroxylation. Post-translational modifications of proteins influence the enzyme activity, protein turnover and localization, protein-protein interactions, modulation for various signaling cascades, DNA repair, and cell division For example, phosphorylation of *B. subtilis* single stranded DNA-binding protein (SsbA) at Tyr82 significantly increases binding affinity with single-stranded DNA *in vitro* (Karve and Cheema, 2011).

Some of the techniques used to identify post-translation modifications are targeted towards identifying specific post-translational modifications. For example, kinase activity assays, phospho-specific antibody development, western blot, mass spectrometry, enzyme-linked immunosorbent assay, intracellular flow cytometry and immunocytochemistry/ immunohistochemistry can be used to assess phosphorylation of proteins (R & D systems, 2002).

The lack of correlation between mRNA abundance and corresponding protein levels can be attributed to the short half-life of mRNA compared to proteins, which are more stable. Moreover, different proteins may vary substantially in their *in vivo* half-lives: regulatory proteins, for example, may have to be produced and degraded very rapidly in order to respond to changing stimuli, whereas structural or housekeeping proteins are more long-lived (Vogel and Marcotte, 2012). In addition, there are many complicated and varied post-transcriptional mechanisms involved in turning mRNA into protein that are not yet sufficiently well defined to be able to compute protein concentrations from mRNA (Greenbaum *et al*., 2003).

Due to cessation or decrease in the level of gene transcription in the VBNC state (Gonzalez-Escalona *et al*, 2006), as well as the short half-life of prokaryotic mRNA (Rauhut and Klug, 1999), it seems reasonable to speculate that VBNC bacteria might contain very little or no mRNA. This was evident in my work when RT-PCR failed to detect mRNAs of BdhA, HspC2, and Lpg0634 proteins that had been detected by SDS-PAGE analysis of VBNC bacteria. In this case, studying gene expression at the level of proteins has the potential to provide a more meaningful understanding of changes in the gene expression. However, studying changes in the protein level after entry into the VBNC state will not clearly indicate the point at which protein levels changed (i.e., prior or after entry into the VBNC state), owing to the long half-lives of proteins (Maier *et al*., 2011). A solution for this problem would be to take protein samples at different time points during the process of entry into the VBNC state. Nevertheless, studying changes in protein level between the VBNC and culturable state might help to understand the mechanism by which bacteria enter this survival state.

For many years, the only option for comparative proteomic studies was staining two-dimensional gels and examining patterns of spots. These types of comparisons are complicated by gel-to-gel differences that can affect spot positions, potentially resulting in false-positives and false-negatives. Even though analysis software has improved greatly, these experiments still require a significant amount of manual intervention, as well as numerous repeats, to ensure trustworthy results (Peck, 2005). Additionally, this approach detects only those proteins that are expressed at relatively high levels and that have long half-lives. Given these limitations, conventional two-dimensional electrophoresis technology has limited potential for large-scale proteome analysis (reviewed in Greenbaum *et al*., 2003).

Recent technological advances, particularly in mass spectrometry, have allowed for large-scale surveys of the proteome (Vogel and Marcotte, 2012). Mass spectrometry, which uses mass analysis for protein characterization, is the most comprehensive and versatile tool in large-scale proteomics. The overall goal of such analysis is to obtain a snapshot of concentrations of proteins associated with different states. In the last few years, the shotgun proteomics approach has become the method of choice for identifying and quantifying proteins in most large-scale studies. This strategy is based on the digestion of proteins into peptides, followed by peptide sequencing using tandem mass spectrometry (MS/MS) and

automated database searching. Compared to two-dimensional gels, shotgun proteomics allows higher data throughput and better protein detection sensitivity (reviewed in Nesvizhskii, 2007).

In shotgun proteomics, a complex protein sample is digested by trypsin and analysed using LC-MS techniques. The heterogeneous sample, with proteins of different chemical and physical behavior, is broken down to a mixture of tryptic peptides complex. Because of the heterogeneous nature of the sample, the intensity of a peptide peak depends on the concentration of the peptide, its propensities for ionization, and post-translational modifications that may result in lower quality MS spectra. In contrast to shotgun proteomic studies, MRM measurements are quantitative analyses strictly targeting a predetermined set of peptides, and depend on specific MRM transitions for each targeted peptide. Therefore, the results from MRM represent more precise measurement of the fold change compared to shotgun proteomics.

In this study, the shotgun proteomics method was applied to screen and quantify the abundance of proteins in the VBNC state in comparison to the culturable bacteria. The shotgun proteomic analysis of MIF-VBNCCs in dd-water revealed a number of proteins with increased and decreased level in comparison to the culturable MIFs (Apendices I and II). Similarly, *E. faecalis* VBNC cells exhibited a different protein profile from that of either starved or exponentially growing bacteria, demonstrating that the VBNC cells are in a distinct physiological state (Heim *et al*., 2002).

In my current work, the majority of proteins with increased expression were those involved in metabolic processes, suggesting that *L. pneumophila* VBNC cells are retaining some metabolic activity in the VBNC state. In VBNC *E. coli*, about 937 genes displayed increased expression using DNA microarray. The induced genes were those necessary for the metabolism of small molecules, energy metabolism, the TCA cycle, and pyruvate dehydrogenase, in addition to genes involved in chemotaxis, mobility, surface structures and flagella biosynthesis (Rozen *et al*., 2002).

In *V. parahaemolyticus*, the protein profile of VBNC also contained a number of proteins that exhibited elevated levels and were suggested to play important roles in the induction or maintenance of VBNC. The proteins with elevated levels are known to be associated with transcription (two homologues of alpha subunit RNA polymerase,

phosphoribosylaminoimidazole carboxamide formyltransferase/IMP cyclohydrolase), translation (ribosomal protein S1, two homologues of elongation factor TU, elongation factor EF-G), ATP synthase (F1 alpha subunit), gluconeogenesis-related metabolism (dihydrolipoamide acetyltransferase, glyceraldehyde 3-phosphate dehydrogenase), and antioxidants (2 homologues of peroxiredoxins, AhpC/Tsa family); the elevated proteins also included a conserved hypothetical protein with unknown function (Lai *et al*., 2009). In this study, NADH dehydrogenase transmembrane protein, putative alpha-amylase, and GTP pyrophosphokinase ((p)ppGpp synthetase I) stringent stress response (RelA) are a few examples of proteins with increased expression that have metabolic activity in *L. pneumophila* MIF-VBNCCs (Apendix I).

In *E. coli* and some other bacteria including *L. pneumophila*, the *relA* gene is the genetic determinant of the stringent response, with the RelA-catalysed increase in the cellular (p)ppGpp leading to the rapid inhibition of stable RNA biosynthesis, ribosome and protein synthesis, and ultimately, to growth arrest (Chatterji and Ojha, 2001). In my work, RelA was shown to have a 1.8-fold increased level in the VBNC state using the quantitative MRM analysis (Table 4.1). In *V. cholerae*, Gonzalez-Escalona *et al*. (2006) found that the expression of *relA* was greater in the VBNC state (induced at 4°C) than in the starvation state (15°C), suggesting that the VBNC bacteria may be capable of adapting to environmental signals via such stringent response. The increased expression of *relA* in the VBNC state suggests that the intracellular level of (p)ppGpp in the VBNC bacteria is higher than that in the culturable cells, which may also lead to the activation of a variety of cellular metabolic events.

The genetic pathways underlying the VBNC response in *E. faecalis* were in part the same as those leading to the starvation response, as indicated by the presence of similar expression profiles for certain proteins. However, major differences in the expression patterns of some proteins (e.g., EF-Ts, fructose-bisphosphate aldolase, and mannose-specific PTS system) were observed, suggesting that the underlying pathways in the starvation and VBNC states do not overlap completely but rather share a few crossing points. Moreover, the VBNC state also differs from other general stress responses, as indicated by the down-regulation of general stress proteins such as the mannose-specific PTS system (Heim *et al*., 2002).

In addition to proteins with metabolic activity, several stress proteins exhibited increased expression in MIF-VBNCCs, as was shown by MRM analysis, including sensory box protein/GGDEF/EAL domains and two small HspC2 heat shock proteins (Lpg2192 and Lpg2493). In my work, MRM analyses showed a 2.34 fold increase in the GGDEF-EAL protein in MIF-VBNC compared to the culturable MIF (Table 4.1). Diguanylate cyclase and phosphodiesterase activities, associated with GGDEF and EAL domains, respectively, controls the intracellular level of of bis-(3'-5')-cyclic dimeric guanosine monophosphate (c-di-GMP), a second messenger that plays a key role in many physiological processes (Romling and Amikam, 2006).

Cyclic-di-GMP can control gene expression and function at a variety of different levels, including transcriptional activity, post-transcriptional activity, enzymatic activity, and protein-protein interactions. For example, c-di-GMP controls the production of *P.aeruginosa* extracellular polysaccharide (EPS) by altering DNA binding of the FleQ transcriptional regulator (Hickman and Harwood, 2008). Cyclic di-GMP also regulates gene expression post-transcriptionally by binding to riboswitches and affecting mRNA translation (Sudarsan *et al*., 2008). In addition to its role in transcriptional and post-transcriptional gene regulation, c-di-GMP allosterically controls enzyme activity: binding of c-di-GMP to the PilZ domain of cellulose synthase, BcsA, is required for optimal activity of this enzyme (Benach *et al*. 2007).

In *S. typhimurium*, mutation in an EAL-domain protein influencing c-di-GMP levels has been linked to the ability of the organism to kill macrophages and resist peroxides (Hisert *et al*., 2005). In *L. pneumophila*, over-expression of several proteins containing domains related to cyclic diguanylate synthesis, hydrolysis, and recognition strongly decreased the ability of *L. pneumophila* to grow inside host cells (Levi *et al*., 2011). Thus, GGDEF/EAL domain proteins might have an influence on the activity or level of other proteins during entry of *L. pneumophila* into the VBNC state, potentially affecting the ability of VBNC *L. pneumophila* to infect host cells.

As shown by MRM analysis, two small heat shock proteins (sHsps) (with accession numbers G8UX67 and G8UYH4) increased 2.05 and 30.66 fold, respectively, in MIF-VBNCCs compared to the culturable MIF (Table 4.1). sHsps have the ability to protect cellular proteins and maintain cellular viability under intensive stress conditions such as heat

shock or chemical treatments. While most chaperone machineries such as DnaK-DnaJ-GrpE or GroEL-GroES consume energy, sHsps are generally believed to be ATP-independent. Nevertheless, sHsps have been added to the catalogue of molecular chaperones because they bind to denatured proteins as holding chaperones and thereby suppress unintended interactions that may lead to the precipitation of aggregates.

This function of sHsps maintains their substrate proteins in a folding-competent state, keeping these proteins amenable to subsequent refolding by the major chaperone machineries. The sHsps have no function in degradation, unlike the general heat shock proteins, which have dual functions as chaperones and proteases (Han *et al*., 2008). Thus, *L. pneumophila* might be responding to environmental stress through these proteins, which in turn might participate in refolding of proteins important for entry of *L. pneumophila* into the VBNC state.

It is noteworthy that the two small HspC2 proteins confirmed by MRM analysis of MIF-VBNCCs in dd-water were also found in the SDS-PAGE and mass spectrometry analysis of SPF-VBNCCs in tap water. Thus, sHsps might play a role in the process of *L. pneumophila* entry into the VBNC state regardless of the bacterial form or the type of water in which the bacteria entered the VBNC state; however more work is needed to explore the role of sHsps in VBNC cell formation. Interestingly, chaperones involved in bacterial envelope stress response, such as the peptidyl-prolyl cis-trans isomerase and the Skp chaperone, decreased in expression in the VBNC state despite exposure of the bacteria to high temperature and changes in the cell wall structure. Probably, their expression was enhanced earlier (prior to entry into the VBNC state) and decreased once the cells were adapted to the stressful environment.

Among the increased-expression proteins, EnhA increased about 1.73-fold in the VBNC state, as validated by MRM analysis. Together with *enhB* and *enhC*, *enhA* is located within the *enh2* locus, which was found to be important for entry of *L. pneumophila* into monocytes (Cirillo *et al*., 2000). Thus, *L. pneumophila* might be retaining its infectivity in the VBNC state, which would facilitate its entry into new host cells and stimulate it to regain culturability.

Proteins related to type IV secretion system were also enhanced. These included IcmW and IcmO/DotL (both important for macrophage infection and killing) (Brand *et al*.,

1994; Segal and Shuman, 1997), IcmL-like protein, IcmK/DotH, Dot/Icm effector Ceg25 (coiled coil domain protein lpg1836 regulated by PmrA (Zusman *et al*., 2008), and Dot/Icm effector LirA (hypothetical protein Lpg1960 which is positively regulated by *rpoS* (Zusman *et al*., 2008). The hypothetical protein Lpg1960 was also found in SDS-PAGE analysis of MIF-VBNCCs in dd-water. The enhancement of type IV secretion system proteins suggest that these proteins might be important in order for VBNC cells to infect an appropriate host cell upon contact. However, this will need further investigation. Moreover, the type of host cell that can resuscitate the MIF-VBNCCs needs further study to be identified.

Two proteins involved in PHB degradation, acetoacetyl CoA synthetase, and 3-hydroxybutyrate dehydrogenase (BdhA), were among the proteins with increased levels in *Legionella* VBNC state (Apendix I), as confirmed by MRM analysis (Table 4.1). In *Sinorhizobium melilot*, acetoacetyl CoA synthetase has been shown to be important for cell survival ability. Viable cell counting after transfer of bacteria to carbon nutrient-free medium indicates that the *S.melilot* acetoacetyl CoA synthetase (*acsA*) mutants do not propagate as well as the wild-type strain (Cai *et al*., 2000). The importance of BdhA for survival is discussed in section 6.7.

On the other hand, in my current study, more proteins with decreased levels were found than with increased levels in the VBNC state. Among proteins with decreased levels in the VBNC state were many involved in metabolic processes, which constitute the major category. This was not a surprising result considering that cells in the VBNC state are thought to be metabolically dormant. Examples of these proteins include malate oxidoreductase, cysteine synthase, enolase, tryptophan synthase, glucose-1-dehydrogenase, phosphoenolpyruvate synthase, and acetyl CoA carboxylase.

In my work, proteins involved in cell division, cell organization and biogenesis were found exclusively among those with decreased levels. In VBNC *E. coli*, about 320 genes exhibited decreased gene expression in seawater as revealed by DNA microarray. The repressed genes included those necessary for cell division in addition to nucleotide biosynthesis. This was not surprising given the lack of cell growth and division in the stressed state (Rozen *et al*., 2002).

As revealed by MRM analysis, proteins involved in cell wall biogenesis and modification decreased in the VBNC state as well. These proteins include D-alanyl-D-

alanine ligase, which catalyzes the ligation of 2 D-alanine into D-alanyl-D-alanine, and D-alanyl-D-alanine carboxypeptidase, which is involved in peptidoglycan remodeling viai removal of terminal D-alanine from muramyl pentapeptide. The lack of these proteins would result in the lack of synthesis of D-ala-D-ala, which has been suggested to be the cause for the insensitivity of *E. faecalis* VBNC cells to vancomycin (Lleo *et al*., 2007).

The shotgun proteomic analysis was applied in this study on MIF-VBNCCs from dd-water because they retained the highest viability among other VBNC *L. pneumophila* samples. Thus, the protein profile will represent mostly the viable cells in the samples. However, in the future it will be recommended that the shotgun proteomics screen analysis be applied on MIF-VBNCCs from tap-water since tap water is a man-made water system that can represent a source of infection to humans by *Legionella*.

6.7. 3-Hydroxybutyrate Dehydrogenase Enhances *L. pneumophila* Survival in Water

Allelic exchange was used to mutate the *bdhA* gene. The reason for not getting highly efficient transformation and allelic exchange when mutating with *aphA3* could be due to degradation of the electroporated fragment in the cytoplasm of the transformed cells or could be due to non-specific integration of the fragment in the *L. pneumophila* chromosome which may lead to genetic change that make bacteria resistance to the antibiotic. Another reason could be could be degradation of the antibiotic during the long period of incubation. Similar explanation could apply for mutation with the *ermAM.*

One of the noticeable morphological changes observed in both SPF-VBNCCs and MIF-VBNCCs in tap water was the lack of PHB inclusion bodies (Fig.3.5 and Fig.3.6B). The level of BdhA enzyme, as observed by SDS-PAGE and shotgun proteomics analysis, increased in the VBNC state, suggesting a role for the degradation of PHB polymer in this survival strategy.

Similar to other PHA-producing bacteria, such as *Azotobacter* spp., *L. pneumophila* possesses most of the enzymes that catalyze synthesis and degradation of PHB, except for PHB depolymerase, as revealed by NCBI blast search. However, homologues proteins involved in PHB regulation such as PhaR and PhaP (phasin) found in *Ralstonia* were not found in *Legionella*. Phasins are present in all PHA-synthesizing bacteria even though they

generally are not conserved in amino acid sequences (Jurasek and Marchessault, 2002). Thus, PHB regulation in *L. pneumophila* might be mediated by some hypothetical proteins.

It has been hypothesized that PHB accumulated by *Rhizobium* prior to infection serves to fuel infection and/or bacteroid differentiation (Lodwig *et al*., 2005). Aurass *et al*. (2009) constructed *Legionella bdhA-patD* double mutant by EZ-Tn*5* and showed that the double mutant *bdhA-patD* was defective for lipolytic activity and degradation of PHB as well as it was defective in infecting amoeba. However, the *L. pneumophila bdhA* mutant constructed in my study by allelic exchange, which was confirmed by lossing the BdhA enzyme activity (Fig. 5.12) and showed no polar effect as confirmed by RT-PCR (Fig. 5.7C), successfully infected amoeba. As well, the mutant differentiated into MIFs, as evident from TEM studies and Giménez staining. These results suggest that *bdhA* plays little or no role in *L. pneumophila* SPF differentiation into MIF.

In contrast to the parent and *bdhA* mutant strains, attempts to infect amoeba using the complemented *bdhA* strains Comp 1P and Comp 4P were unsuccessful. As shown in Figure 5.12, Comp 1P and Comp 4P exhibited maximal BdhA enzyme activity compared to the parent strain. A possible explanation is that *bdhA* is highly expressed from the complementing plasmids in Comp 1P and Comp 4P and this high BdhA level could have an inhibitory effect on *L. pneumophila* infectivity to amoeba. The sequence upstream of the *bdhA* promoter, which contains potential regulatory sites (as predicted using virtual footprint website http://www.prodoric.de/vfp/vfp_promoter.php), might have a role in suppression of *bdhA* expression in the parent strain, whereas its expression in the complementing plasmid is unregulated due to lack of this upstream region and thus may result in constitutive expression of *bdhA*. This hypothesis may also explain the lack of BdhA enzyme activity in the Comp 3P strain whereas both Comp 1P and Comp 4P displayed enzymatic activity.

L. pneumophila is known to utilize PHB reserves during starvation, and such utilization correlated with loss of culturability (James *et al*., 1999). Several studies have shown that the wild-type bacterial strains survived starvation better than either PHA polymerase or PHA depolymerase-negative mutants (Ruiz *et al*., 1999; Ruiz *et al*., 2001; López *et al*., 1995; Povolo and Casella, 2004; Lodwig *et al*., 2005; Tal and Okon, 1985; Kadouri *et al*., 2003a). My results showed that the survival of the *L. pneumophila bdhA* mutant in dd-water was reduced when compared to the parent strain. The mutant SPF in dd-

water lost both culturability and viability in 15 days. In contrast, the parent strain lost culturability at 18 days with a final proportion of VBNCCs of 6.2 ± 0.9%. For the *bdhA* mutant MIF in dd-water, a dramatic decrease in viability was also observed. At day 90, the final mean percentage of VBNCCs was 11.45 ± 0.35% for the mutant strain compared to 73.2 ± 1.69% for the parent strain. Together, these findings indicate that BdhA plays an important role in extending survival of both SPF- and MIF-VBNCCs.

PHA biosynthesis and utilization has been linked to survival strategies in other bacterial forms, such as spore and cyst production. PHB plays an important role in encystment of the genus *Azotobacter*. Encystment is the process of cyst formation under nutrient limiting conditions in which PHAs serve as carbon and energy sources that encourage the extensive formation of cysts (Stevenson and Socolofsky, 1966). In *Bacillus cereus* cells that had accumulated PHA, the polymer disappeared after sporulation, while the degradation products were incorporated into the spore. In that case PHA may serve as a carbon and energy reserve for sporulation (Nakata, 1966).

Moreover, López *et al*. (1995) observed that in a PHA negative mutant of *Bacillus megaterium*, sporulation occurred immediately after exposure to river water, while survival of vegetative cells decreased as compared to wild type, indicating that in an oligotrophic environment, cells with depleted intracellular carbon source may be committed to earlier sporulation than normal cells (López *et al*. 1995). This is similar to what was observed in my work, where the *L. pneumophila* MIF *bdhA* mutant entered the VBNC state earlier than the parent strain.

In contrast to its role in *L. pneumophila* survival in dd-water, BdhA did not seem to play a role when bacteria were suspended in tap water. Aneja *et al.* (2005) suggested that alternate 3-hydroxybutyrate dehydrogenase-independent pathways might exist for utilization of PHB-derived 3-hydroxybutyrate. Thus, *L. pneumophila* might feature an alternative pathway for degradation of PHB, possibly requiring the presence of ions or minerals to catalyze utilization of PHB.

Another suggested reason for the difference could be the osmotic pressure encountered in dd-water—an additional stress that PHB would be required to resist. In *A.brasilense,* for example, after exposure to osmotic pressure, the survival of PHA-rich bacteria was higher than that of PHA-poor ones (Tal and Okon, 1985). In another study, *E.*

coli DH5α with a PHB degradation system exhibited the highest survival rate when treated by a glucose-induced osmotic pressure (Wang *et al*., 2009).

Another possibility for the requirement of BdhA in dd-water might be related to reactive oxygen species (ROS). It is known that in water under the action of various physical factors (ionizing radiation, heat, electromagnetic radiations of extremely high frequencies, and electrolysis), ROS are formed; among these, the most substantial role belongs to hydrogen peroxide, which is the most long-lived species of ROS (cited in Gudkov *et al*., 2012). In tap water the presence of salts and ions might have a role in reducing the effect of ROS, while their absence in dd-water may require the presence of PHB and/or a functioning PHB cycle to minimize the effect of ROS.

In a very special case, PHB serves as an oxidizable substrate which provides respiratory protection to nitrogenase of Azotobacteriaceae (Anderson and Dawes, 1990). The nitrogenase system of *Azotobacter* is very sensitive to oxygen and high concentration of oxygen is inhibitory to this strain. Senior *et al*. (1972) suggested that degradation of PHB could increase the respiratory activity and reduce the concentration of oxygen, thus protecting the nitrogenase system. In this regard, some studies have found that the ability of *phaC* and *phaZ* mutants of *A. brasilense* to tolerate osmotic shock and to grow in the presence of hydrogen peroxide was significantly affected (Kadouri *et al*., 2002; Kadouri *et al*., 2003a; Kadouri *et al*., 2003b).

The mechanisms by which the PHA cycle favours stress alleviation are not yet fully understood. It was found that a rise in ATP and guanosine tetraphosphate (ppGpp) levels occured concomitantly with PHA degradation. This phenomenon was only observed in wild type *Pseudomonas oleovorans* and not in a PHA depolymerase-deficient strain unable to degrade the polymer (Ruiz *et al*., 2001). In my current study, RelA was shown to be at an increased level in the VBNC state, suggesting a similarly elevated ppGpp level. The effector ppGpp was shown to increase the translation of the central stationary phase regulator RpoS (Brown *et al*., 2002), which activates the expression of genes involved in cell survival and provides cross-protection to various environmental insults such as ethanol, H_2O_2, and high temperature (Lange and Hengge-Aronis, 1991; Ramos-Gonzalez and Molin, 1998; Ruiz *et al*., 2001).

In this regard, RT-PCR study showed an increase in *rpoS* expression in wild type *A*.

hydrophila cells compared to a *phbC* mutant when exposed to high temperature (48^{o}C) and changing NaCl concentrations (Zhao *et al*., 2007). In addition, Peralta-Gil *et al*. (2002) showed that one of the promoters that control PHA synthesis in *A. vinelandii* is regulated by RpoS (Peralta-Gil e*t al*., 2002). Thus, it seems that, in order to respond properly to diverse stresses, PHA-producing bacteria require the *rpoS* gene product. This product induces expression of many genes, including the ones responsible for PHA depolymerization, and allows the organism to mediate changes in cellular physiology and structure and to adapt, resist, and survive under stress conditions. While stress response players such as *rpoS* and ppGpp (or their functional homologues in different organisms) are central in determining the type and the strength of the response by redirecting cellular resources to the synthesis of the appropriate effectors, the PHA cycle could be responsible for providing the fuel necessary for this response—and therefore could also determine its intensity (Kadouri *et al*., 2005).

For the best of my knoldege, this the first time to identify gene involved in VBNC formation in *Legionella.* Moreover, This is the first time to mutate a gene in *Legionella* by electroporating a linear DNA fragment into electro-shocked cells.

6.8. Concluding Remarks

L. pneumophila VBNC was largely uncharacterized. In this study, and for the first time, two different *L. pneumophila* developmental forms, SPFs and MIFs, were shown to produce VBNC cells that displayed very different characteristics in terms of morphology, survival, and environmental fitness. These different characteritics depended also on the surrounding water microcosm in which the VBNC cells were produced. This indicates that not all *L. pneumophila* VBNC cells are equal.

L. pneumophila lost its culturability faster in dd-water than in tap water. Moreover, only SPF-CBNCCs produced in tap water could be resuscitated in amoeba. Thus, tap water has an enhancing effect on survival of *L. pneumophila* in the VBNC state that may impose health risks if VBNC cells regain culturability after encountering amoeba in tap water systems. Tap water ions were suggested to play a role in this enhancing effect of tap water on survival of *L. pneumophila*. Several ions were found to have a beneficial effect in extending the culturability of SPFs and improving the yield of SPF-VBNC cells. The role of ions in extending *L. pneumophila* survival in tap water is unknown; however, they might act as co-

factors for some enzymes that participate in catalyzing important processes like utilization of energy sources. A more extensive and comprehensive study would be required to identify the specific ions responsible.

In this study I created a novel way to quantify the number of resuscitated bacteria out of the total VBNC *L. pneumophila* cells, and found that only ~1 out of 10^5 VBNC cells could be resuscitated in amoeba. Moreover, I found that resuscitation of VBNC cells was successful in amoeba but not in macrophages, suggesting that amoeba might be providing molecules essential for this process.

Proteomic shotgun analysis revealed that *L. pneumophila* VBNC cells have a distinct protein profile compared to culturable bacteria. The majority of proteins with increased expression were those involved in metabolic processes, suggesting that *L. pneumophila* VBNC cells are retaining some metabolic activity in the VBNC state. Among proteins with increased expression was RelA, which suggests that the intracellular level of (p)ppGpp in the VBNC bacteria is higher than that in the culturable cells. This, in turn, suggests that the VBNC *L. pneumophila* may be capable of adapting to environmental signals via stringent response. Moreover, shotgun proteomics revealed enhancement in the level of pathogenesis-related proteins such as EnhA, which is involved in entry of *L. pneumophila* into monocytes. Proteins related to the type IV secretion system were also enhanced. Thus, *L. pneumophila* might be retaining its infectivity in the VBNC state, which would facilitate its entry into new host cells and stimulate its regaining of culturability. Further study is required to determine the stage at which these proteins were elevated and to evaluate the role of specific proteins in *L. pneumophila* entry into the VBNC state.

SDS-PAGE and MRM showed that the 3-hydroxybutyrate dehydrogenase (BdhA) enzyme level was more abundant in the MIF-VBNCCs than the culturable MIFs. Moreover, an important change observed in VBNC *L. pneumophila* cells produced in tap water was the lack of PHB inclusions. It seems that *L. pneumophila* degrades PHB in order to maintain an active metabolism and extend its culturability during starvation. Accordingly, to evaluate the role of BdhA in VBNC cell formation, I constructed a *bdhA* mutant and used the mutant to produce VBNC cells. BdhA seems to influence *L. pneumophila* survival and VBNC cell formation. This the first time to identify a gene involved in VBNC formation in *Legionella.* Moreover, This is the first time to mutate a gene in *Legionella* by electroporating a linear

DNA fragment into electro-shocked cells.

The data from the current study widen our knowledge about VBNC *Legionella*, such that VBNC *L. pneumophila* could be integrated into *L. pneumophila* life cycle.

6.9. Future Directions

VBNC cells found in nature might be infectious to humans; therefore, future work should include attempts to infect different human cell lines. Another suggestion would be to monitor viability and culturability of MIFs produced from human macrophages and HeLa cells in water. This will help to determine if the VBNC cells produced from human cells survive better than those produced from amoeba and whether, in human lungs, VBNC cells are produced and expelled to the environment to infect other people.

Guinea pig is the animal model of *Legionella*, and attempts to infect the animal with VBNC have been conducted by intraperitoneal injection (Steinert *et al* .1997). Since humans get the LD by water aerosoles, another suggestion is to use VBNC aerosols to infect guinea pigs.

REFERENCES

Abdelrahman, Y. M. and Belland, R. J. (2005). The chlamydial developmental cycle. *FEMS Microbiology Reviews, 29*(5), 949-959.

Abu Kwaik, Y., Gao, L. Y., Stone, B. J., Venkataraman, C., and Harb, O. S. (1998). Invasion of protozoa by *Legionella pneumophila* and its role in bacterial ecology and pathogenesis. *Applied and Environmental Microbiology, 64*(9), 3127-3133.

Aertsen, A. and Michiels, C. W. (2004). Stress and how bacteria cope with death and survival. *Critical Reviews in Microbiology, 30*(4), 263-273.

Aizenman, E., Engelberg-Kulka, H., and Glaser, G. (1996). An *Escherichia coli* chromosomal "addiction module" regulated by guanosine [corrected] 3',5'-bispyrophosphate: A model for programmed bacterial cell death. *Proceedings of the National Academy of Sciences of the United States of America, 93*(12), 6059-6063.

Albers, U., Tiaden, A., Spirig, T., Al Alam, D., Goyert, S. M., Gangloff, S. C., and Hilbi, H. (2007). Expression of *Legionella pneumophila* paralogous lipid A biosynthesis genes under different growth conditions. *Microbiology,153*(11), 3817-3829.

Albert-Weissenberger, C., Sahr, T., Sismeiro, O., Hacker, J., Heuner, K., and Buchrieser, C. (2010). Control of flagellar gene regulation in *Legionella pneumophila* and its relation to growth phase. *Journal of Bacteriology, 192*(2), 446-455.

Aldor, I. S. and Keasling, J. D. (2003). Process design for microbial plastic factories: Metabolic engineering of polyhydroxyalkanoates. *Current Opinion in Biotechnology, 14*(5), 475-483.

Al-Khodor, S., Kalachikov, S., Morozova, I., Price, C. T., and Abu Kwaik, Y. (2009). The PmrA/PmrB two-component system of *Legionella pneumophila* is a global regulator required for intracellular replication within macrophages and protozoa. *Infection and Immunity, 77*(1), 374-386.

Allegra, S., Berger, F., Berthelot, P., Grattard, F., Pozzetto, B., and Riffard, S. (2008). Use of flow cytometry to monitor *Legionella* viability. *Applied and Environmental Microbiology, 74*(24), 7813-7816.

Allen, P. G. and Dawidowicz, E. A. (1990a). Phagocytosis in *Acanthamoeba* : I. A mannose receptor is responsible for the binding and phagocytosis of yeast. *Journal of Cellular Physiology, 145*(3), 508-513.

Allen, P. G. and Dawidowicz, E. A. (1990b). Phagocytosis in *Acanthamoeba* : II. soluble and insoluble mannose-rich ligands stimulate phosphoinositide metabolism. *Journal of Cellular Physiology, 145*(3), 514-521.

Alleron, L., Merlet, N., Lacombe, C., and Frere, J. (2008). Long-term survival of *Legionella pneumophila* in the viable but nonculturable state after monochloramine treatment. *Current Microbiology, 57*(5), 497-502.

Al-Quadan, T. and Abu Kwaik, Y. (2011). Molecular characterization of exploitation of the polyubiquitination and farnesylation machineries of *Dictyostelium discoideum* by the AnkB F-box effector of *Legionella pneumophila*. *Frontiers in Microbiology, 2*, 23.

Al-Quadan, T., Price, C. T., and Abu Kwaik, Y. (2012). Exploitation of evolutionarily conserved amoeba and mammalian processes by *Legionella* . *Trends in Microbiology, 20*(6), 299-306.

Anderson, A. J. and Dawes, E. A. (1990). Occurrence, metabolism, metabolic role, and industrial uses of bacterial polyhydroxyalkanoates. *Microbiological Reviews, 54*(4), 450-472.

Anderson, I. J., Watkins, R. F., Samuelson, J., Spencer, D. F., Majoros, W. H., Gray, M. W., and Loftus, B.J. (2005). Gene discovery in the *Acanthamoeba castellanii* genome. *Protist, 156*(2), 203-214.

Anderson, M., Bollinger, D., Hagler, A., Hartwell, H., Rivers, B., Ward, K., and Steck, T. R. (2004). Viable but nonculturable bacteria are present in mouse and human urine specimens. *Journal of Clinical Microbiology*, *42*(2), 753-758.

Aneja, P. and Charles, T. C. (1999). Poly-3-hydroxybutyrate degradation in *Rhizobium* (*Sinorhizobium*) *meliloti*: Isolation and characterization of a gene encoding 3-hydroxybutyrate dehydrogenase. *Journal of Bacteriology, 181*(3), 849-857.

Aneja, P., Zachertowska, A., and Charles, T. C. (2005). Comparison of the symbiotic and competition phenotypes of *Sinorhizobium meliloti* PHB synthesis and degradation pathway mutants. *Canadian Journal of Microbiology, 51*(7), 599-604.

Aneja, P. and Charles, T. C. (2005). Characterization of *bdhA*, encoding the enzyme *D*-3-hydroxybutyrate dehydrogenase, from *Sinorhizobium* sp. strain NGR234. *FEMS Microbiology Letters, 242*(1), 87-94.

Anuchin, A. M., Mulyukin, A. L., Suzina, N. E., Duda, V. I., El-Registan, G. I., and Kaprelyants, A. S. (2009). Dormant forms of *Mycobacterium smegmatis* with distinct morphology. *Microbiology, 155*(4), 1071-1079.

Arana, I., Seco, C., Epelde, K., Muela, A., Fernandez-Astorga, A., and Barcina, I. (2004). Relationships between *Escherichia coli* cells and the surrounding medium during survival processes. *Antonie Van Leeuwenhoek, 86*(2), 189-199.

Arasaki, K., Toomre, D. K., and Roy, C. R. (2012). The *Legionella pneumophila* effector DrrA is sufficient to stimulate SNARE-dependent membrane fusion. *Cell Host and Microbe, 11*(1), 46-57.

Artz, R. R., Avery, L. M., Jones, D. L., and Killham, K. (2006). Potential pitfalls in the quantitative molecular detection of *Escherichia coli* O157:H7 in environmental matrices. *Canadian Journal of Microbiology, 52*(5), 482-488.

Asakura, H., Igimi, S., Kawamoto, K., Yamamoto, S., and Makino, S. (2005). Role of *in vivo* passage on the environmental adaptation of enterohemorrhagic *Escherichia coli* O157:H7: Cross-induction of the viable but nonculturable state by osmotic and oxidative stresses. *FEMS Microbiology Letters, 253*(2), 243-249.

Asakura, H., Ishiwa, A., Arakawa, E., Makino, S., Okada, Y., Yamamoto, S., Igimi, S. (2007). Gene expression profile of *Vibrio cholerae* in the cold stress-induced viable but non-culturable state. *Environmental Microbiology*, *9*(4), 869-879.

Asakura, H., Kawamoto, K., Haishima, Y., Igimi, S., Yamamoto, S., and Makino, S. (2008). Differential expression of the outer membrane protein W (OmpW) stress response in enterohemorrhagic *Escherichia coli* O157:H7 corresponds to the viable but non-culturable state. *Research in Microbiology, 159*(9-10), 709-717.

Atlas, R. M., Williams, J. F., and Huntington, M. K. (1995). *Legionella* contamination of dental-unit waters. *Applied and Environmental Microbiology*, *61*(4), 1208-1213.

Aurass, P., Pless, B., Rydzewski, K., Holland, G., Bannert, N., and Flieger, A. (2009). *bdhA-patD* operon as a virulence determinant, revealed by a novel large-scale approach for identification of *Legionella pneumophila* mutants defective for amoeba infection. *Applied and Environmental Microbiology*, *75*(13), 4506-4515.

Aurass, P., Prager, R., and Flieger, A. (2011). EHEC/EAEC O104:H4 strain linked with the 2011 German outbreak of haemolytic uremic syndrome enters into the viable but non-culturable state in response to various stresses and resuscitates upon stress relief. *Environmental Microbiology, 13*(12), 3139-3148.

Ayub, N. D., Pettinari, M. J., Ruiz, J. A., and Lopez, N. I. (2004). A polyhydroxybutyrate-producing *Pseudomonas* sp. isolated from antarctic environments with high stress resistance. *Current Microbiology*, *49*(3), 170-174.

Bachman, M. A. and Swanson, M. S. (2001). RpoS co-operates with other factors to induce *Legionella pneumophila* virulence in the stationary phase. *Molecular Microbiology, 40*(5), 1201-1214.

Bachman, M. A. and Swanson, M. S. (2004a). The LetE protein enhances expression of multiple LetA/LetS-dependent transmission traits by *Legionella pneumophila*. *Infection and Immunity, 72*(6), 3284-3293.

Bachman, M. A. and Swanson, M. S. (2004b). Genetic evidence that *Legionella pneumophila* RpoS modulates expression of the transmission phenotype in both the exponential phase and the stationary phase. *Infection and Immunity, 72*(5), 2468-2476.

Baffone, W., Casaroli, A., Citterio, B., Pierfelici, L., Campana, R., Vittoria, E., Guaglianone, E., Donelli, G. (2006). *Campylobacter jejuni* loss of culturability in aqueous microcosms and ability to resuscitate in a mouse model. *International Journal of Food Microbiology, 107*(1), 83-91.

Baffone, W., Citterio, B., Vittoria, E., Casaroli, A., Campana, R., Falzano, L., Donelli, G. (2003). Retention of virulence in viable but non-culturable halophilic *Vibrio* spp. *International Journal of Food Microbiology*, *89*(1), 31-39.

Baker, C. S., Morozov, I., Suzuki, K., Romeo, T., and Babitzke, P. (2002). CsrA regulates glycogen biosynthesis by preventing translation of *glgC* in *Escherichia coli*. *Molecular Microbiology, 44*(6), 1599-1610.

Barcina, I. and Arana, I. (2009). The viable but nonculturable phenotype: A crossroads in the life-cycle of non-differentiating bacteria? *Rev Environ Sci Biotechnol, 8(3)*, 245-255.

Basaglia, M., Povolo, S., and Casella, S. (2007). Resuscitation of viable but not culturable *Sinorhizobium meliloti* 41 pRP4-luc: Effects of oxygen and host plant. *Current Microbiology, 54*(3), 167-174.

Battesti, A. and Bouveret, E. (2006). Acyl carrier protein/SpoT interaction, the switch linking SpoT-dependent stress response to fatty acid metabolism. *Molecular Microbiology, 62*(4), 1048-1063.

Ben Said, M., Masahiro, O., and Hassen, A. (2010). Detection of viable but non cultivable *Escherichia coli* after UV irradiation using a lytic Qbeta phage. *Annals of Microbiology, 60*(1), 121-127.

Benach, J., Swaminathan, S. S., Tamayo, R., Handelman, S. K., Folta-Stogniew, E., Ramos, J. E., Forouhar, F., Neely, H., Seetharaman, J., Camilli, A., Hunt, J. F. (2007). The structural basis of cyclic diguanylate signal transduction by PilZ domains. *The EMBO Journal, 26*(24), 5153-5166.

Berger, K. H., and Isberg, R. R. (1993). Two distinct defects in intracellular growth complemented by a single genetic locus in *Legionella pneumophila*. *Molecular Microbiology, 7*(1), 7-19.

Bergmeyer, H. U., Gawehn, K., Klotzsch, H., Krebs, H. A., and Williamson, D. H. (1967). Purification and properties of crystalline 3-hydroxybutyrate dehydrogenase from *Rhodopseudomonas spheroides*. *The Biochemical Journal, 102*(2), 423-431.

Berk, S. G., Faulkner, G., Garduno, E., Joy, M. C., Ortiz-Jimenez, M. A., and Garduno, R. A. (2008). Packaging of live *Legionella pneumophila* into pellets expelled by *Tetrahymena* spp. does not require bacterial replication and depends on a Dot/Icm-mediated survival mechanism. *Applied and Environmental Microbiology, 74*(7), 2187-2199.

Berk, S. G., Ting, R. S., Turner, G. W., and Ashburn, R. J. (1998). Production of respirable vesicles containing live *Legionella pneumophila* cells by two *Acanthamoeba* spp. *Applied and Environmental Microbiology, 64*(1), 279-286.

Bernardo, L. M., Johansson, L. U., Solera, D., Skarfstad, E., and Shingler, V. (2006). The guanosine tetraphosphate (ppGpp) alarmone, DksA and promoter affinity for RNA polymerase in regulation of sigma-dependent transcription. *Molecular Microbiology, 60*(3), 749-764.

Bertani, G. (1951). Studies on lysogenesis. I. the mode of phage liberation by lysogenic *Escherichia coli*. *Journal of Bacteriology, 62*(3), 293-300.

Birnboim, H. C., and Doly, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Research, 7*(6), 1513-1523.

Blazquez Garrido, R. M., Espinosa Parra, F. J., Alemany Frances, L., Ramos Guevara, R. M., Sanchez-Nieto, J. M., Segovia Hernandez, M., Serrano Martínez, J. A., Huerta, F. H. (2005). Antimicrobial chemotherapy for Legionnaires disease: Levofloxacin versus macrolides. *Clinical Infectious Diseases : An Official Publication of the Infectious Diseases Society of America, 40*(6), 800-806.

Bloomfield, S. F., Stewart, G. S., Dodd, C. E., Booth, I. R., and Power, E. G. (1998). The viable but non-culturable phenomenon explained? *Microbiology, 144*(1), 1-3.

Boaretti, M., Lleo, M. M., Bonato, B., Signoretto, C., and Canepari, P. (2003). Involvement of *rpoS* in the survival of *Escherichia coli* in the viable but non-culturable state. *Environmental Microbiology, 5*(10), 986-996.

Boehm, A., Kaiser, M., Li, H., Spangler, C., Kasper, C. A., Ackermann, M., Kaever, V., Sourjik, V., Roth, V., Jenal, U. (2010). Second messenger-mediated adjustment of bacterial swimming velocity. *Cell, 141*(1), 107-116.

Borella, P., Guerrieri, E., Marchesi, I., Bondi, M., and Messi, P. (2005). Water ecology of *Legionella* and protozoan: Environmental and public health perspectives. *Biotechnology Annual Review, 11*, 355-380.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry, 72*, 248-254.

Brand, B. C., Sadosky, A. B., and Shuman, H. A. (1994). The *Legionella pneumophila icm* locus: A set of genes required for intracellular multiplication in human macrophages. *Molecular Microbiology, 14*(4), 797-808.

Bremer, H., and Dennis, P. P. (1996). Modulation of chemical composition and other paramters of the cell by growth rate. In R. Curtiss III, Ingraham, J. E.C., Lin, C., Low, K. B., Magasanik, B., Reznikoff, W. S., Riley, M., Schaechter ,M. Umbarger,H.E. Schaechter,M. (Eds.), *Escherichia coli and Salmonella: Cellular and molecular biology* (2nd ed., pp.1553-1569). Washington, D.C. ASM Press.

Brieland, J., McClain, M., Heath, L., Chrisp, C., Huffnagle, G., LeGendre, M., Hurley, M., Fantone, J., Engleberg, C. (1996). Coinoculation with *Hartmannella vermiformis* enhances replicative *Legionella pneumophila* lung infection in a murine model of Legionnaires' disease. *Infection and Immunity, 64*(7), 2449-2456.

Brown, L., Gentry, D., Elliott, T., and Cashel, M. (2002). DksA affects ppGpp induction of RpoS at a translational level. *Journal of Bacteriology, 184*(16), 4455-4465.

Bruggemann, H., Hagman, A., Jules, M., Sismeiro, O., Dillies, M. A., Gouyette, C., Kunst, F., Steinert, M.; Heuner, K., Coppee, J. Y., Buchrieser, C. (2006). Virulence strategies for infecting phagocytes deduced from the *in vivo* transcriptional program of *Legionella pneumophila*. *Cellular Microbiology, 8*(8), 1228-1240.

Byrne, B., and Swanson, M. S. (1998). Expression of *Legionella pneumophila* virulence traits in response to growth conditions. *Infection and Immunity, 66*(7), 3029-3034.

Cai, G. Q., Driscoll, B. T., and Charles, T. C. (2000). Requirement for the enzymes acetoacetyl coenzyme A synthetase and poly-3-hydroxybutyrate (PHB) synthase for growth of *Sinorhizobium meliloti* on PHB cycle intermediates. *Journal of Bacteriology, 182*(8), 2113-2118.

Calva, E., and Oropeza, R. (2006). Two-component signal transduction systems, environmental signals, and virulence. *Microbial Ecology, 51*(2), 166-176.

Cappelier, J. M., Magras, C., Jouve, J. L., and Federighi, M. (1999a). Recovery of viable but non-culturable *Campylobacter jejuni* cells in two animal models. *Food Microbiology, 16(4)*, 375-383.

Cappelier, J. M., Minet, J., Magras, C., Colwell, R. R., and Federighi, M. (1999b). Recovery in embryonated eggs of viable but nonculturable *Campylobacter jejuni* cells and maintenance of ability to adhere to HeLa cells after resuscitation. *Applied and Environmental Microbiology, 65*(11), 5154-5157.

Carvalho, F. R., Nastasi, F. R., Gamba, R. C., Foronda, A. S., and Pellizari, V. H. (2008). Occurrence and diversity of *Legionellaceae* in polar lakes of the antarctic peninsula. *Current Microbiology, 57*(4), 294-300.

Castaneda, M., Guzman, J., Moreno, S., and Espin, G. (2000). The GacS sensor kinase regulates alginate and poly-beta-hydroxybutyrate production in *Azotobacter vinelandii*. *Journal of Bacteriology, 182*(9), 2624-2628.

Catrenich, C. E., and Makin, K. M. (1991). Characterization of the morphologic conversion of *Helicobacter pylori* from bacillary to coccoid forms. *Scandinavian Journal of Gastroenterology.Supplement, 181*, 58-64.

Cazalet, C., Rusniok, C., Bruggemann, H., Zidane, N., Magnier, A., Ma, L.,Tichit, M., Jarraud, S., Bouchier, C., Vandenesch, F., Kunst, F., Etienne, J., Glaser, P., Buchrieser, C. (2004). Evidence in the *Legionella pneumophila* genome for exploitation of host cell functions and high genome plasticity. *Nature Genetics, 36*(11), 1165-1173.

Chaiyanan, S., Chaiyanan, S., Huq, A., Maugel, T., and Colwell, R. R. (2001). Viability of the nonculturable *Vibrio cholerae* O1 and O139. *Systematic and Applied Microbiology, 24*(3), 331-341.

Chang, B., Kura, F., Amemura-Maekawa, J., Koizumi, N., and Watanabe, H. (2005). Identification of a novel adhesion molecule involved in the virulence of *Legionella pneumophila*. *Infection and Immunity, 73*(7), 4272-4280.

Chang, C. W., Hwang, Y. H., Cheng, W. Y., and Chang, C. P. (2007). Effects of chlorination and heat disinfection on long-term starved *Legionella pneumophila* in warm water. *Journal of Applied Microbiology, 102*(6), 1636-1644.

Chang, C. W., Kao, C. H., and Liu, Y. F. (2009). Heterogeneity in chlorine susceptibility for *Legionella pneumophila* released from *Acanthamoeba* and *Hartmannella*. *Journal of Applied Microbiology, 106*(1), 97-105.

Chatterji, D., and Ojha, A. K. (2001). Revisiting the stringent response, ppGpp and starvation signaling. *Current Opinion in Microbiology, 4*(2), 160-165.

Chaveerach, P., ter Huurne, A. A., Lipman, L. J., and van Knapen, F. (2003). Survival and resuscitation of ten strains of *Campylobacter jejuni* and *Campylobacter coli* under acid conditions. *Applied and Environmental Microbiology, 69*(1), 711-714.

Chen, J., de Felipe, K. S., Clarke, M., Lu, H., Anderson, O. R., Segal, G., Shuman, H. A. (2004). *Legionella* effectors that promote nonlytic release from protozoa. *Science, 303*(5662), 1358-1361.

Chien, M., Morozova, I., Shi, S., Sheng, H., Chen, J., Gomez, S. M., Asamani, G., Hill, K., Nuara, J., Feder, M., Rineer, J., Greenberg, J. J., Steshenko, V., Park, S. H., Zhao, B., Teplitskaya, E., Edwards, J. R., Pampou, S., Georghiou, A., Chou, I. C., Iannuccilli, W., Ulz, M. E., Kim, D. H., Geringer-Sameth, A., Goldsberry, C., Morozov, P., Fischer, S. G., Segal, G., Qu, X., Rzhetsky, A., Zhang, P., Cayanis, E., De Jong, P. J., Ju, J., Kalachikov, S., Shuman, H. A., Russo, J. J. (2004). The genomic sequence of the accidental pathogen *Legionella pneumophila*. *Science, 305*(5692), 1966-1968.

Chong, A. (2007). Characterization of the virulence-related roles of the *Legionella pneumophila* chaperonin, HtpB, in mammalian cells. *Dalhousie University (Canada)*

Chong, A., Lima, C. A., Allan, D. S., Nasrallah, G. K., and Garduno, R. A. (2009). The purified and recombinant *Legionella pneumophila* chaperonin alters mitochondrial trafficking and microfilament organization. *Infection and Immunity, 77*(11), 4724-4739.

Cirillo, S. L., Lum, J., and Cirillo, J. D. (2000). Identification of novel loci involved in entry by *Legionella pneumophila*. *Microbiology, 146*(6), 1345-1359.

Clements, M. O., and Foster, S. J. (1998). Starvation recovery of *Staphylococcus aureus* 8325-4. *Microbiology, 144*(7), 1755-1763.

Cohen-Gonsaud, M., Barthe, P., Bagneris, C., Henderson, B., Ward, J., Roumestand, C., Keep, N. H. (2005). The structure of a resuscitation-promoting factor domain from *Mycobacterium tuberculosis* shows homology to lysozymes. *Nature Structural and Molecular Biology, 12*(3), 270-273.

Colbourne, J. S., and Dennis, P. J. (1989). The ecology and survival of *Legionella pneumophila*. *Water and Environment Journal, 3*, 345–350.

Colwell, R. R., Brayton, P., Herrington, D., Tall, B., Huq, A., and Levine, M. M. (1996). Viable but non-culturable *Vibrio cholerae* O1 revert to a cultivable state in the human intestine. *World Journal of Microbiology and Biotechnology, 12(1)*, 28-31.

Colwell, R. R., Brayton, P. R., Grimes, D. J., Roszak, D. R., Huq, S. A., and Palmer, L. M. (1985). Viable but non-culturable *Vibrio cholerae* and related pathogens in the environment: Implications for release of genetically engineered microorganisms. *Nature Biotechnology, 3*, 817-820.

Cook, K. L., and Bolster, C. H. (2007). Survival of *Campylobacter jejuni* and *Escherichia coli* in groundwater during prolonged starvation at low temperatures. *Journal of Applied Microbiology, 103*(3), 573-583.

Costa, J., Tiago, I., da Costa, M. S., and Verissimo, A. (2005). Presence and persistence of *Legionella* spp. in groundwater. *Applied and Environmental Microbiology, 71*(2), 663-671.

Costa, K., Bacher, G., Allmaier, G., Dominguez-Bello, M. G., Engstrand, L., Falk, P., de Pedro, M. A., Garcia-del Portillo, F. (1999). The morphological transition of *Helicobacter pylori* cells from spiral to coccoid is preceded by a substantial modification of the cell wall. *Journal of Bacteriology, 181*(12), 3710-3715.

Costerton, W. J., and Wilson, M. (2004). Introducing biofilms. *Biofilms, 1(1)*, 1-4.

Coutard, F., Pommepuy, M., Loaec, S., and Hervio-Heath, D. (2005). mRNA detection by reverse transcription-PCR for monitoring viability and potential virulence in a pathogenic strain of *Vibrio parahaemolyticus* in viable but nonculturable state. *Journal of Applied Microbiology, 98*(4), 951-961.

Cunningham, E., O'Byrne, C., and Oliver, J. D. (2009). Effect of weak acids on *Listeria monocytogenes* survival: Evidence for a viable but nonculturable state in response to low pH. *Food Control, 20*, 1141-1144.

Cuny, C., Dukan, L., Fraysse, L., Ballesteros, M., and Dukan, S. (2005). Investigation of the first events leading to loss of culturability during *Escherichia coli* starvation: Future nonculturable bacteria form a subpopulation. *Journal of Bacteriology, 187*(7), 2244-2248.

Cursons, R. T., Brown, T. J., and Keys, E. A. (1980). Effect of disinfectants on pathogenic free-living amoebae: In axenic conditions. *Applied and Environmental Microbiology, 40*(1), 62-66.

Dalebroux, Z. D., Yagi, B. F., Sahr, T., Buchrieser, C., and Swanson, M. S. (2010). Distinct roles of ppGpp and DksA in *Legionella pneumophila* differentiation. *Molecular Microbiology, 76*(1), 200-219.

Dame, R. T., and Goosen, N. (2002). HU: Promoting or counteracting DNA compaction? *FEBS Letters, 529*(2-3), 151-156.

Dawes, E. A., and Senior, P. J. (1973). The role and regulation of energy reserve polymers in micro-organisms. *Advances in Microbial Physiology, 10*, 135-266.

Day, A. P., and Oliver, J. D. (2004). Changes in membrane fatty acid composition during entry of *Vibrio vulnificus* into the viable but nonculturable state. *Journal of Microbiology (Seoul, Korea), 42*(2), 69-73.

Declerck, P. (2010). Biofilms: The environmental playground of *Legionella pneumophila*. *Environmental Microbiology, 12*(3), 557-566.

Declerck, P., Behets, J., Margineanu, A., van Hoef, V., De Keersmaecker, B., and Ollevier, F. (2009). Replication of *Legionella pneumophila* in biofilms of water distribution pipes. *Microbiological Research, 164*(6), 593-603.

Declerck, P., Behets, J., van Hoef, V., and Ollevier, F. (2007a). Detection of *Legionella* spp. and some of their amoeba hosts in floating biofilms from anthropogenic and natural aquatic environments. *Water Research, 41*(14), 3159-3167.

Declerck, P., Behets, J., van Hoef, V., and Ollevier, F. (2007b). Replication of *Legionella pneumophila* in floating biofilms. *Current Microbiology, 55*(5), 435-440.

Declerck, P., Behets, J., De Keersmaecker, B., and Ollevier, F. (2007c). Receptor-mediated uptake of *Legionella pneumophila* by *Acanthamoeba castellanii* and *Naegleria lovaniensis*. *Journal of Applied Microbiology, 103*(6), 2697-2703.

Delafield, F. P., Cooksey, K. E., and Doudoroff, M. (1965). β-hydroxybutyric dehydrogenase and dimer hydrolase of *Pseudomonas zemoignei*. *The Journal of Biological Chemistry, 240(10)*, 4023-4028.

Den Boer, J. W., Yzerman, E. P., Schellekens, J., Lettinga, K. D., Boshuizen, H. C., Van Steenbergen, J. E., Bosman, A., Van den Hof, S., Van Vliet, H. A., Peeters, M. F., Van Ketel, R. J., Speelman, P., Kool, J. L., Conyn-Van Spaendonck, M. A. (2002). A large outbreak of Legionnaires' disease at a flower show, the netherlands, 1999. *Emerging Infectious Diseases, 8*(1), 37-43.

Dinu, L. D., and Bach, S. (2011). Induction of viable but nonculturable *Escherichia coli* O157:H7 in the phyllosphere of lettuce: A food safety risk factor. *Applied and Environmental Microbiology, 77*(23), 8295-8302.

Donlan, R. M. (2002). Biofilms: Microbial life on surfaces. *Emerging Infectious Diseases, 8*(9), 881-890.

Dorer, M. S., Kirton, D., Bader, J. S., and Isberg, R. R. (2006). RNA interference analysis of *Legionella* in *Drosophila* cells: Exploitation of early secretory apparatus dynamics. *PLoS Pathog., 2*, e34.

Downing, K. J., Betts, J. C., Young, D. I., McAdam, R. A., Kelly, F., Young, M., Mizrahi, V. (2004). Global expression profiling of strains harbouring null mutations reveals that the five *rpf*-like genes of *Mycobacterium tuberculosis* show functional redundancy. *Tuberculosis, 84*(3-4), 167-179.

Downing, K. J., Mischenko, V. V., Shleeva, M. O., Young, D. I., Young, M., Kaprelyants, A. S., Apt, A. S., Mizrahi, V. (2005). Mutants of *Mycobacterium tuberculosis* lacking three of the five *rpf*-like genes are defective for growth *in vivo* and for resuscitation *in vitro*. *Infection and Immunity, 73*(5), 3038-3043.

D'Souza-Schorey, C., and Chavrier, P. (2006). ARF proteins: Roles in membrane traffic and beyond. *Nature Reviews.Molecular Cell Biology, 7*(5), 347-358.

Du, M., Chen, J., Zhang, X., Li, A., Li, Y., and Wang, Y. (2007). Retention of virulence in a viable but nonculturable *Edwardsiella tarda* isolate. *Applied and Environmental Microbiology, 73*(4), 1349-1354.

Dusserre, E., Ginevra, C., Hallier-Soulier, S., Vandenesch, F., Festoc, G., Etienne, J., Jarraud, S., Molmeret, M. (2008). A PCR-based method for monitoring *Legionella pneumophila* in water samples detects viable but noncultivable *Legionellae* that can recover their cultivability. *Applied and Environmental Microbiology, 74*(15), 4817-4824.

Edwards, R. L., Bryan, A., Jules, M., Harada, K., Buchrieser, C., and Swanson, M. S. (2013). Nicotinic acid modulates *Legionella pneumophila* gene expression and induces virulence traits. *Infection and Immunity, 81*(3), 945-955.

Edwards, R. L., Jules, M., Sahr, T., Buchrieser, C., and Swanson, M. S. (2010). The *Legionella pneumophila* LetA/LetS two-component system exhibits rheostat-like behavior. *Infection and Immunity, 78*(6), 2571-2583.

Ehrlich, G. D., Veeh, R., Wang, X., Costerton, J. W., Hayes, J. D., Hu, F. Z., Daigle, B. J., Ehrlich, M. D., Post, J. C. (2002). Mucosal biofilm formation on middle-ear mucosa in the Chinchilla model of otitis media. *JAMA : The Journal of the American Medical Association, 287*(13), 1710-1715.

England, A. C.,3rd, Fraser, D. W., Mallison, G. F., Mackel, D. C., Skaliy, P., and Gorman, G. W. (1982). Failure of *Legionella pneumophila* sensitivities to predict culture results from disinfectant-treated air-conditioning cooling towers. *Applied and Environmental Microbiology, 43*(1), 240-244.

Ericsson, M., Hanstorp, D., Hagberg, P., Enger, J., and Nystrom, T. (2000). Sorting out bacterial viability with optical tweezers. *Journal of Bacteriology, 182*(19), 5551-5555.

Errington, J. (2003). Regulation of endospore formation in *Bacillus subtilis*. *Nat Rev Microbiol. 1*(2), 117-26

Faulkner, G., Berk, S. G., Garduno, E., Ortiz-Jimenez, M. A., and Garduno, R. A. (2008). Passage through *Tetrahymena tropicalis* triggers a rapid morphological differentiation in *Legionella pneumophila*. *Journal of Bacteriology, 190*(23), 7728-7738.

Faulkner, G., and Garduno, R. A. (2002). Ultrastructural analysis of differentiation in *Legionella pneumophila*. *Journal of Bacteriology, 184*(24), 7025-7041.

Feller, C., Gunther, R., Hofmann, H. J., and Grunow, M. (2006). Molecular basis of substrate recognition in D-3-hydroxybutyrate dehydrogenase from *Pseudomonas putida*. *Chembiochem: A European Journal of Chemical Biology, 7*(9), 1410-1418.

Fernandez-Moreira, E., Helbig, J. H., and Swanson, M. S. (2006). Membrane vesicles shed by *Legionella pneumophila* inhibit fusion of phagosomes with lysosomes. *Infection and Immunity, 74*(6), 3285-3295.

Fettes, P. S., Forsbach-Birk, V., Lynch, D., and Marre, R. (2001). Overexpresssion of a *Legionella pneumophila* homologue of the *E. coli* regulator *csrA* affects cell size, flagellation, and pigmentation. *International Journal of Medical Microbiology : IJMM, 291*(5), 353-360.

Fields, B. S., Benson, R. F., and Besser, R. E. (2002). *Legionella* and Legionnaires' disease: 25 years of investigation. *Clinical Microbiology Reviews, 15*(3), 506-526.

Fields, B. S. (1993). *Legionella* and protozoa: Interaction of a pathogen and its natural host . In J. M. Barbaree, R. F. Breiman and A. P. Dufour (Eds.), *Legionella: Current status and emerging perspectives* (pp. 129-136). Washington: American Society of Microbiology.

Fields, B. S. (1996). The molecular ecology of *Legionellae*. *Trends in Microbiology, 4*(7), 286-290.

Fields, B. S., Barbaree, J. M., Shotts, E. B.,Jr, Feeley, J. C., Morrill, W. E., Sanden, G. N., Dykstra, M. J. (1986). Comparison of guinea pig and protozoan models for determining virulence of *Legionella* species. *Infection and Immunity, 53*(3), 553-559.

Fischer-Le Saux, M., Hervio-Heath, D., Loaec, S., Colwell, R. R., and Pommepuy, M. (2002). Detection of cytotoxin-hemolysin mRNA in nonculturable populations of environmental and clinical *Vibrio vulnificus* strains in artificial seawater. *Applied and Environmental Microbiology, 68*(11), 5641-5646.

Fisman, D. N., Lim, S., Wellenius, G. A., Johnson, C., Britz, P., Gaskins, M., Maher, J., Mittleman, M. A., Spain, C. V., Haas, C. N., Newbern, C. (2005). It's not the heat, it's the humidity: Wet weather increases Legionellosis risk in the greater philadelphia metropolitan area. *The Journal of Infectious Diseases, 192*(12), 2066-2073.

Fliermans, C. B., Cherry, W. B., Orrison, L. H., Smith, S. J., Tison, D. L., and Pope, D. H. (1981a). Ecological distribution of *Legionella pneumophila*. *Applied and Environmental Microbiology, 41*(1), 9-16.

Fliermans, C.,B., Soracco, R.,J., and Pope, D.,H. (1981b). Measure of *Legionella pneumophila* activity *in situ*. *Current Microbiology,* (2), 89-94.

Forsbach-Birk, V., McNealy, T., Shi, C., Lynch, D., and Marre, R. (2004). Reduced expression of the global regulator protein CsrA in *Legionella pneumophila* affects virulence-associated regulators and growth in *Acanthamoeba castellanii*. *International Journal of Medical Microbiology: IJMM, 294*(1), 15-25.

Fraser, D. W., Tsai, T. R., Orenstein, W., Parkin, W. E., Beecham, H. J., Sharrar, R. G., Sharrar, R. G., Harris, J., Mallison, G. F., Martin, S. M., McDade, J. E., Shepard, C. C., Brachman, P. S. (1977). Legionnaires' disease: Description of an epidemic of pneumonia. *The New England Journal of Medicine, 297*(22), 1189-1197.

Freestone, P. P., Haigh, R. D., Williams, P. H., and Lyte, M. (1999). Stimulation of bacterial growth by heat-stable, norepinephrine-induced autoinducers. *FEMS Microbiology Letters, 172*(1), 53-60.

Galka, F., Wai, S. N., Kusch, H., Engelmann, S., Hecker, M., Schmeck, B., Hippenstiel, S., Uhlin, B. E., Steinert, M. (2008). Proteomic characterization of the whole secretome of *Legionella pneumophila* and functional analysis of outer membrane vesicles. *Infection and Immunity, 76*(5), 1825-1836.

Gao, L. Y., Harb, O. S., and Abu Kwaik, Y. (1997). Utilization of similar mechanisms by *Legionella pneumophila* to parasitize two evolutionarily distant host cells, mammalian macrophages and protozoa. *Infection and Immunity, 65*(11), 4738-4746.

Garcia, M. T., Jones, S., Pelaz, C., Millar, R. D., and Abu Kwaik, Y. (2007). *Acanthamoeba polyphaga* resuscitates viable non-culturable *Legionella pneumophila* after disinfection. *Environmental Microbiology, 9*(5), 1267-1277.

Garcia-Fulgueiras, A., Navarro, C., Fenoll, D., Garcia, J., Gonzalez-Diego, P., Jimenez-Bunuales, T., Rodriguez, M., Lopez, R., Pacheco, F., Ruiz, J., Segovia, M., Balandron, B., Pelaz,C. (2003). Legionnaires' disease outbreak in Murcia, Spain. *Emerging Infectious Diseases, 9*(8), 915-921.

Garduno, R. A., Chong, A., Faulkner, G. (2008). Developmental Cycle-Differentiation of *Legionella pneumophila.* In K. Heuner and M. S. Swanson (Eds.), *Legionella*: Molecular Microbiology (pp.55-74). Caister Academic Press

Garduno, R. A., Garduno, E., Hiltz, M., and Hoffman, P. S. (2002a). Intracellular growth of *Legionella pneumophila* gives rise to a differentiated form dissimilar to stationary-phase forms. *Infection and Immunity, 70*(11), 6273-6283.

Garduno, R. A., Garduno, E., Hiltz, M., Allan, D., and Hoffman, P. S. (2002b). Morphological and physiological evidence for a developmental cycle in *Legionella pneumophila*. In R. Marre, Y. Abu Kwaik, C. Bartlett, N. P. Cianciotto, B.S. Fields, M. Frosch, J. Hacker, P.C. Luck (Eds.), *Legionella* (pp. 82-85). Washington, D.C.: ASM Press.

Garduno, R. A., Quinn, F. D., and Hoffman, P. S. (1998a). HeLa cells as a model to study the invasiveness and biology of *Legionella pneumophila*. *Canadian Journal of Microbiology, 44*(5), 430-440.

Garduno, R. A., Garduno, E., and Hoffman, P. S. (1998b). Surface-associated hsp60 chaperonin of *Legionella pneumophila* mediates invasion in a HeLa cell model. *Infection and Immunity, 66*(10), 4602-4610.

Garduno, R. A., Faulkner, G., Trevors, M. A., Vats, N., and Hoffman, P. S. (1998c). Immunolocalization of Hsp60 in *Legionella pneumophila*. *Journal of Bacteriology, 180*(3), 505-513.

Garin, J., Diez, R., Kieffer, S., Dermine, J. F., Duclos, S., Gagnon, E., Sadoul, R., Rondeau, C., Desjardins, M. (2001). The phagosome proteome: Insight into phagosome functions. *The Journal of Cell Biology, 152*(1), 165-180.

Gast, R. J., Moran, D. M., Dennett, M. R., Wurtsbaugh, W. A., and Amaral-Zettler, L. A. (2011). Amoebae and *Legionella pneumophila* in saline environments. *Journal of Water and Health, 9*(1), 37-52.

Gentry, D. R., Hernandez, V. J., Nguyen, L. H., Jensen, D. B., and Cashel, M. (1993). Synthesis of the stationary-phase sigma factor sigma S is positively regulated by ppGpp. *Journal of Bacteriology, 175*(24), 7982-7989.

Ghezzi, J. I., and Steck, T. R. (1999). Induction of the viable but non-culturable condition in *Xanthomonas campestris* pv. campestris in liquid microcosms and sterile soil. *FEMS Microbiology Ecology, 30*(3), 203-208.

Gilbert, S. E., and Rose, L. J. (2012). Survival and persistence of nonspore-forming biothreat agents in water. *Letters in Applied Microbiology, 55*(3), 189-194.

Glick, T. H., Gregg, M. B., Berman, B., Mallison, G., Rhodes, W. W.,Jr, and Kassanoff, I. (1978). Pontiac fever. An epidemic of unknown etiology in a health department: I. clinical and epidemiologic aspects. *American Journal of Epidemiology, 107*(2), 149-160.

Glockner, G., Albert-Weissenberger, C., Weinmann, E., Jacobi, S., Schunder, E., Steinert, M., Hacker, J., Heuner, K. (2008). Identification and characterization of a new conjugation/type IVA secretion system (*trb/tra*) of *Legionella pneumophila* corby localized on two mobile genomic islands. *International Journal of Medical Microbiology: IJMM, 298*(5-6), 411-428.

Gobin, I., Newton, P. R., Hartland, E. L., and Newton, H. J. (2009). Infections caused by nonpneumophila species of *Legionella. Rev.Med.Microbiol., 20*(1), 1-11.

Gonzalez-Escalona, N., Fey, A., Hofle, M. G., Espejo, R. T., and A Guzman, C. (2006). Quantitative reverse transcription polymerase chain reaction analysis of *Vibrio cholerae* cells entering the viable but non-culturable state and starvation in response to cold shock. *Environmental Microbiology, 8*(4), 658-666.

Goosen, N., and van de Putte, P. (1995). The regulation of transcription initiation by integration host factor. *Molecular Microbiology, 16*(1), 1-7.

Gottesman, S. (2004). The small RNA regulators of *Escherichia coli*: Roles and mechanisms. *Annual Review of Microbiology, 58*, 303-328.

Gourmelon, M., Cillard, J., and Pommepuy, M. (1994). Visible light damage to *Escherichia coli* in seawater: Oxidative stress hypothesis. *The Journal of Applied Bacteriology, 77*(1), 105-112.

Greenbaum, D., Colangelo, C., Williams, K., and Gerstein, M. (2003). Comparing protein abundance and mRNA expression levels on a genomic scale. *Genome Biology, 4*(9), 117.

Greub, G., and Raoult, D. (2003). Morphology of *Legionella pneumophila* according to their location within *Hartmanella vermiformis*. *Research in Microbiology, 154*(9), 619-621.

Greub, G., and Raoult, D. (2004). Microorganisms resistant to free-living amoebae. *Clinical Microbiology Reviews, 17*(2), 413-433.

Grey, B. and Steck, T. R. (2001a). Concentrations of copper thought to be toxic to *Escherichia coli* can induce the viable but nonculturable condition. *Applied and Environmental Microbiology, 67*(11), 5325-5327.

Grey, B. E., and Steck, T. R. (2001b). The viable but nonculturable state of *Ralstonia solanacearum* may be involved in long-term survival and plant infection. *Applied and Environmental Microbiology, 67*(9), 3866-3872.

Griebel, R., Smith, Z., and Merrick, J. M. (1968). Metabolism of poly-beta-hydroxybutyrate. I. purification, composition, and properties of native poly-beta-hydroxybutyrate granules from *Bacillus megaterium*. *Biochemistry, 7*(10), 3676-3681.

Griebel, R. J., and Merrick, J. M. (1971). Metabolism of poly-beta-hydroxybutyrate: Effect of mild alkaline extraction on native poly-beta-hydroxybutyrate granules. *Journal of Bacteriology, 108*(2), 782-789.

Groothuis, D. G., and Veenendaal, H. R. (1983). Heat treatment as an aid for the isolation of *Legionella pneumophila* from clinical and environmental samples. *Zentralbl Bakteriol Mikrobiol Hyg A. 255*(1), 39-43.

Gudkov, S. V., Karp, O. E., Garmash, S. A., Ivanov, V. E., Chernikov, A. V., Manokhin, A. A., Astashev, M. E., Iaguzhinskii, L. S., Bruskov,V.I. (2012). Generation of reactive oxygen species in water under exposure of visible or infrared irradiation at absorption band of molecular oxygen. *Biofizika, 57*(1), 5-13.

Gunasekera, T. S., Sorensen, A., Attfield, P. V., Sorensen, S. J., and Veal, D. A. (2002). Inducible gene expression by nonculturable bacteria in milk after pasteurization. *Applied and Environmental Microbiology, 68*(4), 1988-1993.

Gupte, A. R., De Rezende, C. L., and Joseph, S. W. (2003). Induction and resuscitation of viable but nonculturable *Salmonella enterica* serovar Typhimurium DT104. *Applied and Environmental Microbiology, 69*(11), 6669-6675.

Guyard, C., and Low, D. E. (2011). *Legionella* infections and travel associated Legionellosis. *Travel Medicine and Infectious Disease, 9*(4), 176-186.

Halifax Regional Water Comission (2006). *2005/06 water quality report.* (WWW.document) (www.halifax.ca/hrwc/documents/WaterReport200506.pdf)

Hall-Stoodley, L. and Stoodley, P. (2009). Evolving concepts in biofilm infections. *Cellular Microbiology, 11*(7), 1034-1043

Hambleton, P., Broster, M. G., Dennis, P. J., Henstridge, R., Fitzgeorge, R., and Conlan, J. W. (1983). Survival of virulent *Legionella pneumophila* in aerosols. *The Journal of Hygiene, 90*(3), 451-460.

Hammer, B. K., and Swanson, M. S. (1999). Co-ordination of *Legionella pneumophila* virulence with entry into stationary phase by ppGpp. *Molecular Microbiology, 33*(4), 721-731.

Hammer, B. K., Tateda, E. S., and Swanson, M. S. (2002). A two-component regulator induces the transmission phenotype of stationary-phase *Legionella pneumophila*. *Molecular Microbiology, 44*(1), 107-118.

Han, M. J., Yun, H., and Lee, S. Y. (2008). Microbial small heat shock proteins and their use in biotechnology. *Biotechnology Advances, 26*(6), 591-609.

Handrick, R., Reinhardt, S., and Jendrossek, D. (2000). Mobilization of poly(3-hydroxybutyrate) in *Ralstonia eutropha*. *Journal of Bacteriology, 182*(20), 5916-5918.

Harb, O. S., Venkataraman, C., Haack, B. J., Gao, L. Y., and Kwaik, Y. A. (1998). Heterogeneity in the attachment and uptake mechanisms of the Legionnaires' disease bacterium, *Legionella pneumophila*, by protozoan hosts. *Applied and Environmental Microbiology, 64*(1), 126-132.

Haugen, S. P., Ross, W., and Gourse, R. L. (2008). Advances in bacterial promoter recognition and its control by factors that do not bind DNA. *Nature Reviews. Microbiology, 6*(7), 507-519.

Hautemaniere, A., Remen, T., Mathieu, L., Deloge-Abarkan, M., Hartemann, P., Zmirou-Navier, D. (2011). Pontiac fever among retirement home nurses associated with airborne *Legionella*. *J Hosp Infect. 78*(4), 269-73.

Hawn, T. R., Verbon, A., Janer, M., Zhao, L. P., Beutler, B., and Aderem, A. (2005). Toll-like receptor 4 polymorphisms are associated with resistance to Legionnaires' disease. *Proceedings of the National Academy of Sciences of the United States of America, 102*(7), 2487-2489.

Hawn, T. R., Verbon, A., Lettinga, K. D., Zhao, L. P., Li, S. S., Laws, R. J., Skerrett, S. J., Beutler, B., Schroeder, L., Nachman, A., Ozinsky, A., Smith, K. D., Aderem, A. (2003). A common dominant TLR5 stop codon polymorphism abolishes flagellin signaling and is associated with susceptibility to Legionnaires' disease. *The Journal of Experimental Medicine, 198*(10), 1563-1572.

Hayashi, T., Nakamichi, M., Naitou, H., Ohashi, N., Imai, Y., and Miyake, M. (2010). Proteomic analysis of growth phase-dependent expression of *Legionella pneumophila* proteins which involves regulation of bacterial virulence traits. *PloS One, 5*(7), e11718.

Heeb, S., Kuehne, S. A., Bycroft, M., Crivii, S., Allen, M. D., Haas, D., Camara, M., Williams, P. (2006). Functional analysis of the post-transcriptional regulator RsmA reveals a novel RNA-binding site. *Journal of Molecular Biology, 355*(5), 1026-1036.

Heim, S., Lleo, M., Bonato, B., Guzman, C. A., and Canepari, P. (2002). The viable but nonculturable state and starvation are different stress responses of *Enterococcus faecalis*, as determined by proteome analysis. *Journal of Bacteriology, 184*(23), 6739-6745.

Heinzen, R. A., Hackstadt, T., and Samuel, J. E. (1999). Developmental biology of *Coxiella burnettii*. *Trends in Microbiology, 7*(4), 149-154.

Heller, R., Holler, C., Sussmuth, R., and Gundermann, K. O. (1998). Effect of salt concentration and temperature on survival of *Legionella pneumophila*. *Letters in Applied Microbiology, 26*(1), 64-68.

Heuner, K., Dietrich, C., Skriwan, C., Steinert, M., and Hacker, J. (2002). Influence of the alternative sigma(28) factor on virulence and flagellum expression of *Legionella pneumophila*. *Infection and Immunity, 70*(3), 1604-1608.

Hickman, J. W., and Harwood, C. S. (2008). Identification of FleQ from *Pseudomonas aeruginosa* as a c-di-GMP-responsive transcription factor. *Molecular Microbiology, 69*(2), 376-389.

Hicks, L. A., Rose, C. E.,Jr, Fields, B. S., Drees, M. L., Engel, J. P., Jenkins, P. R., Rouse, B. S., Blythe, D., Khalifah, A. P., Feikin, D. R., Whitney, C. G. (2007). Increased rainfall is associated with increased risk for legionellosis. *Epidemiology and Infection, 135*(5), 811-817.

Higgins, D. A., Pomianek, M. E., Kraml, C. M., Taylor, R. K., Semmelhack, M. F., and Bassler, B. L. (2007). The major *Vibrio cholerae* autoinducer and its role in virulence factor production. *Nature, 450*, 883-886.

Hilbi, H., Hoffmann, C., and Harrison, C. F. (2011). *Legionella* spp. outdoors: Colonization, communication and persistence. *Environmental Microbiology Reports, 3*, 286–296.

Hilbi, H., Segal, G., and Shuman, H. A. (2001). Icm/dot-dependent upregulation of phagocytosis by *Legionella pneumophila*. *Molecular Microbiology, 42*(3), 603-617.

Hiltz, M. F., Sisson, G. R., Brassinga, A. K., Garduno, E., Garduno, R. A., and Hoffman, P. S. (2004). Expression of *magA* in *Legionella pneumophila* philadelphia-1 is developmentally regulated and a marker of formation of mature intracellular forms. *Journal of Bacteriology, 186*(10), 3038-3045.

Hindre, T., Bruggemann, H., Buchrieser, C., and Hechard, Y. (2008). Transcriptional profiling of *Legionella pneumophila* biofilm cells and the influence of iron on biofilm formation. *Microbiology (Reading, England), 154*(Pt 1), 30-41.

Hisert, K. B., MacCoss, M., Shiloh, M. U., Darwin, K. H., Singh, S., Jones, R. A., Ehrt, S., Zhang, Z., Gaffney, B.L., Gandotra, S., Holden, D. W., Murray, D., Nathan, C. (2005). A glutamate-alanine-leucine (EAL) domain protein of *Salmonella* controls bacterial survival in mice, antioxidant defence and killing of macrophages: Role of cyclic diGMP. *Molecular Microbiology, 56*(5), 1234-1245.

Hofmann, K., Heinz, E. B., Charles, T. C., Hoppert, M., Liebl, W., and Streit, W. R. (2000). *Sinorhizobium meliloti* strain 1021 bioS and *bdhA* gene transcriptions are both affected by biotin available in defined medium. *FEMS Microbiology Letters, 182*(1), 41-44.

Horwitz, M. A. (1983). The Legionnaires' disease bacterium (*Legionella pneumophila*) inhibits phagosome-lysosome fusion in human monocytes. *The Journal of Experimental Medicine, 158*(6), 2108-2126.

Hovel-Miner, G., Pampou, S., Faucher, S. P., Clarke, M., Morozova, I., Morozov, P., Russo, J. J., Shuman, H. A., Kalachikov, S. (2009). SigmaS controls multiple pathways associated with intracellular multiplication of *Legionella pneumophila*. *Journal of Bacteriology, 191*(8), 2461-2473.

Hubber, A., and Roy, C. R. (2010). Modulation of host cell function by *Legionella pneumophila* type IV effectors. *Annual Review of Cell and Developmental Biology, 26*, 261-283.

Hussong, D., Colwell, R. R., O'Brien, M., Weiss, E., Pearson, A. D., Weiner, R. M., Burge, W. D. (1987). Viable *Legionella pneumophila* not detectable by culture on agar media. *Nat Biotech, 5*(9), 947-950.

Hwang, M. G., Katayama, H., and Ohgaki, S. (2006). Effect of intracellular resuscitation of *Legionella pneumophila* in *Acanthamoeba polyphaga* cells on the antimicrobial properties of silver and copper. *Environmental Science and Technology, 40*(23), 7434-7439.

James, B. W., Mauchline, W. S., Dennis, P. J., Keevil, C. W., and Wait, R. (1999). Poly-3-hydroxybutyrate in *Legionella pneumophila*, an energy source for survival in low-nutrient environments. *Applied and Environmental Microbiology, 65*(2), 822-827.

Jendrossek, D., Schirmer, A., and Schlegel, H. G. (1996). Biodegradation of polyhydroxyalkanoic acids. *Applied Microbiology and Biotechnology, 46*(5-6), 451-463.

Jiang, X., and Chai, T. J. (1996). Survival of *Vibrio parahaemolyticus* at low temperatures under starvation conditions and subsequent resuscitation of viable, nonculturable cells. *Applied and Environmental Microbiology, 62*(4), 1300-1305.

Jones, D. M., Sutcliffe, E. M., and Curry, A. (1991). Recovery of viable but non-culturable *Campylobacter jejuni*. *Journal of General Microbiology, 137*(10), 2477-2482.

Joseph, C. A., and Ricketts, K.D., on behalf of the European Working Group for *Legionella* Infections. (2010). Legionnaires' disease in europe 2007–2008. *Euro Surveill., 15(8)*,19493.

Jurasek, L., and Marchessault, R. H. (2002). The role of phasins in the morphogenesis of poly(3-hydroxybutyrate) granules. *Biomacromolecules, 3*(2), 256-261.

Justice, S. S., Hunstad, D. A., Cegelski, L., and Hultgren, S. J. (2008). Morphological plasticity as a bacterial survival strategy. *Nature Reviews.Microbiology, 6*(2), 162-168.

Kadouri, D., Burdman, S., Jurkevitch, E., and Okon, Y. (2002). Identification and isolation of genes involved in poly(beta-hydroxybutyrate) biosynthesis in *Azospirillum brasilense* and characterization of a *phbC* mutant. *Applied and Environmental Microbiology, 68*(6), 2943-2949.

Kadouri, D., Jurkevitch, E., and Okon, Y. (2003a). Involvement of the reserve material poly-beta-hydroxybutyrate in *Azospirillum brasilense* stress endurance and root colonization. *Applied and Environmental Microbiology, 69*(6), 3244-3250.

Kadouri, D., Jurkevitch, E., and Okon, Y. (2003b). Poly beta-hydroxybutyrate depolymerase (PhaZ) in *Azospirillum brasilense* and characterization of a *phaZ* mutant. *Archives of Microbiology, 180*(5), 309-318.

Kadouri, D., Jurkevitch, E., Okon, Y., and Castro-Sowinski, S. (2005). Ecological and agricultural significance of bacterial polyhydroxyalkanoates. *Critical Reviews in Microbiology, 31*(2), 55-67.

Kagan, J. C., and Roy, C. R. (2002). *Legionella* phagosomes intercept vesicular traffic from endoplasmic reticulum exit sites. *Nature Cell Biology, 4*(12), 945-954.

Kagan, J. C., Stein, M. P., Pypaert, M., and Roy, C. R. (2004). *Legionella* subvert the functions of Rab1 and Sec22b to create a replicative organelle. *The Journal of Experimental Medicine, 199*(9), 1201-1211.

Kallberg, Y., Oppermann, U., Jornvall, H., Persson, B. (2002). Short-chain dehydrogenases/ reductases (SDRs) Coenzyme-based functional assignments in completed genomes. *Eur.J.Biochem. 269*, 4409-4417.

Kaminsky, Y. (1985). Some regulatory properties of 3-hydroxybutyrate dehydrogenase in frozen-thawed rat liver mitochondria. *Comparative Biochemistry and Physiology.B, Comparative Biochemistry, 82*(2), 378-384.

Kana, B. D., Gordhan, B. G., Downing, K. J., Sung, N., Vostroktunova, G., Machowski, E. E., Tsenova, L., Young, M., Kaprelyants, A., Kaplan, G., Mizrahi,V. (2008). The resuscitation-promoting factors of *Mycobacterium tuberculosis* are required for virulence and resuscitation from dormancy but are collectively dispensable for growth *in vitro*. *Molecular Microbiology, 67*(3), 672-684.

Kave, T., and Cheema, A. (2011). Small Changes Huge Impact: The Role of Protein Posttranslational Modifications in Cellular Homeostasis and Disease. *Journal of Amino Acids, 2011*, 1-13.

Khanna, S., and Srivastava, A. K. (2005). Recent advances in microbial polyhydroxyalkanoates. *Process Biochemistry. 40*(2), 607-619

Kimura, S., Tateda, K., Ishii, Y., Horikawa, M., Miyairi, S., Gotoh, N., Ishiguro, M., Yamaguchi, K. (2009). *Pseudomonas aeruginosa* las quorum sensing autoinducer suppresses growth and biofilm production in *Legionella* species. *Microbiology, 155*(6), 1934-1939.

King, C. H., Fields, B. S., Shotts, E. B.,Jr, and White, E. H. (1991). Effects of cytochalasin D and methylamine on intracellular growth of *Legionella pneumophila* in amoebae and human monocyte-like cells. *Infection and Immunity, 59*(3), 758-763.

Kobayashi, T., Uchino, K., Abe, T., Yamazaki, Y., and Saito, T. (2005). Novel intracellular 3-hydroxybutyrate-oligomer hydrolase in *Wautersia eutropha* H16. *Journal of Bacteriology, 187*(15), 5129-5135.

Koch, A. L. (1996). What size should a bacterium be? A question of scale. *Annual Review of Microbiology, 50*, 317-348.

Kong, I. S., Bates, T. C., Hulsmann, A., Hassan, H., Smith, B. E., and Oliver, J. D. (2004). Role of catalase and *oxyR* in the viable but nonculturable state of *Vibrio vulnificus*. *FEMS Microbiology Ecology, 50*(3), 133-142.

Koubar, M., Rodier, M. H., Garduno, R. A., and Frere, J. (2011). Passage through *Tetrahymena tropicalis* enhances the resistance to stress and the infectivity of *Legionella pneumophila*. *FEMS Microbiology Letters, 325*(1), 10-15.

Kruger, K., Lang, G., weidner, T., and Engel, A. M. (1999). Cloning and functional expression of the *D*-β-hydroxybutyrate dehydrogenase gene of *Rhodobacter* sp. DSMZ 12077. *Applied Microbiology and Biotechnology, 52(5)*, 666-669.

Kuehl, B., Marten, S. M., Bischoff, Y., Brenner-Weiss, G., and Obst, U. (2011). MALDI-ToF mass spectrometry-multivariate data analysis as a tool for classification of reactivation and non-culturable states of bacteria. *Analytical and Bioanalytical Chemistry, 401*(5), 1593-1600.

Kuiper, M. W., Wullings, B. A., Akkermans, A. D., Beumer, R. R., and van der Kooij, D. (2004). Intracellular proliferation of *Legionella pneumophila* in *Hartmannella vermiformis* in aquatic biofilms grown on plasticized polyvinyl chloride. *Applied and Environmental Microbiology, 70*(11), 6826-6833.

Kunst, F., Ogasawara, N., Moszer, I., Albertini, A. M., Alloni, G., Azevedo, V., Bertero, M. G., Bessieres, P., Bolotin, A., Borchert, S., Borriss, R., Boursier, L., Brans, A., Braun, M., Brignell, S. C., Bron, S., Brouillet, S., Bruschi, C. V., Caldwell, B., Capuano,V., Carter, N. M., Choi, S. K., Codani, J. J., Connerton, I. F., Danchin, A. (1997). The complete genome sequence of the Gram-positive bacterium *Bacillus subtilis*. *Nature, 390*(6657), 249-256.

Kurokawa, M., Nukina, M., Nakanishi, H., Tomita, S., Tamura, T., and Shimoyama, T. (1999). Resuscitation from the viable but nonculturable state of *Helicobacter pylori*. *Kansenshogaku Zasshi.the Journal of the Japanese Association for Infectious Diseases, 73*(1), 15-19.

Kusnetsov, J. M., Ottoila, E., and Martikainen, P. J. (1996). Growth, respiration and survival of *Legionella pneumophila* at high temperatures. *The Journal of Applied Bacteriology, 81*(4), 341-347.

Lai, C. J., Chen, S. Y., Lin, I. H., Chang, C. H., and Wong, H. C. (2009). Change of protein profiles in the induction of the viable but nonculturable state of *Vibrio parahaemolyticus*. *International Journal of Food Microbiology, 135*(2), 118-124.

Lam, M. C., Ang, L. W., Tan, A. L., James, L., and Goh, K. T. (2011). Epidemiology and control of Legionellosis, singapore. *Emerging Infectious Diseases, 17*(7), 1209-1215.

Lange, R., and Hengge-Aronis, R. (1991). Identification of a central regulator of stationary-phase gene expression in *Escherichia coli*. *Molecular Microbiology, 5*(1), 49-59.

Lasheras, A., Boulestreau, H., Rogues, A. M., Ohayon-Courtes, C., Labadie, J. C., and Gachie, J. P. (2006). Influence of amoebae and physical and chemical characteristics of water on presence and proliferation of *Legionella* species in hospital water systems. *American Journal of Infection Control, 34*(8), 520-525.

Lau, H. Y., and Ashbolt, N. J. (2009). The role of biofilms and protozoa in *Legionella* pathogenesis: Implications for drinking water. *Journal of Applied Microbiology, 107*(2), 368-378.

Lee, S. Y. (1996). Bacterial polyhydroxyalkanoates. *Biotechnology and Bioengineering, 49*(1), 1-14.

Levi, A., Folcher, M., Jenal, U., and Shuman, H. A. (2011). Cyclic diguanylate signaling proteins control intracellular growth of *Legionella pneumophila*. *Mbio, 2*(1), e00316-10.

Li, Y., Meng, Q., Wang, W., and Sun, X. (2012). Differential expression of ATP-dependent RNA helicase gene in viable but nonculturable *Salmonella pullorum*. *African Journal of Biotechnology, 11*(11), 2625-2630.

Lindback, T., Rottenberg, M. E., Roche, S. M., and Rorvik, L. M. (2010). The ability to enter into an avirulent viable but non-culturable (VBNC) form is widespread among *Listeria monocytogenes* isolates from salmon, patients and environment. *Veterinary Research, 41*(1), 8.

Linder, K., and Oliver, J. D. (1989). Membrane fatty acid and virulence changes in the viable but nonculturable state of *Vibrio vulnificus*. *Applied and Environmental Microbiology, 55*(11), 2837-2842.

Linehan, S. A., Martinez-Pomares, L., and Gordon, S. (2000). Macrophage lectins in host defence. *Microbes and Infection /Institut Pasteur, 2*(3), 279-288.

Liu, Y., Wang, C., Tyrrell, G., Hrudey, S. E., and Li, X. F. (2009). Induction of *Escherichia coli* O157:H7 into the viable but non-culturable state by chloraminated water and river water, and subsequent resuscitation. *Environmental Microbiology Reports, 1*, 155-161.

Liu, M., Conover, G. M., and Isberg, R. R. (2008). *Legionella pneumophila* EnhC is required for efficient replication in tumour necrosis factor alpha-stimulated macrophages. *Cellular Microbiology, 10*(9), 1906-1923. doi:10.1111/j.1462-5822.2008.01180.x

Liu, Z., Lin, Y. E., Stout, J. E., Hwang, C. C., Vidic, R. D., and Yu, V. L. (2006). Effect of flow regimes on the presence of *Legionella* within the biofilm of a model plumbing system. *Journal of Applied Microbiology, 101*(2), 437-442.

Lleo, M. M., Benedetti, D., Tafi, M. C., Signoretto, C., and Canepari, P. (2007). Inhibition of the resuscitation from the viable but non-culturable state in *Enterococcus faecalis*. *Environmental Microbiology, 9*(9), 2313-2320.

Lleo, M. M., Bonato, B., Tafi, M. C., Signoretto, C., Boaretti, M., and Canepari, P. (2001). Resuscitation rate in different *Enterococcal* species in the viable but non-culturable state. *Journal of Applied Microbiology, 91*(6), 1095-1102.

Lleo, M. M., Pierobon, S., Tafi, M. C., Signoretto, C., and Canepari, P. (2000). mRNA detection by reverse transcription-PCR for monitoring viability over time in an *Enterococcus faecalis* viable but nonculturable population maintained in a laboratory microcosm. *Applied and Environmental Microbiology, 66*(10), 4564-4567.

Lock, R., Ohman, L., and Dahlgren, C. (1987). Phagocytic recognition mechanisms in human granulocytes and *Acanthamoeba castellanii* using type 1 fimbriated *Escherichia coli* as phagocytic prey. *FEMS Microbiol Lett, 44*, 135-140.

Lodwig, E. M., Leonard, M., Marroqui, S., Wheeler, T. R., Findlay, K., Downie, J. A., Poole, P. S. (2005). Role of polyhydroxybutyrate and glycogen as carbon storage compounds in pea and bean bacteroids. *Molecular Plant-Microbe Interactions: MPMI, 18*(1), 67-74.

Loo, C. Y., and Sudesh, K. (2007). Biosynthesis and native granule characteristics of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) in *Delftia acidovorans*. *International Journal of Biological Macromolecules, 40*(5), 466-471.

López, N. L., Floccari, M. E., Steinbüchel, A., García, A. F., and Méndez, B. S. (1995). Effect of poly(3-hydroxybutyrate) (PHB) content on the starvation-survival of bacteria in natural waters. *FEMS Microbiology Ecology, 16(2)*, 95–101.

Lothigius, A., Sjoling, A., Svennerholm, A. M., and Bolin, I. (2010). Survival and gene expression of enterotoxigenic *Escherichia coli* during long-term incubation in sea water and freshwater. *Journal of Applied Microbiology, 108*(4), 1441-1449.

Lundgren, D. G., Pfister, R. M., and Merrick, J. M. (1964). Structure of poly-beta-hydroxybutyric acid granules. *Journal of General Microbiology, 34*, 441-446.

Lurie-Weinberger, M. N., Gomez-Valero, L., Merault, N., Glockner, G., Buchrieser, C., and Gophna, U. (2010). The origins of eukaryotic-like proteins in *Legionella pneumophila*. *International Journal of Medical Microbiology: IJMM, 300*(7), 470-481.

Lynch, D., Fieser, N., Gloggler, K., Forsbach-Birk, V., and Marre, R. (2003). The response regulator LetA regulates the stationary-phase stress response in *Legionella pneumophila* and is required for efficient infection of *Acanthamoeba castellanii*. *FEMS Microbiology Letters, 219*(2), 241-248.

Lyte, M., Frank, C. D., and Green, B. T. (1996). Production of an autoinducer of growth by norepinephrine cultured *Escherichia coli* O157:H7. *FEMS Microbiology Letters, 139*(2-3), 155-159.

Maalej, S., Gdoura, R., Dukan, S., Hammami, A., and Bouain, A. (2004). Maintenance of pathogenicity during entry into and resuscitation from viable but nonculturable state in *Aeromonas hydrophila* exposed to natural seawater at low temperature. *Journal of Applied Microbiology, 97*(3), 557-565.

Machner, M. P., and Isberg, R. R. (2006). Targeting of host rab GTPase function by the intravacuolar pathogen *Legionella pneumophila*. *Developmental Cell, 11*(1), 47-56.

Magariños, B., Romalde, J. L., Cid, A., and Toranzo, A. E. (1997). Viability of starved *Pasteurella piscicida* in seawater monitored by flow cytometry and the effect of antibiotics on its resuscitation. *Letters in Applied Microbiology, 24*, 122–126.

Magnusson, L. U., Farewell, A., and Nystrom, T. (2005). ppGpp: A global regulator in *Escherichia coli*. *Trends in Microbiology, 13*(5), 236-242.

Maier, T., Schmidt, A., Guell, M., Kuhner, S., Gavin, A. C., Aebersold, R., Serrano, L. (2011). Quantification of mRNA and protein and integration with protein turnover in a bacterium. *Molecular Systems Biology, 7*, 511.

Mallegol, J., Duncan, C., Prashar, A., So, J., Low, D. E., Terebeznik, M., Guyard, C. (2012). Essential roles and regulation of the *Legionella pneumophila* collagen-like adhesin during biofilm formation. *Plos One, 7(9)*, e46462.

Mallette, M. F. (1969). Evaluation of growth by physical and chemical means. In J. R. Norris, and D. W. Ribbons (Eds.), *Methods in microbiology* (pp. 521-566). New York, NY. Academic Press, Inc.

Marden, P., Tunlid, A., Malmcrona-Friberg, K., Odham, G., and Kjelleberg, S. (1985). Physiological and morphological changes during short term starvation of marine bacterial isolates . *Arch. Microbiol., 142*, 326-332.

Marra, A., Blander, S. J., Horwitz, M. A., and Shuman, H. A. (1992). Identification of a *Legionella pneumophila* locus required for intracellular multiplication in human macrophages. *Proc. Natl. Acad. Sci. USA, 89*, 9607-9611.

Marston, B. J., Lipman, H. B., and Breiman, R. F. (1994). Surveillance for Legionnaires' disease. Risk factors for morbidity and mortality. *Archives of Internal Medicine, 154*(21), 2417-2422.

Mashburn, L. M., and Whiteley, M. (2005). Membrane vesicles traffic signals and facilitate group activities in a prokaryote. *Nature, 437*(7057), 422-425.

Masmoudi, S., Denis, M., and Maalej, S. (2010). Inactivation of the gene *katA* or *sodA* affects the transient entry into the viable but non-culturable response of *Staphylococcus aureus* in natural seawater at low temperature. *Marine Pollution Bulletin, 60*(12), 2209-2214.

Matin, A., Veldhuis, C., Stegeman, V., and Veenhuis, M. (1979). Selective advantage of a *Spirillum* sp. in a carbon-limited environment. Accumulation of poly-beta-hydroxybutyric acid and its role in starvation. *Journal of General Microbiology, 112*(2), 349-355.

Mauchline, W. S., Araujo, R., Wait, R., Dowsett, A. B., Dennis, P. J., and Keevil, C. W. (1992). Physiology and morphology of *Legionella pneumophila* in continuous culture at low oxygen concentration. *J. Gen. Microbiol, 138*, 2371-2380.

May, C., Preisig-Muller, R., Hohne, M., Gnau, P., and Kindl, H. (1998). A phospholipase A2 is transiently synthesized during seed germination and localized to lipid bodies. *Biochimica Et Biophysica Acta, 1393*(2-3), 267-276.

Mayer, F., Madkour, M. H., Piper-Fürst, U., Wieczorek, R., Liebergesell, M., and Steinbüchel, A. (1996). Electron microscopic observations on the macromolecular organization of the boundary layer of bacterial PHA inclusion bodies. *J. Gen. Appl. Microbiol., 42(6)*, 445-455.

McDade, J. E. (1979). Primary isolation using guinea pigs and embryonated eggs. In G. L. Jones, and G. A. Hébert (Eds.), *" Ligionnaires' " the disease, the bacterium and methodology* (pp. 70-76). Atlanta, Ga.: Center for Disease Control, Bureau of Laboratories, U.S. Public Health Service, U.S. Department of Health, Education, and Welfare.

McIntyre, J. O., Bock, H. G., and Fleischer, S. (1978). The orientation of *D*-beta-hydroxybutyrate dehydrogenase in the mitochondrial inner membrane. *Biochimica Et Biophysica Acta, 513*(2), 255-267.

McIntyre, J. O., Churchill, P., Maurer, A., Berenski, C. J., Jung, C. Y., and Fleischer, S. (1983). Target size of *D*-beta-hydroxybutyrate dehydrogenase. Functional and structural molecular weight based on radiation inactivation. *The Journal of Biological Chemistry, 258*(2), 953-959.

McNealy, T. L., Forsbach-Birk, V., Shi, C., and Marre, R. (2005). The *hfq* homolog in *Legionella pneumophila* demonstrates regulation by LetA and RpoS and interacts with the global regulator CsrA. *Journal of Bacteriology, 187*(4), 1527-1532.

Mellman, I., and Warren, G. (2000). The road taken: Past and future foundations of membrane traffic. *Cell, 100*(1), 99-112.

Miller, J. F., Roy, C. R., and Falkow, S. (1989). Analysis of *Bordetella pertussis* virulence gene regulation by use of transcriptional fusions in *Escherichia coli*. *Journal of Bacteriology, 171*(11), 6345-6348.

Milohanic, E., Glaser, P., Coppee, J. Y., Frangeul, L., Vega, Y., Vazquez-Boland, J. A., Kunst, F., Cossart, P., Buchrieser, C. (2003). Transcriptome analysis of *Listeria monocytogenes* identifies three groups of genes differently regulated by PrfA. *Molecular Microbiology, 47*(6), 1613-1625.

Mishra, A., Taneja, N., and Sharma, M. (2012). Viability kinetics, induction, resuscitation and quantitative real-time polymerase chain reaction analyses of viable but nonculturable vibrio cholerae O1 in freshwater microcosm. *Journal of Applied Microbiology, 112*(5), 945-953.

Mizunoe, Y., Wai, S. N., Ishikawa, T., Takade, A., and Yoshida, S. (2000). Resuscitation of viable but nonculturable cells of *Vibrio parahaemolyticus* induced at low temperature under starvation. *FEMS Microbiology Letters, 186*(1), 115-120.

Molofsky, A. B., Shetron-Rama, L. M., and Swanson, M. S. (2005). Components of the *Legionella pneumophila* flagellar regulon contribute to multiple virulence traits, including lysosome avoidance and macrophage death. *Infection and Immunity, 73*(9), 5720-5734.

Molofsky, A. B., and Swanson, M. S. (2003). *Legionella pneumophila* CsrA is a pivotal repressor of transmission traits and activator of replication. *Molecular Microbiology, 50*(2), 445-461.

Molofsky, A. B., and Swanson, M. S. (2004). Differentiate to thrive: Lessons from the *Legionella pneumophila* life cycle. *Molecular Microbiology, 53*(1), 29-40.

Morash, M. G. (2006). Roles of sodium and integration host factor in the virulence and developmental cycle of *Legionella pneumophila*. *Dalhousie University (Canada).*

Morash, M. G., Brassinga, A. K., Warthan, M., Gourabathini, P., Garduno, R. A., Goodman, S. D., Hoffman, P. S. (2009). Reciprocal expression of integration host factor and HU in the developmental cycle and infectivity of *Legionella pneumophila*. *Applied and Environmental Microbiology, 75*(7), 1826-1837.

Muela, A., Seco, C., Camafeita, E., Arana, I., Orruno, M., Lopez, J. A., Barcina, I. (2008). Changes in *Escherichia coli* outer membrane subproteome under environmental conditions inducing the viable but nonculturable state. *FEMS Microbiology Ecology, 64*(1), 28-36.

Mukamolova, G. V., Kaprelyants, A. S., Kell, D. B., and Young, M. (2003). Adoption of the transiently non-culturable state--a bacterial survival strategy? *Advances in Microbial Physiology, 47*, 65-129.

Mukamolova, G. V., Kormer, S. S., Kell, D. B., and Kaprelyants, A. S. (1999). Stimulation of the multiplication of *Micrococcus luteus* by an autocrine growth factor. *Archives of Microbiology, 172*(1), 9-14.

Mukamolova, G. V., Turapov, O. A., Kazarian, K., Telkov, M., Kaprelyants, A. S., Kell, D. B., Young, M. (2002a). The *rpf* gene of *Micrococcus luteus* encodes an essential secreted growth factor. *Molecular Microbiology, 46*(3), 611-621.

Mukamolova, G. V., Turapov, O. A., Young, D. I., Kaprelyants, A. S., Kell, D. B., and Young, M. (2002b). A family of autocrine growth factors in *Mycobacterium tuberculosis*. *Molecular Microbiology, 46*(3), 623-635.

Mukamolova, G. V., Yanopolskaya, N. D., Kell, D. B., and Kaprelyants, A. S. (1998). On resuscitation from the dormant state of *Micrococcus luteus*. *Antonie Van Leeuwenhoek, 73*(3), 237-243.

Murga, R., Forster, T. S., Brown, E., Pruckler, J. M., Fields, B. S., and Donlan, R. M. (2001). Role of biofilms in the survival of *Legionella pneumophila* in a model potable-water system. *Microbiology, 147*(11), 3121-3126.

Nagai, H., Kagan, J. C., Zhu, X., Kahn, R. A., and Roy, C. R. (2002). A bacterial guanine nucleotide exchange factor activates ARF on *Legionella phagosomes*. *Science, 295*(5555), 679-682.

Nakada, T., Fukui, T., Saito, T., Miki, K., Oji, C., Matsuda, S., Ushijima, A., Tomita, K. (1981). Purification and properties of *D*-beta-hydroxybutyrate dehydrogenase from *Zoogloea ramigera* I-16-M. *Journal of Biochemistry, 89*(2), 625-635.

Nakata, H. M. (1966). Role of acetate in sporogenesis of *Bacillus cereus*. *Journal of Bacteriology, 91*(2), 784-788.

Neff, R. J. (1957). Purification, axenic cultivation, and description of a soil amoeba, *Acanthamoeba* sp. *Journal of Eukaryotic Microbiology, 4*(3), 176-182.

Nesvizhskii, A. I. (2007). Protein identification by tandem mass spectrometry and sequence database searching. *Methods in Molecular Biology, 367*, 87-119.

Newton, H. J., Ang, D. K., van Driel, I. R., and Hartland, E. L. (2010). Molecular pathogenesis of infections caused by *Legionella pneumophila*. *Clinical Microbiology Reviews, 23*(2), 274-298.

Newton, H. J., Sansom, F. M., Dao, J., Cazalet, C., Bruggemann, H., Albert-Weissenberger, C., Buchrieser, C., Cianciotto, N. P., Hartland, E. L. (2008). Significant role for *ladC* in initiation of *Legionella pneumophila* infection. *Infection and Immunity, 76*(7), 3075-3085.

Newton, H. J., Sansom, F. M., Dao, J., McAlister, A. D., Sloan, J., Cianciotto, N. P., Hartland, E. L. (2007). Sel1 repeat protein LpnE is a *Legionella pneumophila* virulence determinant that influences vacuolar trafficking. *Infection and Immunity, 75*(12), 5575-5585.

Nichols, C. E., Johnson, C., Lockyer, M., Charles, I. G., Lamb, H. K., Hawkins, A. R., Stammers, D. K. (2006). Structural characterization of *Salmonella typhimurium* YeaZ, an M22 O-sialoglycoprotein endopeptidase homolog. *Proteins, 64*(1), 111-123.

Nikitushkin, V. D., Demina, G. R., Shleeva, M. O., and Kaprelyants, A. S. (2013). Peptidoglycan fragments stimulate resuscitation of "non-culturable" *Mycobacteria*. *Antonie Van Leeuwenhoek, 103*(1), 37-46.

Noll, F., May, C., and Kindl, H. (2000). Phospholipid monolayer of plant lipid bodies attacked by phospholipase A2 shows 80 nm holes analyzed by atomic force microscopy. *Biophysical Chemistry, 86*(1), 29-35.

Nygard, K., Werner-Johansen, O., Ronsen, S., Caugant, D. A., Simonsen, O., Kanestrom, A., Ask, E., Ringstad, J., Odegard, R., Jensen, T., Krogh, T., Hoiby, E. A., Ragnhildstveit, E., Aaberge, I. S., Aavitsland, P. (2008). An outbreak of Legionnaires disease caused by long-distance spread from an industrial air scrubber in sarpsborg, norway. *Clinical Infectious Diseases : An Official Publication of the Infectious Diseases Society of America, 46*(1), 61-69.

Nystrom, T. (2003). Nonculturable bacteria: Programmed survival forms or cells at death's door? *BioEssays : News and Reviews in Molecular, Cellular and Developmental Biology, 25*(3), 204-211.

Oeding, V. and Schlegel, H. G. (1973). Beta-ketothiolase from *Hydrogenomonas eutropha* H16 and its significance in the regulation of poly-beta-hydroxybutyrate metabolism. *The Biochemical Journal, 134*(1), 239-248.

Ohno, A., Kato, N., Yamada, K., and Yamaguchi, K. (2003). Factors influencing survival of *Legionella pneumophila* serotype 1 in hot spring water and tap water. *Applied and Environmental Microbiology, 69*(5), 2540-2547.

Ohtomo, R., and Saito, M. (2001). Increase in the culturable cell number of *Escherichia coli* during recovery from saline stress: Possible implication for resuscitation from the VBNC state. *Microbial Ecology, 42*(2), 208-214.

Oliver, J. D. (2000). The viable but nonculturable state and cellular resuscitation. In C. R. Bell, M. Brylinsky and P. Johnson-Green (Eds.), *Microbial biosystems: New frontiers* (pp. 723-730). Halifax, Canada: Atlantic Canada Soc. Microb. Ecol.

Oliver, J. D. (2005). The viable but nonculturable state in bacteria. *Journal of Microbiology, 43*, 93-100.

Oliver, J. D. (2010). Recent findings on the viable but nonculturable state in pathogenic bacteria. *FEMS Microbiology Reviews, 34*(4), 415-425.

Oliver, J. D., and Bockian, R. (1995*). In vivo* resuscitation, and virulence towards mice, of viable but nonculturable cells of *Vibrio vulnificus*. *Applied and Environmental Microbiology, 61*(7), 2620-2623.

Oliver, J. D., Dagher, M., and Linden, K. (2005). Induction of *Escherichia coli* and *Salmonella typhimurium* into the viable but nonculturable state following chlorination of wastewater. *Journal of Water and Health, 3*(3), 249-257.

Oliver, J. D., Nilsson, L., and Kjelleberg, S. (1991). Formation of nonculturable *Vibrio vulnificus* cells and its relationship to the starvation state. *Applied and Environmental Microbiology, 57*(9), 2640-2644.

O'Loughlin, R. E., Kightlinger, L., Werpy, M. C., Brown, E., Stevens, V., Hepper, C., Keane, T., Benson, R. F., Fields, B. S., Moore, M. R. (2007). Restaurant outbreak of Legionnaires' disease associated with a decorative fountain: An environmental and case-control study. *BMC Infectious Diseases, 7*, 93.

Oppermann, U., Filling, C., Hult, M., Shafqat, N., Wu, X., Lindh, M., Shafqat, J., Nordling, E., Kallberg, Y., Persson, B., Jörnvall, H. (2003). Short-chain dehydrogenases/reductases (SDR): The 2002 update. *Chem Biol Interact, 143-144*, 247-253.

Ordax, M., Marco-Noales, E., Lopez, M. M., and Biosca, E. G. (2006). Survival strategy of *Erwinia amylovora* against copper: Induction of the viable-but-nonculturable state. *Applied and Environmental Microbiology, 72*(5), 3482-3488.

Osterhout, W. J. V. (1906). On the importance of physiologically balanced solutions for plants. I. marine plants. *Botanical Gazette, 42*(2), 127-134.

Pai, S. R., Actor, J. K., Sepulveda, E., Hunter, R. L.,Jr, and Jagannath, C. (2000). Identification of viable and non-viable *Mycobacterium tuberculosis* in mouse organs by directed RT-PCR for antigen 85B mRNA. *Microbial Pathogenesis, 28*(6), 335-342.

Panutdaporn, N., Kawamoto, K., Asakura, H., and Makino, S. I. (2006). Resuscitation of the viable but non-culturable state of *Salmonella enterica* serovar Oranienburg by recombinant resuscitation-promoting factor derived from *Salmonella* typhimurium strain LT2. *International Journal of Food Microbiology, 106*(3), 241-247.

Papageorgiou, A. C., Hermawan, S., Singh, C. B., and Jendrossek, D. (2008). Structural basis of poly(3-hydroxybutyrate) hydrolysis by PhaZ7 depolymerase from *Paucimonas lemoignei*. *Journal of Molecular Biology, 382*(5), 1184-1194.

Pardanani, A., Wieben, E. D., Spelsberg, T. C., and Tefferi, A. (2002). Primer on medical genomics. part IV: Expression proteomics. *Mayo Clinic Proceedings.Mayo Clinic, 77*(11), 1185-1196.

Pasculle, A. W., Feeley, J. C., Gibson, R. J., Cordes, L. G., Myerowitz, R. L., Patton, C. M., Gorman, G. W., Carmack, C. L., Ezzell, J. W., Dowling, J. N. (1980). Pittsburgh pneumonia agent: Direct isolation from human lung tissue. *The Journal of Infectious Diseases, 141*(6), 727-732.

Paszko-Kolva, C., Shahamat, M., and Colwell, R. R. (1992). Long-term survival of *Legionella pneumophila* serogroup 1 under low-nutrient conditions and associated morphological changes. *FEMS Microbiology Letters, 102*(1), 45-55.

Paul, B. J., Barker, M. M., Ross, W., Schneider, D. A., Webb, C., Foster, J. W., Gourse, R. L. (2004). DksA: A critical component of the transcription initiation machinery that potentiates the regulation of rRNA promoters by ppGpp and the initiating NTP. *Cell, 118*(3), 311-322.

Pawlowski, D. R., Metzger, D. J., Raslawsky, A., Howlett, A., Siebert, G., Karalus, R. J., Garrett, S., Whitehouse, C. A. (2011). Entry of *Yersinia pestis* into the viable but nonculturable state in a low-temperature tap water microcosm. *Plos One, 6(3)*, e17585.

Peck, S. C. (2005). Update on proteomics in Aarabidopsis. where do we go from here? *Plant Physiology, 138*(2), 591-599.

Pedersen, K., Christensen, S. K., and Gerdes, K. (2002). Rapid induction and reversal of a bacteriostatic condition by controlled expression of toxins and antitoxins. *Molecular Microbiology, 45*(2), 501-510.

Peralta-Gil, M., Segura, D., Guzman, J., Servin-Gonzalez, L., and Espin, G. (2002). Expression of the *Azotobacter vinelandii* poly-beta-hydroxybutyrate biosynthetic *phbBAC* operon is driven by two overlapping promoters and is dependent on the transcriptional activator PhbR. *Journal of Bacteriology, 184*(20), 5672-5677.

Piao, Z., Sze, C. C., Barysheva, O., Iida, K., and Yoshida, S. (2006). Temperature-regulated formation of mycelial mat-like biofilms by *Legionella pneumophila*. *Applied and Environmental Microbiology, 72*(2), 1613-1622.

Pommepuy, M., Butin, M., Derrien, A., Gourmelon, M., Colwell, R. R., and Cormier, M. (1996). Retention of enteropathogenicity by viable but nonculturable *Escherichia coli* exposed to seawater and sunlight. *Applied and Environmental Microbiology, 62*(12), 4621-4626.

Pope, D. H., Soracco, R. J., Gill, H. K., and Fliermans, C. B. (1982). Growth of *Legionella pneumophila* in two-membered cultures with green algae and Cyanobacteria. *Curr Microbiol, 7*, 319-321.

Pope, C. D., Dhand, L., and Cianciotto, N. P. (1994). Random mutagenesis of *Legionella pneumophila* with mini-Tn10. *FEMS Microbiology Letters, 124*(1), 107-111.

Potrykus, K., and Cashel, M. (2008). (p)ppGpp: Still magical? *Annual Review of Microbiology, 62*, 35-51.

Pötter, M. and Steinbüchel, A. (2005). Poly(3-hydroxybutyrate) granule-associated proteins: Impacts on poly(3-hydroxybutyrate) synthesis and degradation. *Biomacromolecules, 6*(2), 552-560.

Povolo, S., and Casella, S. (2004). Poly-3-hydroxybutyrate has an important role for the survival of *Rhizobium tropici* under starvation. *Annals of Microbiology, 54(3)*, 307-316.

Prashar, A., Bhatia, S., Tabatabaeiyazdi, Z., Duncan, C., Garduno, R. A., Tang, P., Low, D. E., Guyard, C., Terebiznik, M. R. (2012). Mechanism of invasion of lung epithelial cells by filamentous *Legionella pneumophila*. *Cellular Microbiology, 14*(10), 1632-1655.

Price, C. T., Al-Khodor, S., Al-Quadan, T., and Abu Kwaik, Y. (2010). Indispensable role for the eukaryotic-like ankyrin domains of the ankyrin B effector of *Legionella pneumophila* within macrophages and amoebae. *Infection and Immunity, 78*(5), 2079-2088.

Price, C. T., Al-Khodor, S., Al-Quadan, T., Santic, M., Habyarimana, F., Kalia, A., Kwaik, Y. A. (2009). Molecular mimicry by an F-box effector of *Legionella pneumophila* hijacks a conserved polyubiquitination machinery within macrophages and protozoa. *PLoS Pathogens, 5*(12), e1000704.

Price, C. T., Al-Quadan, T., Santic, M., Rosenshine, I., and Abu Kwaik, Y. (2011). Host proteasomal degradation generates amino acids essential for intracellular bacterial growth. *Science, 334*(6062), 1553-1557.

Prieto, M. A., Buhler, B., Jung, K., Witholt, B., and Kessler, B. (1999). PhaF, a polyhydroxyalkanoate-granule-associated protein of *Pseudomonas oleovorans* GPo1 involved in the regulatory expression system for *pha* genes. *Journal of Bacteriology, 181*(3), 858-868.

Provasoli, L. (1958). A catalogue of laboratory strains of free-living and parasitic protozoa. *Journal of Eukaryotic Microbiology, 5*(1), 1-38.

Pruzzo, C., Tarsi, R., Lleo, M. M., Signoretto, C., Zampini, M., Colwell, R. R., Canepari, P. (2002). *In vitro* adhesion to human cells by viable but nonculturable *Enterococcus faecalis*. *Current Microbiology, 45*(2), 105-110.

Pyla, R., Kim, T. J., Silva, J. L., and Jung, Y. S. (2010). Proteome analysis of *Azotobacter vinelandii arrF* mutant that overproduces poly-beta-hydroxybutyrate polymer. *Applied Microbiology and Biotechnology, 88*(6), 1343-1354.

R & D systems (2002). Methods for Detecting Protein Phosphorylation. *R & D systems*. (WWW.document)(www.rndsystems.com/mini_review_detail_objectname_MR08_ProteinPhosphorylation.aspx)

Rahman, I., Shahamat, M., Chowdhury, M. A., and Colwell, R. R. (1996). Potential virulence of viable but nonculturable *Shigella dysenteriae* type 1. *Applied and Environmental Microbiology, 62*(1), 115-120.

Rahman, I., Shahamat, M., Kirchman, P. A., Russek-Cohen, E., and Colwell, R. R. (1994). Methionine uptake and cytopathogenicity of viable but nonculturable *Shigella dysenteriae* type 1. *Applied and Environmental Microbiology, 60*(10), 3573-3578.

Rahman, M., Abd, H., Romling, U., Sandstrom, G., and Mollby, R. (2008). *Aeromonas-Acanthamoeba* interaction and early shift to a viable but nonculturable state of *Aeromonas* by *Acanthamoeba*. *Journal of Applied Microbiology, 104*(5), 1449-1457.

Ramaiah, N., Ravel, J., Straube, W. L., Hill, R. T., and Colwell, R. R. (2002). Entry of *Vibrio harveyi* and *Vibrio fischeri* into the viable but nonculturable state. *Journal of Applied Microbiology, 93*(1), 108-116.

Ramos-Gonzalez, M. I., and Molin, S. (1998). Cloning, sequencing, and phenotypic characterization of the *rpoS* gene from *Pseudomonas putida* KT2440. *Journal of Bacteriology, 180*(13), 3421-3431.

Rasis, M., and Segal, G. (2009). The LetA-RsmYZ-CsrA regulatory cascade, together with RpoS and PmrA, post-transcriptionally regulates stationary phase activation of *Legionella pneumophila* Icm/Dot effectors. *Molecular Microbiology, 72*(4), 995-1010.

Rauhut, R., and Klug, G. (1999). mRNA degradation in bacteria. *FEMS Microbiology, 23,* 353-370.

Ravdin, J. I., Petri, W. A., Murphy, C. F., and Smith, R. D. (1986). Production of mouse monoclonal antibodies which inhibit *in vitro* adherence of *Entamoeba histolytica* trophozoites. *Infection and Immunity, 53*(1), 1-5.

Ravel, J., Hill, R. T., and Colwell, R. R. (1994). Isolation of a *Vibrio cholerae* transposon-mutant with an altered viable but nonculturable response. *FEMS Microbiology Letters, 120*(1-2), 57-61.

Reddy, C. S. K., Ghai, R., and Kalia, V. C. (2003). Polyhydroxyalkanoates: An overview. *Bioresour Technol. 87*(2),137-46.

Reeves, M. W., Pine, L., Hutner, S. H., George, J. R., and Harrell, W. K. (1981). Metal requirements of *Legionella pneumophila*. *Journal of Clinical Microbiology, 13*(4), 688-695.

Reissbrodt, R., Rienaecker, I., Romanova, J. M., Freestone, P. P., Haigh, R. D., Lyte, M., Tschape, H., Williams, P. H. (2002). Resuscitation of *Salmonella enterica* serovar Typhimurium and enterohemorrhagic *Escherichia coli* from the viable but nonculturable state by heat-stable enterobacterial autoinducer. *Applied and Environmental Microbiology, 68*(10), 4788-4794.

Remen, T., Mathieu, L., Hautemaniere, A., Deloge-Abarkan, M., Hartemann, P., and Zmirou-Navier, D. (2011). Pontiac fever among retirement home nurses associated with airborne *Legionella* . *The Journal of Hospital Infection, 78*(4), 269-273.

Retzlaff, C., Yamamoto, Y., Okubo, S., Hoffman, P. S., Friedman, H., and Klein, T. W. (1996). *Legionella pneumophila* heat-shock protein-induced increase of interleukin-1 beta mRNA involves protein kinase C signalling in macrophages. *Immunology, 89*(2), 281-288.

Rivers, B., and Steck, T. R. (2001). Viable but nonculturable uropathogenic bacteria are present in the mouse urinary tract following urinary tract infection and antibiotic therapy. *Urological Research, 29*(1), 60-66.

Rogers, J., and Keevil, C. W. (1992). Immunogold and fluorescein immunolabelling of *Legionella pneumophila* within an aquatic biofilm visualized by using episcopic differential interference contrast microscopy. *Applied and Environmental Microbiology, 58*(7), 2326-2330.

Romling, U., and Amikam, D. (2006). Cyclic di-GMP as a second messenger. *Current Opinion in Microbiology, 9*(2), 218-228.

Rossmann, M. G., Moras, D., and Olsen, K. W. (1974). Chemical and biological evolution of nucleotide-binding protein. *Nature, 250*(463), 194-199.

Roth, W. G., Leckie, M. P., and Dietzler, D. N. (1988). Restoration of colony-forming activity in osmotically stressed *Escherichia coli* by betaine. *Applied and Environmental Microbiology, 54*(12), 3142-3146.

Rowbotham, T. J. (1983). Isolation of *Legionella pneumophila* from clinical specimens via amoebae, and the interaction of those and other isolates with amoebae. *Journal of Clinical Pathology, 36*(9), 978-986.

Rowbotham, T. J. (1986). Current views on the relationships between amoebae, *Legionellae* and man. *Israel Journal of Medical Sciences, 22*(9), 678-689.

Rowe, M. T., Dunstall, G., Kirk, R., Loughney, C. F., Cooke, J. L., and Brown, S. R. (1998). Development of an image system for the study of viable but non-culturable forms of *Campylobacter jejuni* and its use to determine their resistance to disinfectants. *Food Microbiology, 15(5)*, 491-498.

Rozen, Y., Larossa, R. A., Templeton, L. J., Smulski, D. R., and Belkin, S. (2002). Gene expression analysis of the response by *Escherichia coli* to seawater. *Antonie Van Leeuwenhoek, 81*(1-4), 15-25.

Ruggiero, A., Tizzano, B., Pedone, E., Pedone, C., Wilmanns, M., and Berisio, R. (2009). Crystal structure of the resuscitation-promoting factor (DeltaDUF) RpfB from *M. tuberculosis*. *Journal of Molecular Biology, 385*(1), 153-162.

Ruiz, J. A., Lopez, N. I., Fernandez, R. O., and Mendez, B. S. (2001). Polyhydroxyalkanoate degradation is associated with nucleotide accumulation and enhances stress resistance and survival of *Pseudomonas oleovorans* in natural water microcosms. *Applied and Environmental Microbiology, 67*(1), 225-230.

Ruiz, J. A., Lopez, N. I., and Mendez, B. S. (1999). Polyhydroxyalkanoates degradation affects survival of *Pseudomonas oleovorans* in river water microcosms. *Revista Argentina De Microbiologia, 31*(4), 201-204.

Ruiz, J. A., Lopez, N. I., and Mendez, B. S. (2004). *rpoS* gene expression in carbon-starved cultures of the polyhydroxyalkanoate-accumulating species *Pseudomonas oleovorans*. *Current Microbiology, 48*(6), 396-400.

Ruth, K., de Roo, G., Egli, T., and Ren, Q. (2008). Identification of two acyl-CoA synthetases from *Pseudomonas putida* GPo1: One is located at the surface of polyhydroxyalkanoates granules. *Biomacromolecules, 9*(6), 1652-1659.

Rydel, T. J., Williams, J. M., Krieger, E., Moshiri, F., Stallings, W. C., Brown, S. M., Pershing, J. C., Purcell, J. P., Alibhai, M. F. (2003). The crystal structure, mutagenesis, and activity studies reveal that patatin is a lipid acyl hydrolase with a Ser-Asp catalytic dyad. *Biochemistry, 42*(22), 6696-6708.

Sadosky, A. B., Wiater, L. A., and Shuman, H. A. (1993). Identification of *Legionella pneumophila* genes required for growth within and killing of human macrophages. *Infection and Immunity, 61*(12), 5361-5373.

Sardesai, N., and Babu, C. R. (2001). Poly-beta-hydroxybutyrate metabolism is affected by changes in respiratory enzymatic activities due to cold stress in two psychrotrophic strains of *Rhizobium*. *Current Microbiology, 42*(1), 53-58.

Sarniguet, A., Kraus, J., Henkels, M. D., Muehlchen, A. M., and Loper, J. E. (1995). The sigma factor sigma S affects antibiotic production and biological control activity of pseudomonas fluorescens pf-5. *Proceedings of the National Academy of Sciences of the United States of America, 92*(26), 12255-12259.

Sauer, J. D., Bachman, M. A., and Swanson, M. S. (2005). The phagosomal transporter A couples threonine acquisition to differentiation and replication of *Legionella pneumophila* in macrophages. *Proceedings of the National Academy of Sciences of the United States of America, 102*(28), 9924-9929.

Schofield, G. M. (1985). A note on the survival of *Legionella pneumophila* in stagnant tap water. *The Journal of Applied Bacteriology, 59*(4), 333-335.

Scott, C. C., Botelho, R. J., and Grinstein, S. (2003). Phagosome maturation: A few bugs in the system. *The Journal of Membrane Biology, 193*(3), 137-152.

Segal, G., and Shuman, H. A. (1997). Characterization of a new region required for macrophage killing by *Legionella pneumophila*. *Infection and Immunity, 65*(12), 5057-5066.

Segura, D., and Espin, G. (2004). Inactivation of pycA, encoding pyruvate carboxylase activity, increases poly-beta-hydroxybutyrate accumulation in *Azotobacter vinelandii* on solid medium. *Applied Microbiology and Biotechnology, 65*(4), 414-418.

Senior, P. J., Beech, G. A., Ritchie, G. A., and Dawes, E. A. (1972). The role of oxygen limitation in the formation of poly-beta-hydroxybutyrate during batch and continuous culture of *Azotobacter beijerinckii*. *The Biochemical Journal, 128*(5), 1193-1201.

Senior, P. J., and Dawes, E. A. (1973). The regulation of poly-beta-hydroxybutyrate metabolism in *Azotobacter beijerinckii*. *The Biochemical Journal,134*(1), 225-238.

Senoh, M., Ghosh-Banerjee, J., Ramamurthy, T., Colwell, R. R., Miyoshi, S., Nair, G. B., Takeda, Y. (2012). Conversion of viable but nonculturable enteric bacteria to culturable by co-culture with eukaryotic cells. *Microbiology and Immunology, 56*(5), 342-345.

Shelton, B. G., Kerbel, W., Witherell, L., and Millar, J. D. (2000). Review of Legionnaires' disease. *AIHAJ: A Journal for the Science of Occupational and Environmental Health and Safety, 61*(5), 738-742.

Shevchuk, O., Jager, J., and Steinert, M. (2011). Virulence properties of the *Legionella pneumophila* cell envelope. *Frontiers in Microbiology, 2*, 74.

Shi, C., Forsbach-Birk, V., Marre, R., and McNealy, T. L. (2006). The *Legionella pneumophila* global regulatory protein LetA affects DotA and *mip*. *International Journal of Medical Microbiology: IJMM, 296*(1), 15-24.

Shiraki, M., Endo, T., and Saito, T. (2006). Fermentative production of (R)-(-)-3-hydroxybutyrate using 3-hydroxybutyrate dehydrogenase null mutant of *Ralstonia eutropha* and recombinant *Escherichia coli*. *Journal of Bioscience and Bioengineering, 102*(6), 529-534.

Shively, J. M. (1974). Inclusion bodies of prokaryotes. *Annual Review of Microbiology, 28*(0), 167-187.

Shoff, M. E., Rogerson, A., Kessler, K., Schatz, S., and Seal, D. V. (2008). Prevalence of *Acanthamoeba* and other naked amoebae in south florida domestic water. *Journal of Water and Health, 6*(1), 99-104.

Signoretto, C., Lleo, M., and Canepari, P. (2002). Modification of the peptidoglycan of *Escherichia coli* in the viable but nonculturable state. *Current Microbiology, 44*(2), 125-131.

Signoretto, C., Lleo, M. M., Tafi, M. C., and Canepari, P. (2000). Cell wall chemical composition of *Enterococcus faecalis* in the viable but nonculturable state. *Applied and Environmental Microbiology, 66*(5), 1953-1959.

Simpson, L. M., White, V. K., Zane, S. F., and Oliver, J. D. (1987). Correlation between virulence and colony morphology in *Vibrio vulnificus*. *Infection and Immunity, 55*(1), 269-272.

Skaliy, P., and McEachern, H. V. (1979). Survival of the Legionnaires' disease bacterium in water. *Annals of Internal Medicine, 90*(4), 662-663.

Slater, S. C., Voige, W. H., and Dennis, D. E. (1988). Cloning and expression in *Escherichia coli* of the alcaligenes eutrophus H16 poly-beta-hydroxybutyrate biosynthetic pathway. *Journal of Bacteriology, 170*(10), 4431-4436.

Smith, J. J., Howington, J. P., and McFeters, G. A. (1994). Survival, physiological response and recovery of enteric bacteria exposed to a polar marine environment. *Applied and Environmental Microbiology, 60*(8), 2977-2984.

Soderberg, M. A., and Cianciotto, N. P. (2010). Mediators of lipid A modification, RNA degradation, and central intermediary metabolism facilitate the growth of *Legionella pneumophila* at low temperatures. *Current Microbiology, 60*(1), 59-65.

Soderberg, M. A., Dao, J., Starkenburg, S. R., and Cianciotto, N. P. (2008). Importance of type II secretion for survival of *Legionella pneumophila* in tap water and in amoebae at low temperatures. *Applied and Environmental Microbiology, 74*(17), 5583-5588.

Solomon, J. M., and Isberg, R. R. (2000). Growth of *Legionella pneumophila* in *Dictyostelium discoideum*: A novel system for genetic analysis of host-pathogen interactions. *Trends in Microbiology, 8*(10), 478-480.

Sperandio, V., Torres, A. G., Jarvis, B., Nataro, J. P., and Kaper, J. B. (2003). Bacteria-host communication: The language of hormones. *Proceedings of the National Academy of Sciences of the United States of America, 100*(15), 8951-8956.

Spirig, T., Tiaden, A., Kiefer, P., Buchrieser, C., Vorholt, J. A., and Hilbi, H. (2008). The *Legionella* autoinducer synthase LqsA produces an alpha-hydroxyketone signaling molecule. *The Journal of Biological Chemistry, 283*(26), 18113-18123.

Srivatsan, A., and Wang, J. D. (2008). Control of bacterial transcription, translation and replication by (p)ppGpp. *Current Opinion in Microbiology, 11*(2), 100-105.

States, S. J., Conley, L. F., Ceraso, M., Stephenson, T. E., Wolford, R. S., Wadowsky, R. M., McNamara, A. M., Yee, R. B. (1985). Effects of metals on *Legionella pneumophila* growth in drinking water plumbing systems. *Applied and Environmental Microbiology, 50*(5), 1149-1154.

Steinbüchel, A., Aerts, K., Babel, W., Follner, C., Liebergesell, M., Madkour, M. H., Mayer, F., Pieper-Furst, U., Pries, A., Valentin, H. E. (1995). Considerations on the structure and biochemistry of bacterial polyhydroxyalkanoic acid inclusions. *Canadian Journal of Microbiology, 41 Suppl 1*, 94-105.

Steinbüchel, A., and Schlegel, H. G. (1991). Physiology and molecular genetics of poly(beta-hydroxy-alkanoic acid) synthesis in *Alcaligenes eutrophus*. *Molecular Microbiology, 5*(3), 535-542.

Steinert, M., Emody, L., Amann, R., and Hacker, J. (1997). Resuscitation of viable but nonculturable *Legionella pneumophila* philadelphia JR32 by *Acanthamoeba castellanii*. *Applied and Environmental Microbiology, 63*(5), 2047-2053.

Steinert, M., Hentschel, U., and Hacker, J. (2002). *Legionella pneumophila*: An aquatic microbe goes astray. *FEMS Microbiology Reviews, 26*(2), 149-162.

Steinert, M., and Heuner, K. (2005). Dictyostelium as host model for pathogenesis. *Cellular Microbiology, 7*(3), 307-314.

Stenmark, H. (2009). Rab GTPases as coordinators of vesicle traffic. *Nature Reviews.Molecular Cell Biology, 10*(8), 513-525.

Stevenson, L. H., and Socolofsky, M. D. (1966). Cyst formation and poly-beta-hydroxybutyric acid accumulation in *Azotobacter*. *Journal of Bacteriology, 91*(1), 304-310.

Stewart, C. R., Burnside, D. M., and Cianciotto, N. P. (2011). The surfactant of *Legionella pneumophila* is secreted in a TolC-dependent manner and is antagonistic toward other *Legionella* species. *Journal of Bacteriology, 193*(21), 5971-5984.

Stewart, C. R., Rossier, O., and Cianciotto, N. P. (2009). Surface translocation by *Legionella pneumophila*: A form of sliding motility that is dependent upon type II protein secretion. *Journal of Bacteriology, 191*(5), 1537-1546.

Stone, B. J., and Abu Kwaik, Y. (1998). Expression of multiple pili by *Legionella pneumophila*: Identification and characterization of a type IV pilin gene and its role in adherence to mammalian and protozoan cells. *Infection and Immunity, 66*(4), 1768-1775.

Storey, M. V., Winiecka-Krusnell, J., Ashbolt, N. J., and Stenstrom, T. A. (2004). The efficacy of heat and chlorine treatment against thermotolerant *Acanthamoebae* and *Legionellae*. *Scandinavian Journal of Infectious Diseases, 36*(9), 656-662.

Su, C. P., Jane, W. N., and Wong, H. C. (2013). Changes of ultrastructure and stress tolerance of *Vibrio parahaemolyticus* upon entering viable but nonculturable state. *International Journal of Food Microbiology, 160*(3), 360-366.

Sudarsan, N., Lee, E. R., Weinberg, Z., Moy, R. H., Kim, J. N., Link, K. H., Breaker, R. R. (2008). Riboswitches in eubacteria sense the second messenger cyclic di-GMP. *Science, 321*(5887), 411-413.

Sun, F., Chen, J., Zhong, L., Zhang, X. H., Wang, R., Guo, Q., Dong, Y. (2008). Characterization and virulence retention of viable but nonculturable *Vibrio harveyi*. *FEMS Microbiology Ecology, 64*(1), 37-44.

Sussman, M., Loya, Y., Fine, M., and Rosenberg, E. (2003). The marine fireworm *Hermodice carunculata* is a winter reservoir and spring-summer vector for the coral-bleaching pathogen *Vibrio shiloi*. *Environmental Microbiology, 5*(4), 250-255.

Sutherland, E. E., and Berk, S. G. (1996). Survival of protozoa in cooling tower biocides. *Journal of Industrial Microbiology, 16(1)*, 73-78.

Sutherland, D. R., Abdullah, K. M., Cyopick, P., and Mellors, A. (1992). Cleavage of the cell-surface O-sialoglycoproteins CD34, CD43, CD44, and CD45 by a novel glycoprotease from *Pasteurella haemolytica*. *Journal of Immunology, 148*(5), 1458-1464.

Swanson, M. S., and Hammer, B. K. (2000). *Legionella pneumophila* pathogesesis: A fateful journey from amoebae to macrophages. *Annual Review of Microbiology, 54*, 567-613.

Swanson, M. S., and Isberg, R. R. (1995). Association of *Legionella pneumophila* with the macrophage endoplasmic reticulum. *Infection and Immunity, 63*(9), 3609-3620.

Szalewska-Palasz, A., Johansson, L. U., Bernardo, L. M., Skarfstad, E., Stec, E., Brannstrom, K., Shingler, V. (2007). Properties of RNA polymerase bypass mutants: Implications for the role of ppGpp and its co-factor DksA in controlling transcription dependent on sigma54. *The Journal of Biological Chemistry, 282*(25), 18046-18056.

Tal, S., and Okon, Y. (1985). Production of the reserve material poly-phydroxybutyrate and its function in *Azospirillum brasilense* cd . *Canadian Journal of Microbiology, 31*, 608-613.

Tal, S., Smirnoff, P., and Okon, Y. (1990). Purification and characterization of D(-)-β-hydroxybutyrate dehydrogenase from *Azospirillum brasilense* cd. *Journal of General Microbiology, 136*, 645-649.

Tanio, T., Fukui, T., Shirakura, Y., Saito, T., Tomita, K., Kaiho, T., Masamune, S. (1982). An extracellular poly(3-hydroxybutyrate) depolymerase from *Alcaligenes faecalis*. *European Journal of Biochemistry/FEBS, 124*(1), 71-77.

Taylor, M., Ross, K., and Bentham, R. (2009). *Legionella*, protozoa, and biofilms: Interactions within complex microbial systems. *Microbial Ecology, 58*(3), 538-547.

Temmerman, R., Vervaeren, H., Noseda, B., Boon, N., and Verstraete, W. (2006). Necrotrophic growth of *Legionella pneumophila*. *Applied and Environmental Microbiology, 72*(6), 4323-4328.

Testerman, T. L., Vazquez-Torres, A., Xu, Y., Jones-Carson, J., Libby, S. J., and Fang, F. C. (2002). The alternative sigma factor sigmaE controls antioxidant defences required for *Salmonella* virulence and stationary-phase survival. *Molecular Microbiology, 43*(3), 771-782.

Thomas, V., Bouchez, T., Nicolas, V., Robert, S., Loret, J. F., and Levi, Y. (2004). Amoebae in domestic water systems: Resistance to disinfection treatments and implication in *Legionella* persistence. *Journal of Applied Microbiology, 97*(5), 950-963.

Tiaden, A., Spirig, T., Weber, S. S., Bruggemann, H., Bosshard, R., Buchrieser, C., Hilbi, H. (2007). The *Legionella pneumophila* response regulator LqsR promotes host cell interactions as an element of the virulence regulatory network controlled by RpoS and LetA. *Cellular Microbiology, 9*(12), 2903-2920.

Tilney, L. G., Harb, O. S., Connelly, P. S., Robinson, C. G., and Roy, C. R. (2001). How the parasitic bacterium *Legionella pneumophila* modifies its phagosome and transforms it into rough ER: Implications for conversion of plasma membrane to the ER membrane. *Journal of Cell Science, 114*(24), 4637-4650.

Tison, D. L., Pope, D. H., Cherry, W. B., and Fliermans, C. B. (1980). Growth of *Legionella pneumophila* in association with blue-green algae (cyanobacteria). *Applied and Environmental Microbiology, 39*(2), 456-459.

Trevors, J. T. (2011). Viable but non-culturable (VBNC) bacteria: Gene expression in planktonic and biofilm cells. *Journal of Microbiological Methods, 86*(2), 266-273.

Trevors, J. T., Elsas, J. D., and Bej, A. K. (2012). The molecularly crowded cytoplasm of bacterial cells: Dividing cells contrasted with viable but non-culturable (VBNC) bacterial cells. *Current Issues in Molecular Biology, 15*(1), 1-6.

Trotsenko, Y. A., and Belova, L. L. (2000). Biosynthesis of poly(3-hydroxybutyrate) and poly(3-hydroxybutyrate-co-3-hydroxyvalerate) and its regulation in bacteria *Microbiology, 69(6)*, 635-645.

Turetgen, I. (2008). Induction of viable but nonculturable (VBNC) state and the effect of multiple subculturing on the survival of *Legionella pneumophila* strains in the presence of monochloramine. *Annals of Microbiology, 58(1)*, 153-156.

Uchino, K., Saito, T., Gebauer, B., and Jendrossek, D. (2007). Isolated poly(3-hydroxybutyrate) (PHB) granules are complex bacterial organelles catalyzing formation of PHB from acetyl coenzyme A (CoA) and degradation of PHB to acetyl-CoA. *Journal of Bacteriology, 189*(22), 8250-8256.

Uchino, K., Saito, T., and Jendrossek, D. (2008). Poly(3-hydroxybutyrate) (PHB) depolymerase PhaZa1 is involved in mobilization of accumulated PHB in *Ralstonia eutropha* H16. *Applied and Environmental Microbiology, 74*(4), 1058-1063.

Utsumi, R., Katayama, S., Taniguchi, M., Horie, T., Ikeda, M., Igaki, S., Nakagawa, H., Miwa, A., Tanabe, H., Noda, M. (1994). Newly identified genes involved in the signal transduction of *Escherichia coli* K-12. *Gene, 140*(1), 73-77.

Valverde, C., Heeb, S., Keel, C., and Haas, D. (2003). RsmY, a small regulatory RNA, is required in concert with RsmZ for GacA-dependent expression of biocontrol traits in *Pseudomonas fluorescens* CHA0. *Molecular Microbiology, 50*(4), 1361-1379.

van Elsas, J. D., Kastelein, P., van Bekkum, P., van der Wolf, J. M., de Vries, P. M., and van Overbeek, L. S. (2000). Survival of *Ralstonia solanacearum* biovar 2, the causative agent of potato brown rot, in field and microcosm soils in temperate climates. *Phytopathology, 90*(12), 1358-1366.

Vandenesch, F., Surgot, M., Bornstein, N., Paucod, J. C., Marmet, D., Isoard, P., Fleurette, J. (1990). Relationship between free amoeba and *Legionella*: Studies *in vitro* and *in vivo*. *Zentralblatt Fur Bakteriologie: International Journal of Medical Microbiology, 272*(3), 265-275.

Venkataraman, C., Gao, L. Y., Bondada, S., and Kwaik, Y. A. (1998). Identification of putative cytoskeletal protein homologues in the protozoan host *Hartmannella vermiformis* as substrates for induced tyrosine phosphatase activity upon attachment to the Legionnaires' disease bacterium, *Legionella pneumophila*. *The Journal of Experimental Medicine, 188*(3), 505-514.

Venkataraman, C., Haack, B. J., Bondada, S., and Abu Kwaik, Y. (1997). Identification of a Gal/GalNAc lectin in the protozoan *Hartmannella vermiformis* as a potential receptor for attachment and invasion by the Legionnaires' disease bacterium. *The Journal of Experimental Medicine, 186*(4), 537-547.

Viswanathan, V. K., Edelstein, P. H., Pope, C. D., and Cianciotto, N. P. (2000). The *Legionella pneumophila iraAB* locus is required for iron assimilation, intracellular infection, and virulence. *Infection and Immunity, 68*(3), 1069-1079.

Vogel, C., and Marcotte, E. M. (2012). Insights into the regulation of protein abundance from proteomic and transcriptomic analyses. *Nature Reviews.Genetics, 13*(4), 227-232.

von Baum, H., Ewig, S., Marre, R., Suttorp, N., Gonschior, S., Welte, T., Luck, C., Competence Network for Community Acquired Pneumonia Study Group. (2008). Community-acquired *Legionella pneumonia*: New insights from the German competence network for community acquired pneumonia. *Clinical Infectious Diseases: An Official Publication of the Infectious Diseases Society of America, 46*(9), 1356-1364.

Vora, G. J., Meador, C. E., Bird, M. M., Bopp, C. A., Andreadis, J. D., and Stenger, D. A. (2005). Microarray-based detection of genetic heterogeneity, antimicrobial resistance, and the viable but nonculturable state in human pathogenic *Vibrio* spp. *Proceedings of the National Academy of Sciences of the United States of America, 102*(52), 19109-19114.

Wadowsky, R. M., Butler, L. J., Cook, M. K., Verma, S. M., Paul, M. A., Fields, B. S., Keleti, G., Sykora, J. L., Yee, R. B. (1988). Growth-supporting activity for *Legionella pneumophila* in tap water cultures and implication of *Hartmannellid* amoebae as growth factors. *Applied and Environmental Microbiology, 54*(11), 2677-2682.

Wadowsky, R. M., Wolford, R., McNamara, A. M., and Yee, R. B. (1985). Effect of temperature, pH, and oxygen level on the multiplication of naturally occurring *Legionella pneumophila* in potable water. *Applied and Environmental Microbiology, 49*(5), 1197-1205.

Wadowsky, R. M., and Yee, R. B. (1983). Satellite growth of *Legionella pneumophila* with an environmental isolate of *Flavobacterium breve*. *Applied and Environmental Microbiology, 46*(6), 1447-1449.

Wagner, C., Kronert, C., Luck, P. C., Jacobs, E., Cianciotto, N. P., and Helbig, J. H. (2007). Random mutagenesis of *Legionella pneumophila* reveals genes associated with lipopolysaccharide synthesis and recognition by typing monoclonal antibodies. *Journal of Applied Microbiology, 103*(5), 1975-1982.

Wai, S. N., Mizunoe, Y., Takade, A., and Yoshida, S. (2000). A comparison of solid and liquid media for resuscitation of starvation- and low-temperature-induced nonculturable cells of *Aeromonas hydrophila*. *Archives of Microbiology, 173*(4), 307-310.

Wallis, L., and Robinson, P. (2005). Soil as a source of *Legionella pneumophila* serogroup 1 (Lp1). *Australian and New Zealand Journal of Public Health, 29*(6), 518-520.

Wang, Q., Yu, H., Xia, Y., Kang, Z., and Qi, Q. (2009). Complete PHB mobilization in *Escherichia coli* enhances the stress tolerance: A potential biotechnological application. *Microbial Cell Factories, 8*, 47-2859-8-47.

Wang, X., Dubey, A. K., Suzuki, K., Baker, C. S., Babitzke, P., and Romeo, T. (2005). CsrA post-transcriptionally represses *pgaABCD*, responsible for synthesis of a biofilm polysaccharide adhesin of *Escherichia coli*. *Molecular Microbiology, 56*(6), 1648-1663.

Weichart, D., and Kjelleberg, S. (1996). Stress resistance and recovery potential of culturable and viable but nonculturable cells of *Vibrio vulnificus*. *Microbiology (Reading, England), 142*(4), 845-853.

Weng, L., Biswas, I., and Morrison, D. A. (2009). A self-deleting *cre-lox-ermAM* cassette, cheshire, for marker-less gene deletion in *Streptococcus pneumoniae*. *Journal of Microbiological Methods, 79*(3), 353-357.

White, A. P., Gibson, D. L., Collinson, S. K., Banser, P. A., and Kay, W. W. (2003). Extracellular polysaccharides associated with thin aggregative fimbriae of *Salmonella enterica* serovar Enteritidis. *Journal of Bacteriology, 185*(18), 5398-5407.

White, A. P., Gibson, D. L., Kim, W., Kay, W. W., and Surette, M. G. (2006). Thin aggregative fimbriae and cellulose enhance long-term survival and persistence of *Salmonella*. *Journal of Bacteriology, 188*(9), 3219-3227.

Whitesides, M. D., and Oliver, J. D. (1997). Resuscitation of *Vibrio vulnificus* from the viable but nonculturable state. *Applied and Environmental Microbiology, 63*(3), 1002-1005.

Winn, W. C.,Jr. (1988). Legionnaires disease: Historical perspective. *Clinical Microbiology Reviews, 1*(1), 60-81.

Wong, H. C., and Liu, S. H. (2008). Characterization of the low-salinity stress in *Vibrio vulnificus*. *Journal of Food Protection, 71*(2), 416-419.

Wong, H. C., and Wang, P. (2004). Induction of viable but nonculturable state in *Vibrio parahaemolyticus* and its susceptibility to environmental stresses. *Journal of Applied Microbiology, 96*(2), 359-366.

Wong, H. C., Wang, P., Chen, S. Y., and Chiu, S. W. (2004a). Resuscitation of viable but non-culturable *Vibrio parahaemolyticus* in a minimum salt medium. *FEMS Microbiology Letters, 233*(2), 269-275.

Wong, H. C., Shen, C. T., Chang, C. N., Lee, Y. S., and Oliver, J. D. (2004b). Biochemical and virulence characterization of viable but nonculturable cells of *Vibrio parahaemolyticus*. *Journal of Food Protection, 67*(11), 2430-2435.

Wu, G., Moir, A. J., Sawers, G., Hill, S., and Poole, R. K. (2001). Biosynthesis of poly-beta-hydroxybutyrate (PHB) is controlled by CydR (*fnr*) in the obligate aerobe *Azotobacter vinelandii*. *FEMS Microbiology Letters, 194*(2), 215-220.

Xu, H., Roberts, N., Singleton, F. L., Attwell, R. W., Grimes, D. J., and Colwell, R. R. (1982). Survival and viability of nonculturable *Escherichia coli* and *Vibrio cholerae* in the estuarine and marine environment. *Microbial Ecology, 8(4)*, 313-323.

Xu, L., and Luo, Z. Q. (2013). Cell biology of infection by *Legionella pneumophila*. *Microbes and Infection /Institut Pasteur, 15*(2), 157-167.

Yamamoto, H., Hashimoto, Y., and Ezaki, T. (1996). Study of nonculturable *Legionella pneumophila* cells during multiple-nutrient starvation. *FEMS Microbiology Ecology, 20*, 149-154.

Yee, R. B., and Wadowsky, R. M. (1982). Multiplication of *Legionella pneumophila* in unsterilized tap water. *Applied and Environmental Microbiology, 43*(6), 1330-1334.

York, G. M., Lupberger, J., Tian, J., Lawrence, A. G., Stubbe, J., and Sinskey, A. J. (2003). *Ralstonia eutropha* H16 encodes two and possibly three intracellular poly[*D*-(-)-3-hydroxybutyrate] depolymerase genes. *Journal of Bacteriology, 185*(13), 3788-3794.

Young, D. B., Gideon, H. P., and Wilkinson, R. J. (2009). Eliminating latent tuberculosis. *Trends in Microbiology, 17*(5), 183-188.

Yu, V. L., Plouffe, J. F., Pastoris, M. C., Stout, J. E., Schousboe, M., Widmer, A., Summersgill, J., File, T., Heath, C. M., Paterson, D. L., Chereshsky, A. (2002). Distribution of *Legionella* species and serogroups isolated by culture in patients with sporadic community-acquired legionellosis: An international collaborative survey. *The Journal of Infectious Diseases, 186*(1), 127-128.

Zandri, G., Pasquaroli, S., Vignaroli, C., Talevi, S., Manso, E., Donelli, G., Biavasco, F. (2012). Detection of viable but non-culturable *Staphylococci* in biofilms from central venous catheters negative on standard microbiological assays. *Clinical Microbiology and Infection: The Official Publication of the European Society of Clinical Microbiology and Infectious Diseases, 18*(7), E259-61.

Zhao, L., and Mathews, K. R. (2000). Influence of starvation, temperature, and pH on culturability of *Escherichia coli* O157:H7. *Journal of Food Safety, 20*, 193–206.

Zhao, Y. H., Li, H. M., Qin, L. F., Wang, H. H., and Chen, G. Q. (2007). Disruption of the polyhydroxyalkanoate synthase gene in *Aeromonas hydrophila* reduces its survival ability under stress conditions. *FEMS Microbiology Letters, 276*(1), 34-41.

Zhu, W., Banga, S., Tan, Y., Zheng, C., Stephenson, R., Gately, J., Luo, Z. Q. (2011). Comprehensive identification of protein substrates of the Dot/Icm type IV transporter of *Legionella pneumophila*. *PloS One, 6*(3), e17638.

Zusman, T., Degtyar, E., and Segal, G. (2008). Identification of a hypervariable region containing new *Legionella pneumophila* Icm/Dot translocated substrates by using the conserved *icmQ* regulatory signature. *Infection and Immunity, 76*(10), 4581-4591.

APPENDIX I

Proteins with increased level in MIF-VBNCCs as compared to the culturable MIFs according to shotgun proteomic screen

Accession	Description	Molecular Function	Cellular Component	Biological Process	fold change MIF-VBNCCs/ culturable MIFs
G8UXQ5	DNA integration/recombination/inversion protein OS=*Legionella pneumophila* subsp. pneumophila ATCC 43290	catalytic activity; nucleotide binding, ATP binding, nucleoside-triphosphatase activity		metabolic process	100.000
E1XZ23	Global stress protein GspA OS=*Legionella pneumophila*			stress response	100.000
Q5ZST9	Malonate decarboxylase acyl carrier protein OS=*Legionella pneumophila* subsp. pneumophila (strain Philadelphia 1 / ATCC 33152 / DSM 7513)	catalytic activity, ligase activity, transferase activity	cytoplasm	metabolic process	100.000
G8UXT7	Putative uncharacterized protein OS=*Legionella pneumophila* subsp. pneumophila ATCC 43290				100.000
A5IGS9	Type IV fimbrial biogenesis PilY1-related protein OS=*Legionella pneumophila* (strain Corby)	protein binding			100.000
Q5WVG6	Putative uncharacterized protein OS=*Legionella pneumophila* (strain Lens)				96.603
E1XZ77	Putative uncharacterized protein OS=*Legionella pneumophila*	catalytic activity; metal ion binding		metabolic process	95.260
G8UYH4	Small HspC2 heat shock protein OS=*Legionella pneumophila* subsp. pneumophila ATCC 43290			response to stimulus, stress response	87.048
G8UYH3	Global stress protein GspA OS=*Legionella pneumophila* subsp. pneumophila ATCC 43290			stress response	94.444
A5IBJ7	TPR repeat protein OS=*Legionella pneumophila* (strain Corby)	protein binding			55.024
G8UWF6	Putative uncharacterized protein OS=*Legionella pneumophila* subsp. pneumophila ATCC 43290				42.375
G8UXA3	Alpha-amylase, putative OS=*Legionella pneumophila* subsp. pneumophila ATCC 43290	catalytic activity, cation binding		metabolic proces, carbohydrate metabolic processs	33.064
D5TDI2	GabD succinate semialdehyde dehyrogenase (NADP) OS=*Legionella pneumophila* serogroup 1 (strain 2300/99 Alcoy)	catalytic activity, Oxidoreductase	membrane	metabolic process	30.692
A5IB57	Phosphoribosyltransferase OS=*Legionella pneumophila* (strain Corby)	catalytic activity, Glycosyltransferase, Transferase activity		metabolic process, nucleoside metabolic process	27.408
Q5ZVE9	Putative uncharacterized protein OS=*Legionella pneumophila* subsp. pneumophila (strain Philadelphia 1 / ATCC 33152 / DSM 7513)				26.464

Accession	Description	Molecular Function	Cellular Component	Biological Process	fold change MIF-VBNCCs/ culturable MIFs
Q6RCR0	SdeA OS=*Legionella pneumophila*	substrate of the Dot/Icm system			25.203
G8UYD6	Sid related protein-like protein OS=*Legionella pneumophila* subsp. pneumophila ATCC 43290	Toxin production / other pathogen functions			22.709
G8UX45	Putative uncharacterized protein OS=Legionella pneumophila subsp. pneumophila ATCC 43290 GN=lp12_2139 PE=4 SV=1 - [G8UX45_LEGPN]				22.673
G8US05	Putative uncharacterized protein OS=*Legionella pneumophila* subsp. pneumophila ATCC 43290	nucleotide binding			22.590
Q5ZYW6	NAD-glutamate dehydrogenase OS=*Legionella pneumophila* subsp. pneumophila (strain Philadelphia 1 / ATCC 33152 / DSM 7513)	nucleotide binding			21.591
G8URP1	IcmL-like protein OS=*Legionella pneumophila* subsp. pneumophila ATCC 43290	Transport and binding, Toxin production / other pathogen functions, Protein fate / hydrolases / secretion			21.486
E1Y1C5	Putative uncharacterized protein OS=*Legionella pneumophila*	catalytic activity; protein binding		metabolic process	21.257
G8UV16	Putative uncharacterized protein OS=*Legionella pneumophila* subsp. pneumophila ATCC 43290	nucleotide binding			19.265
E1XXE2	Putative uncharacterized protein OS=*Legionella pneumophila*	catalytic activity		metabolic process	17.901
Q5ZZ16	Putative uncharacterized protein OS=*Legionella pneumophila* subsp. pneumophila (strain Philadelphia 1 / ATCC 33152				16.397
E1Y0E7	Putative uncharacterized protein OS=*Legionella pneumophila*	catalytic activity	cytoplasm	cellular homeostasis; metabolic process; regulation of biological process	15.639
A5IG24	Sensory box protein/GGDEF/EAL domains OS=*Legionella pneumophila* (strain Corby)	catalytic activity; protein binding; receptor activity; signal transducer activity, phosphorus-oxygen lyase activity, two-component sensor activity	intracellular	cell communication; metabolic process; regulation of biological process; response to stimulus, cyclic nucleotide biosynthetic process,intracellular signal transduction, regulation of transcription, DNA-dependent, signal transduction by phosphorylation	15.523
G8UU62	VipE OS=*Legionella pneumophila* subsp. pneumophila ATCC 43290				15.416
E1XWZ8	Putative uncharacterized protein OS=*Legionella pneumophila*				14.947
Q5X610	Putative uncharacterized protein OS=*Legionella pneumophila* (strain Paris)				14.134
A5IAB4	Glutaminase OS=*Legionella pneumophila* (strain Corby)	catalytic activity, glutaminase activity, hydrolase		metabolic process, glutamine metabolic process	13.739
A5IAX4	Putative uncharacterized protein OS=*Legionella pneumophila* (strain Corby)				13.180

Accession	Description	Molecular Function	Cellular Component	Biological Process	fold change MIF-VBNCCs/ culturable MIFs
G8UX67	Small heat shock protein HspC2 OS=*Legionella pneumophila* subsp. pneumophila ATCC 43290			response to stimulus, Stress response	11.217
A5IE02	Replication factor C subunit (Activator I) OS=*Legionella pneumophila* (strain Corby)				11.162
G8UXA2	Putative uncharacterized protein OS=*Legionella pneumophila* subsp. pneumophila ATCC 43290				10.352
Q5X001	Putative uncharacterized protein OS=*Legionella pneumophila* (strain Lens)				10.171
G8UZ12	Guanine nucleotide exchange protein OS=*Legionella pneumophila* subsp. pneumophila ATCC 43290	enzyme regulator activity, ARF guanyl-nucleotide exchange factor activity	intracellular	cell communication; regulation of biological process; response to stimulus, regulation of ARF protein signal transduction	10.139
A5IGT2	Putative uncharacterized protein OS=*Legionella pneumophila* (strain Corby)	protein binding			9.497
Q5ZWQ0	Copper efflux ATPase OS=Legionella pneumophila subsp. pneumophila (strain Philadelphia 1 / ATCC 33152 / DSM 7513) GN=pcoA PE=4 SV=1 - [Q5ZWQ0_LEGPH]	catalytic activity; metal ion binding, copper ion binding, oxidoreductase activity	membrane	cellular homeostasis; metabolic process, cellular copper ion homeostasis	9.298
E1XZ20	Putative uncharacterized protein OS=*Legionella pneumophila*				8.247
G8UV69	Putative uncharacterized protein OS=*Legionella pneumophila* subsp. pneumophila ATCC 43290			metabolic process	7.813
G8UUB0	Coiled-coil containing protein OS=*Legionella pneumophila* subsp. pneumophila ATCC 43290				7.441
I7HVW5	Uncharacterized protein OS=*Legionella pneumophila* subsp. pneumophila				7.333
A5ICG7	Alpha-amylase, putative OS=*Legionella pneumophila* (strain Corby)	catalytic activity, cation binding		metabolic process, carbohydrate metabolic process	7.303
G8UZ99	Interaptin OS=*Legionella pneumophila* subsp. pneumophila ATCC 43290		membrane	transport	6.333
A5IAT5	Enhanced entry protein EnhA OS=*Legionella pneumophila* (strain Corby)	catalytic activity, transferase activity			6.252
E1XX67	Dot/Icm T4SS effector OS=*Legionella pneumophila*	Dot/Icm T4SS effector			6.141
E1XYI2	Putative uncharacterized protein OS=*Legionella pneumophila*		membrane		5.959
A5IHT5	Putative uncharacterized protein OS=*Legionella pneumophila* (strain Corby)				5.455
Q0ZHG2	IcmO (Fragment) OS=*Legionella pneumophila*	IcmO	membrane		5.383
Q5X855	30S ribosomal protein S19 OS=*Legionella pneumophila* (strain Paris)	RNA binding; structural molecule activity	cytoplasm; ribosome	metabolic process, translation	5.377
G8UY02	Putative uncharacterized protein OS=*Legionella pneumophila* subsp. pneumophila ATCC 43290				5.067

Accession	Description	Molecular Function	Cellular Component	Biological Process	fold change MIF-VBNCCs/ culturable MIFs
G8UYL1	Saframycin Mx1 synthetase B OS=*Legionella pneumophila* subsp. pneumophila ATCC 43290	catalytic activity		metabolic process	5.057
G8UX86	SidC, interaptin OS=*Legionella pneumophila* subsp. pneumophila ATCC 43290	Toxin production / other pathogen functions			5.009
E1Y1K8	Putative uncharacterized protein OS=*Legionella pneumophila*	catalytic activity; metal ion binding		metabolic process	4.920
Q48800	IcmW OS=*Legionella pneumophila*	IcmW, intracellular multiplication			4.847
G8URB3	Diguanylate cyclase/phosphodiesterase domain 2 OS=*Legionella pneumophila* subsp. pneumophila ATCC 43290	catalytic activity, phosphorus-oxygen lyase activity		cell communication; metabolic process; regulation of biological process; response to stimulus, cyclic nucleotide biosynthetic process, intracellular signal transduction	4.830
I7I4V0	Predicted esterase of the alpha-beta hydrolase superfamily OS=*Legionella pneumophila* subsp. pneumophila	hydrolase activity		lipid metabolic process	4.815
G8UT31	Putative uncharacterized protein OS=*Legionella pneumophila* subsp. pneumophila ATCC 43290				4.811
G8UYD8	Putative uncharacterized protein OS=*Legionella pneumophila* subsp. pneumophila ATCC 43290				4.774
D5T7D2	Putative conserved protein OS=*Legionella pneumophila* serogroup 1 (strain 2300/99 Alcoy)				4.646
I7HL34	Predicted large extracellular alpha-helical protein OS=*Legionella pneumophila* subsp. pneumophila	endopeptidase inhibitor activity		negative regulation of endopeptidase activity	4.097
D5TBI7	Transmembrane protein OS=*Legionella pneumophila* serogroup 1 (strain 2300/99 Alcoy)	NfeD, Membrane-bound serine protease (ClpP class) [Posttranslational modification, protein turnover, chaperones]	membrane		3.984
Q6QFW5	IcmK protein (Fragment) OS=*Legionella pneumophila*	IcmK protein			2.553
E1Y1R4	Putative uncharacterized protein OS=*Legionella pneumophila*				3.655
A5ICD5	Putative uncharacterized protein OS=*Legionella pneumophila* (strain Corby)				3.654
G8UUZ7	Putative glucosyltransferase Lgt1 OS=*Legionella pneumophila* subsp. pneumophila ATCC 43290	catalytic activity, transferase activity			3.653
G8UWF7	Putative uncharacterized protein OS=*Legionella pneumophila* subsp. pneumophila ATCC 43290				3.630
E1Y1R3	Putative uncharacterized protein OS=*Legionella pneumophila*			metabolic process	3.578
G8UYK9	Acetyl CoA carboxylase alpha subunit OS=*Legionella pneumophila* subsp. pneumophila ATCC 43290	catalytic activity, ligase activity			3.534

Accession	Description	Molecular Function	Cellular Component	Biological Process	fold change MIF-VBNCCs/ culturable MIFs
Q8RNQ1	Putative uncharacterized protein OS=*Legionella pneumophila*				3.505
E1XWS7	Dot/Icm T4SS effector OS=*Legionella pneumophila*	Dot/Icm T4SS effector			3.438
G8UZ01	Polyketide synthase, type I OS=*Legionella pneumophila* subsp. pneumophila ATCC 43290	catalytic activity, transferase activity		metabolic process	3.335
I7HKJ2	17 kDa surface antigen OS=*Legionella pneumophila* subsp. pneumophila		outer membrane		3.284
D5T789	Betaine-aldehyde dehydrogenase OS=*Legionella pneumophila* serogroup 1 (strain 2300/99 Alcoy)	catalytic activity, Oxidoreductase	membrane	metabolic process	3.083
E1Y172	Putative uncharacterized protein OS=*Legionella pneumophila*	catalytic activity; metal ion binding		cellular homeostasis; metabolic process; response to stimulus	3.028
Q5ZXQ9	Acetoacetate decarboxylase ADC OS=*Legionella pneumophila* subsp. pneumophila (strain Philadelphia 1 / ATCC 33152 / DSM 7513)	catalytic activity, Lyase, acetoacetate decarboxylase activity			2.996
A5IBU8	GTP pyrophosphokinase ((P)ppGpp synthetase I) stringent stress response RelA OS=*Legionella pneumophila* (strain Corby)	catalytic activity, kinase activity, transferase activity, nucleotide binding		metabolic process, guanosine tetraphosphate metabolic process	2.024
A5ICE4	Putative uncharacterized protein OS=*Legionella pneumophila* (strain Corby)				2.889
E1Y2T8	Putative uncharacterized protein OS=*Legionella pneumophila*	catalytic activity; metal ion binding		cellular homeostasis; metabolic process	2.855
E1XZ29	Putative uncharacterized protein OS=*Legionella pneumophila*	catalytic activity; protein binding		metabolic process	2.501
G8UYV2	Lipase OS=*Legionella pneumophila* subsp. pneumophila ATCC 43290	catalytic activity, hydrolase activity		metabolic process	2.490
I7HXG1	Uncharacterized protein OS=*Legionella pneumophila* subsp. pneumophila				2.476
E1XYJ4	Putative uncharacterized protein OS=*Legionella pneumophila*	catalytic activity; metal ion binding; nucleotide binding		metabolic process	2.335
G8UVI8	Putative virulence protein OS=*Legionella pneumophila* subsp. pneumophila ATCC 43290	Toxin production / other pathogen functions			2.320
G8UY94	Acetoacetyl CoA synthetase OS=*Legionella pneumophila* subsp. Pneumophila ATCC 43290	catalytic activity, acetoacetate-CoA ligase activity		metabolic process, lipid metabolic process	2.308
A5IGN7	NADH dehydrogenase transmembrane protein OS=*Legionella pneumophila* (strain Corby)	catalytic activity, flavin adenine dinucleotide binding, oxidoreductase activity	membrane	metabolic process	2.019
G8UTX4	3-hydroxybutyrate dehydrogenase OS=*Legionella pneumophila* subsp. pneumophila ATCC 43290	catalytic activity; nucleotide binding		metabolic process	2.375

APPENDIX II

Proteins with decreased level in MIF-VBNCCs as compared to the culturable MIFs according to shotgun proteomic screen

Accession	Description	Molecular Function	Cellular Component	Biological Process	fold change MIF-VBNCCs/ culturable MIFs
J7S544	Uncharacterized protein OS=Legionella pneumophila serogroup 1 PE=4 SV=1 - [J7S544_LEGPN]				0.010
A5IG68	UDP-N-acetylglucosamine 1-carboxyvinyltransferase 1 OS=*Legionella pneumophila* (strain Corby)	catalytic activity, Transferase	cytoplasm	cell division; cell organization and biogenesis; metabolic process; regulation of biological process, UDP-N-acetylgalactosamine biosynthetic process, cell cycle, cellular cell wall organization, peptidoglycan biosynthetic process, regulation of cell shape	0.010
G8UT76	Putative uncharacterized protein OS=*Legionella pneumophila* subsp. pneumophila ATCC 43290				0.010
G8UR48	Putative uncharacterized protein OS=*Legionella pneumophila* subsp. pneumophila ATCC 43290				0.010
E1XWL0	Putative uncharacterized protein OS=*Legionella pneumophila*				0.010
E1XV95	Putative uncharacterized protein OS=*Legionella pneumophila*	catalytic activity		metabolic process	0.010
E1XV74	Putative uncharacterized protein OS=*Legionella pneumophila*	catalytic activity		metabolic process	0.010
E1XV06	Putative uncharacterized protein OS=*Legionella pneumophila*	catalytic activity; nucleotide binding	cytoplasm	metabolic process	0.010
A5II47	Putative uncharacterized protein OS=*Legionella pneumophila* (strain Corby)				0.010
A5ID13	Putative uncharacterized protein OS=*Legionella pneumophila* (strain Corby)	catalytic activity		metabolic process; transport	0.010
A5IDM7	Outer membrane protein OS=*Legionella pneumophila* (strain Corby)	protein binding	membrane		0.010
D5TDA5	D-alanyl-D-alanine carboxypeptidase OS=*Legionella pneumophila* serogroup 1 (strain 2300/99 Alcoy)	catalytic activity, Carboxypeptidase, Hydrolase, Protease		metabolic process, proteolysis, Cell envelope biogenesis, outer membrane	0.010
G8UYR6	Cell division protein FtsZ OS=*Legionella pneumophila* subsp. pneumophila ATCC 43290	catalytic activity; nucleotide binding, GTP binding, GTPase activity	cytoplasm	cell division; cell organization and biogenesis; metabolic process, Cell cycle, Cell division, Septation	0.010
E1Y0P8	Acyl carrier protein 1 OS=*Legionella pneumophila*		cytoplasm	metabolic process, fatty acid biosynthetic process	0.010
A5IFI9	Acetoacetyl CoA reductase OS=*Legionella pneumophila* (strain Corby)	catalytic activity; nucleotide binding	cytoplasm	metabolic process, poly-hydroxybutyrate biosynthetic process	0.010

Accession	Description	Molecular Function	Cellular Component	Biological Process	fold change MIF-VBNCCs/ culturable MIFs
Q05358	Protein RecA OS=*Legionella pneumophila*	catalytic activity; DNA binding; nucleotide binding, ATP binding	cytoplasm	cell communication; metabolic process; response to stimulus, DNA damage, DNA recombination, DNA repair, SOS response	0.010
I7HKY7	Putative lipoprotein OS=*Legionella pneumophila* subsp. pneumophila		membrane	Gram-negative-bacterium-type cell outer membrane assembly, protein insertion into membrane	0.012
Q5X7B7	Putative uncharacterized protein OS=*Legionella pneumophila* (strain Paris)				0.013
A5IIH6	Putative uncharacterized protein OS=*Legionella pneumophila* (strain Corby)				0.014
G8UYB4	Putative uncharacterized protein OS=*Legionella pneumophila* subsp. pneumophila ATCC 43290				0.016
E1Y3G0	Putative uncharacterized protein OS=*Legionella pneumophila*	catalytic activity		metabolic process	0.016
G8UUW9	Flagellin OS=*Legionella pneumophila* subsp. pneumophila ATCC 43290	structural molecule activity		cell organization and biogenesis; cellular component movemen, ciliary or flagellar motility, flagellum organizationt	0.018
I7I6Y8	Cold shock protein Qin prophage OS=*Legionella pneumophila* subsp. pneumophila	DNA binding	cytoplasm	regulation of transcription	0.018
Q9RDY3	Glucose-1-phosphate thymidylyltransferase OS=*Legionella pneumophila*	catalytic activity; metal ion binding		metabolic process, extracellular polysaccharide biosynthetic process	0.019
A5IFZ0	Electron transfer flavoprotein, alpha subunit OS=*Legionella pneumophila* (strain Corby)	electron carrier activity, flavin adenine dinucleotide binding			0.019
E1Y093	Zinc metalloprotein OS=*Legionella pneumophila*	catalytic activity, metallopeptidase activity, hydrolase		metabolic process, proteolysis	0.019
A5I9U2	Thiol:disulfide interchange protein DsbA OS=*Legionella pneumophila* (strain Corby)	catalytic activity, protein disulfide oxidoreductase activity	periplasmic space	cellular homeostasis; regulation of biological process, cell redox homeostasis	0.019
G8URL5	Adenosine deaminase OS=*Legionella pneumophila* subsp. pneumophila ATCC 43290	catalytic activity, deaminase activity		metabolic process, Nucleotide Metabolism	0.023
A5IG15	Putative uncharacterized protein OS=*Legionella pneumophila* (strain Corby)		membrane		0.027
A5ICS9	Ribonucleoside-diphosphate reductase OS=*Legionella pneumophila* (strain Corby)	catalytic activity; nucleotide binding, ATP binding	cytoplasm	metabolic process, DNA replication	0.027
A5IGF8	Imidazole glycerol phosphate synthase subunit hisF OS=*Legionella pneumophila* (strain Corby)	catalytic activity	cytoplasm	metabolic process, Amino-acid biosynthesis	0.028
I7HSL5	Putative Outer membrane chaperone Skp (OmpH) OS=*Legionella pneumophila* subsp. pneumophila		membrane?		0.028
A5IBR2	Cytidylate kinase OS=*Legionella pneumophila* (strain Corby)	catalytic activity; nucleotide binding, ATP binding	cytoplasm	metabolic process, pyrimidine nucleotide metabolic process	0.029
I7HKK9	Uncharacterized protein OS=*Legionella pneumophila* subsp. pneumophila				0.030

Accession	Description	Molecular Function	Cellular Component	Biological Process	fold change MIF-VBNCCs/ culturable MIFs
G8UTA1	Putative uncharacterized protein OS=*Legionella pneumophila* subsp. pneumophila ATCC 43290	catalytic activity		metabolic process	0.037
I7I5D1	Alpha-amylase OS=*Legionella pneumophila* subsp. pneumophila	catalytic activity, cation binding		carbohydrate metabolic process	0.038
A5IBJ4	Piperidine-6-carboxylate dehydrogenase OS=*Legionella pneumophila* (strain Corby)	catalytic activity, oxidoreductase activity		metabolic process	0.038
A5I9T3	Probable glycine dehydrogenase [decarboxylating] subunit 2 OS=*Legionella pneumophila* (strain Corby)	catalytic activity, Oxidoreductase		metabolic process, glycine catabolic process	0.039
A5IGA3	Putative uncharacterized protein OS=*Legionella pneumophila* (strain Corby)	protein binding		cell organization and biogenesis	0.045
I7I4P6	Methylated DNA protein cysteine S-methyltransferase OS=*Legionella pneumophila* subsp. pneumophila	Methyltransferase		DNA repair	0.045
A5IE81	Glu/Leu/Phe/Val dehydrogenase OS=*Legionella pneumophila* (strain Corby)	catalytic activity; nucleotide binding		metabolic process	0.047
E1Y3F1	Putative uncharacterized protein OS=*Legionella pneumophila*	catalytic activity		metabolic process	0.049
E1XWT5	Proline iminopeptidase OS=*Legionella pneumophila*	catalytic activity	cytoplasm	metabolic process	0.051
A5ICK3	N-succinylglutamate 5-semialdehyde dehydrogenase OS=*Legionella pneumophila* (strain Corby)	catalytic activity		metabolic process	0.057
A5IBF9	Glutamine--tRNA ligase OS=*Legionella pneumophila* (strain Corby)	catalytic activity; nucleotide binding	cytoplasm	metabolic process	0.064
A5IFU7	DNA polymerase III, alpha subunit OS=*Legionella pneumophila* (strain Corby)	catalytic activity; DNA binding	cytoplasm	metabolic process	0.065
G8URQ9	Glyceraldehyde 3-phosphate dehydrogenase OS=*Legionella pneumophila* subsp. pneumophila ATCC 43290	catalytic activity; nucleotide binding		metabolic process	0.069
I7HM34	D-alanine--D-alanine ligase A OS=*Legionella pneumophila* subsp. pneumophila	ATP binding,D-alanine-D-alanine ligase activity, magnesium ion binding, manganese ion binding	cytoplasm	Cell shape, Cell wall biogenesis/degradation, Peptidoglycan synthesis	0.073
I7I1Z1	Stress protein, member of the CspA-family OS=*Legionella pneumophila* subsp. pneumophila	DNA binding	cytoplasm	regulation of transcription	0.077
G8UX84	SdeD OS=*Legionella pneumophila* subsp. pneumophila ATCC 43290	Toxin production / other pathogen functions			0.079
A5IAL4	Phenylalanine--tRNA ligase beta subunit OS=*Legionella pneumophila* (strain Corby)	catalytic activity; metal ion binding; nucleotide binding; RNA binding	cytoplasm	metabolic process	0.080
A5IGW0	ABC transporter, permease OS=*Legionella pneumophila* (strain Corby)			cell organization and biogenesis	0.081

Accession	Description	Molecular Function	Cellular Component	Biological Process	fold change MIF-VBNCCs/ culturable MIFs
I7I4P8	Putative Carboxymuconolactone decarboxylase OS=*Legionella pneumophila* subsp. pneumophila	peroxidase activity			0.082
E1XVK1	Putative uncharacterized protein OS=*Legionella pneumophila*				0.083
G8UUN2	Alanine dehydrogenase OS=*Legionella pneumophila* subsp. pneumophila ATCC 43290	catalytic activity		metabolic process	0.084
A5IDN0	ABC transport system periplasmic substrate binding protein OS=*Legionella pneumophila* (strain Corby)				0.095
A5IAU5	Probable cytosol aminopeptidase OS=*Legionella pneumophila* (strain Corby)	catalytic activity; metal ion binding	cytoplasm	metabolic process	0.096
G8UYU0	Putative uncharacterized protein OS=*Legionella pneumophila* subsp. pneumophila ATCC 43290				0.097
A5IFW4	2-oxoglutarate ferredoxin oxidoreductase alpha subunit OS=*Legionella pneumophila* (strain Corby)	catalytic activity, oxidoreductase activity		metabolic process	0.102
G8UY35	2-methylcitrate dehydratase PrpD OS=*Legionella pneumophila* subsp. pneumophila ATCC 43290	catalytic activity, 2 iron, 2 sulfur cluster binding, 2-methylcitrate dehydratase activity		metabolic process, propionate catabolic process, 2-methylcitrate cycle	0.102
A5IC49	Long chain fatty acid-CoA ligase OS=*Legionella pneumophila* (strain Corby)	catalytic activity		metabolic process	0.112
E1XVB9	ATP-dependent Clp protease ATP-binding subunit ClpA OS=*Legionella pneumophila*	catalytic activity; nucleotide binding		metabolic process	0.105
A5IAS2	GTP-dependent nucleic acid-binding protein OS=*Legionella pneumophila* (strain Corby)	nucleotide binding; transporter activity	membrane	transport	0.106
E1Y1U2	Malate oxidoreductase OS=*Legionella pneumophila*	catalytic activity; metal ion binding; nucleotide binding		metabolic process	0.112
I7HKT8	Saccharopine dehydrogenase OS=*Legionella pneumophila* subsp. pneumophila	nucleotide binding, oxidoreductase activity			0.112
G8UT80	Hydrolase, isochorismatase family OS=*Legionella pneumophila* subsp. pneumophila ATCC 43290	catalytic activity		metabolic process	0.113
I7HRI6	HU, DNA-binding transcriptional regulator, beta subunit OS=*Legionella pneumophila* subsp. pneumophila	DNA binding		chromosome condensation	0.113
A5IC33	Poly(A) polymerase (PAP) (Plasmid copy number protein) OS=*Legionella pneumophila* (strain Corby)	catalytic activity; nucleotide binding; RNA binding		metabolic process	0.113
A5IC39	Histidine--tRNA ligase OS=*Legionella pneumophila* (strain Corby)	catalytic activity; nucleotide binding	cytoplasm	metabolic process	0.114
D5T5Y5	Guanosine-3',5'-bis(Diphosphate) 3'-pyrophosphohydrolase OS=*Legionella pneumophila* serogroup 1 (strain 2300/99 Alcoy)	catalytic activity; metal ion binding	cytoplasm; endoplasmic reticulum; membrane	metabolic process	0.237

Accession	Description	Molecular Function	Cellular Component	Biological Process	fold change MIF-VBNCCs/ culturable MIFs
E1XWM2	Site-determining protein OS=*Legionella pneumophila*	catalytic activity; nucleotide binding		metabolic process	0.117
I7HXZ8	DNA-binding response regulator in two-component regulatory system with CpxA OS=*Legionella pneumophila* subsp. pneumophila	DNA binding, phosphorelay response regulator activity		intracellular signal transduction, regulation of transcription	0.117
A5ID71	6-phosphofructokinase OS=*Legionella pneumophila* (strain Corby)	catalytic activity	cytoplasm; cytosol	metabolic process	0.119
E1Y2B5	Putative uncharacterized protein OS=*Legionella pneumophila*	catalytic activity		metabolic process	0.119
G8URN4	Putative uncharacterized protein OS=*Legionella pneumophila* subsp. pneumophila ATCC 43290				0.119
E1XWQ1	Putative uncharacterized protein OS=*Legionella pneumophila*	catalytic activity		metabolic process	0.120
D5T6B3	Cytosolic IMP-GMP specific 5'-nucleotidase OS=*Legionella pneumophila* serogroup 1 (strain 2300/99 Alcoy)	metal ion binding	membrane		0.121
A5IHS6	Transcription antitermination protein nusG OS=*Legionella pneumophila* (strain Corby)			cell organization and biogenesis; metabolic process; regulation of biological process	0.124
A5IH98	Carboxy-terminal protease OS=*Legionella pneumophila* (strain Corby)	catalytic activity; protein binding		metabolic process	0.124
A5IIF3	Cysteine synthase OS=*Legionella pneumophila* (strain Corby)	catalytic activity		metabolic process	0.128
A5IDU5	Sid related protein-like protein OS=*Legionella pneumophila* (strain Corby)				0.129
G8USD0	Putative uncharacterized protein OS=*Legionella pneumophila* subsp. pneumophila ATCC 43290	catalytic activity		metabolic process	0.131
A5IGV6	Nitrogen fixation protein (Fe-S cluster formation) NifU OS=*Legionella pneumophila* (strain Corby)	metal ion binding		cell organization and biogenesis	0.133
Q5X6H1	Shikimate kinase OS=*Legionella pneumophila* (strain Paris)	catalytic activity; metal ion binding; nucleotide binding	cytoplasm	metabolic process	0.136
E1Y3P2	Lipoprotein DotD OS=*Legionella pneumophila*	Component of the Dot/Icm secretion system. Lipoprotein			0.146
A5IFW5	Pyridoxine 5'-phosphate synthase OS=*Legionella pneumophila* (strain Corby)	catalytic activity	cytoplasm	metabolic process	0.148
E1XY92	Methionine--tRNA ligase OS=*Legionella pneumophila*	catalytic activity; metal ion binding; nucleotide binding; RNA binding	cytoplasm	metabolic process	0.149
D5TEC5	Membrane bound lytic murein transglycosylase OS=*Legionella pneumophila* serogroup 1 (strain 2300/99 Alcoy)	membrane bound lytic murein transglycosylase	membrane		0.153
E1Y2H1	Putative uncharacterized protein OS=*Legionella pneumophila*	catalytic activity		metabolic process	0.153

Accession	Description	Molecular Function	Cellular Component	Biological Process	fold change MIF-VBNCCs/ culturable MIFs
A5IAM4	Sugar kinase OS=*Legionella pneumophila* (strain Corby)	catalytic activity		metabolic process	0.154
A5I9J4	Putative uncharacterized protein OS=*Legionella pneumophila* (strain Corby)				0.155
G8UY86	Putative uncharacterized protein OS=*Legionella pneumophila* subsp. pneumophila ATCC 43290	catalytic activity		metabolic process	0.158
I7I555	Serine endoprotease (Protease Do), membrane-associated OS=*Legionella pneumophila* subsp. Pneumophila	Hydrolase, Protease		Proteolysis	0.163
E1Y252	3-oxoacyl-[acyl-carrier-protein] synthase 2 OS=*Legionella pneumophila*	catalytic activity, Acyltransferase, Transferase		metabolic process, fatty acid biosynthetic process	0.165
E1Y2L0	Putative uncharacterized protein OS=*Legionella pneumophila*	catalytic activity		metabolic process	0.170
E1XWQ0	Putative uncharacterized protein OS=*Legionella pneumophila*		membrane		0.171
A5IFZ1	Electron transfer flavoprotein, beta subunit OS=*Legionella pneumophila* (strain Corby)	electron carrier activity			0.174
A5ICM9	Site-determining protein OS=*Legionella pneumophila* (strain Corby)	catalytic activity; nucleotide binding		cell division; cell organization and biogenesis; metabolic process	0.175
A5IEB1	Putative uncharacterized protein OS=*Legionella pneumophila* (strain Corby)				0.175
A5ICH5	Phosphoribosylformylglycinamidine cyclo-ligase OS=*Legionella pneumophila* (strain Corby)	catalytic activity; nucleotide binding	cytoplasm	metabolic process	0.176
A5II14	Inosine 5'-monophosphate dehydrogenase OS=*Legionella pneumophila* (strain Corby)	catalytic activity		metabolic process	0.180
A5IG02	Type III pantothenate kinase OS=*Legionella pneumophila* (strain Corby)	catalytic activity; metal ion binding; nucleotide binding	cytoplasm	metabolic process; regulation of biological process	0.183
A5IBC9	Electron transferring flavoprotein dehydrogenase OS=*Legionella pneumophila* (strain Corby)	catalytic activity		metabolic process	0.184
E1XYJ1	Acetylornithine aminotransferase OS=*Legionella pneumophila*	catalytic activity		metabolic process	0.186
D5T616	Enolase OS=*Legionella pneumophila* serogroup 1 (strain 2300/99 Alcoy)	catalytic activity; metal ion binding	cell surface; cytoplasm; cytosol; extracellular; membrane	metabolic process	0.191
I7I833	Peptidyl-prolyl cis-trans isomerase OS=*Legionella pneumophila* subsp. pneumophila	peptidyl-prolyl cis-trans isomerase activity		protein folding, protein peptidyl-prolyl isomerization	0.194
A5II93	Probable ubiquinone biosynthesis protein UbiB OS=*Legionella pneumophila* (strain Corby)	catalytic activity	membrane	metabolic process	0.196
A5ICC3	Putative secreted protein OS=*Legionella pneumophila* (strain Corby)		membrane		0.197

Accession	Description	Molecular Function	Cellular Component	Biological Process	fold change MIF-VBNCCs/ culturable MIFs
Q5X153	Glucosamine--fructose-6-phosphate aminotransferase [isomerizing] OS=*Legionella pneumophila* (strain Paris)	catalytic activity	cytoplasm	metabolic process	0.198
A5IET5	Structural toxin protein (Hemagglutinin/hemolysin) RtxA OS=*Legionella pneumophila* (strain Corby)				0.200
E1XVI8	Putative uncharacterized protein OS=*Legionella pneumophila*	catalytic activity		metabolic process	0.201
A5IHW9	ATP-dependent zinc metalloprotease FtsH OS=*Legionella pneumophila* (strain Corby)	catalytic activity; metal ion binding; nucleotide binding	membrane	cell communication; cell division; metabolic process; regulation of biological process; response to stimulus	0.212
A5IHA9	Adenylosuccinate synthetase OS=*Legionella pneumophila* (strain Corby)	catalytic activity; metal ion binding; nucleotide binding	cytoplasm	metabolic process	0.215
A5IC55	Dihydrolipoamide acetyltransferase OS=*Legionella pneumophila* (strain Corby)	catalytic activity	cytoplasm	metabolic process	0.221
A5IBQ3	Adenylate kinase OS=*Legionella pneumophila* (strain Corby)	catalytic activity; nucleotide binding	cytoplasm	metabolic process	0.224
A5IGK7	L-threonine 3-dehydrogenase OS=*Legionella pneumophila* (strain Corby)	catalytic activity; metal ion binding; nucleotide binding	cytoplasm	metabolic process	0.228
A5II05	Transcription accessory protein OS=*Legionella pneumophila* (strain Corby)	catalytic activity; RNA binding		metabolic process	0.231
D5TEA2	Lipoprotein VacJ-like protein OS=*Legionella pneumophila* serogroup 1 (strain 2300/99 Alcoy)		membrane		0.234
E1XZI1	DNA gyrase subunit B OS=*Legionella pneumophila*	catalytic activity; DNA binding; nucleotide binding	chromosome; cytoplasm	metabolic process	0.234
G8UW51	Putative uncharacterized protein OS=*Legionella pneumophila* subsp. pneumophila ATCC 43290		membrane		0.237
I7HWP9	UDP-N-acetylmuramate:L-alanine ligase OS=*Legionella pneumophila* subsp. pneumophila	ATP binding, UDP-N-acetylmuramate-L-alanine ligase activity	cytoplasm	Cell cycle,Cell division, Cell shape, Cell wall biogenesis/degradation, Peptidoglycan synthesis	0.091
A5IBJ1	Leucine--tRNA ligase OS=*Legionella pneumophila* (strain Corby)	catalytic activity; nucleotide binding	cytoplasm	metabolic process; regulation of biological process	0.239
G8UT94	Cytochrome D ubiquinol oxidase, subunit I OS=*Legionella pneumophila* subsp. pneumophila ATCC 43290		membrane	Energy Metabolism	0.244
I7HYU2	Uncharacterized protein OS=*Legionella pneumophila* subsp. pneumophila				0.248
G8UYH6	(Beta)-carbonic anhydrase OS=*Legionella pneumophila* subsp. pneumophila ATCC 43290	catalytic activity; metal ion binding، carbonate dehydratase activity، zinc ion binding		Energy Metabolism	0.248
A5IGB3	Adenylosuccinate lyase OS=*Legionella pneumophila* (strain Corby)	catalytic activity		metabolic process	0.249

Accession	Description	Molecular Function	Cellular Component	Biological Process	fold change MIF-VBNCCs/ culturable MIFs
I7I5I2	Glutamyl-tRNA synthetase OS=*Legionella pneumophila* subsp. pneumophila	ATP binding, glutamate-tRNA ligase activity	cytoplasm	Protein biosynthesis	0.254
G8USE2	Putative uncharacterized protein OS=*Legionella pneumophila* subsp. pneumophila ATCC 43290				0.257
A5I9U7	3-hydroxyisobutyrate dehydrogenase OS=*Legionella pneumophila* (strain Corby)	catalytic activity; nucleotide binding		metabolic process	0.259
G8UTK1	Enoyl CoA hydratase OS=*Legionella pneumophila* subsp. pneumophila ATCC 43290	catalytic activity; nucleotide binding		metabolic process	0.261
A5ICK8	Uridylate kinase OS=*Legionella pneumophila* (strain Corby)	catalytic activity; nucleotide binding	cytoplasm	metabolic process	0.265
G8UU91	PhoH protein (Phosphate starvation inducible protein) OS=*Legionella pneumophila* subsp. pneumophila ATCC 43290	nucleotide binding			0.274
A5IE89	ABC transporter, ATP binding protein OS=*Legionella pneumophila* (strain Corby)	catalytic activity; nucleotide binding; transporter activity	membrane	metabolic process; transport	0.277
A5IDL4	Arginine--tRNA ligase OS=*Legionella pneumophila* (strain Corby)	catalytic activity; nucleotide binding	cytoplasm	metabolic process	0.280
Q5ZVY4	Tryptophan synthase beta chain OS=*Legionella pneumophila* subsp. pneumophila (strain Philadelphia 1 / ATCC 33152 / DSM 7513)	catalytic activity		metabolic process	0.283
A5IGY5	Glutathione reductase OS=*Legionella pneumophila* (strain Corby)	antioxidant activity; catalytic activity; nucleotide binding	cytoplasm	cellular homeostasis; metabolic process; regulation of biological process	0.284
A5IAS9	Phenylalanine-4-hydroxylase OS=*Legionella pneumophila* (strain Corby)	catalytic activity; metal ion binding		metabolic process	0.285
I7HS47	Inorganic pyrophosphatase OS=*Legionella pneumophila* subsp. pneumophila	inorganic diphosphatase activity, magnesium ion binding	cytoplasm	phosphate-containing compound metabolic process	0.286
A5IIB3	Lytic murein transglycosylase OS=*Legionella pneumophila* (strain Corby)	lytic murein transglycosylase			0.288
E1XWK6	Thioredoxin reductase OS=*Legionella pneumophila* GN=LPW_17941 PE=3 SV=1 - [E1XWK6_LEGPN]	antioxidant activity; catalytic activity	cytoplasm	metabolic process; response to stimulus	0.288
A5ICT2	Lysine--tRNA ligase OS=*Legionella pneumophila* (strain Corby)	catalytic activity; metal ion binding; nucleotide binding	cytoplasm	metabolic process	0.288
I7HS12	Peptide deformylase OS=*Legionella pneumophila* subsp. pneumophila	iron ion binding, peptide deformylase activity, Hydrolase		Protein biosynthesis	0.290
A5IHN7	Glucose-1-dehydrogenase OS=*Legionella pneumophila* (strain Corby)	catalytic activity; nucleotide binding		metabolic process	0.290
E1Y273	DNA gyrase subunit A OS=*Legionella pneumophila*	catalytic activity; DNA binding; nucleotide binding	chromosome; cytoplasm	metabolic process	0.298
A5IGA9	Phosphoenolpyruvate synthase OS=*Legionella pneumophila* (strain Corby)	catalytic activity; nucleotide binding		metabolic process	0.299

Accession	Description	Molecular Function	Cellular Component	Biological Process	fold change MIF-VBNCCs/ culturable MIFs
G8UW46	Transcription termination factor NusB OS=*Legionella pneumophila* subsp. pneumophila ATCC 43290	RNA binding		metabolic process; regulation of biological process	0.300
I7I586	Aspartyl-tRNA synthetase OS=*Legionella pneumophila* subsp. pneumophila	ATP binding, aspartate-tRNA ligase activity, nucleic acid binding	cytoplasm	Protein biosynthesis	0.301
Q5WYQ9	ATP-dependent protease subunit HslV OS=*Legionella pneumophila* (strain Lens)	catalytic activity; metal ion binding	cytoplasm; proteasome	metabolic process; response to stimulus	0.303
A5I9U8	Methylmalonate-semialdehyde dehydrogenase OS=*Legionella pneumophila* (strain Corby)	catalytic activity		metabolic process	0.303
E1Y2B9	Protein translocase subunit SecA OS=*Legionella pneumophila*	catalytic activity; metal ion binding; nucleotide binding	cytoplasm; membrane	transport	0.305
G8UUB9	Phosphoenolpyruvate protein phosphotransferase PtsP OS=*Legionella pneumophila* subsp. pneumophila ATCC 43290	catalytic activity; metal ion binding; protein binding; transporter activity	cytoplasm; membrane	metabolic process; transport	0.309
Q5ZUS0	GMP synthase [glutamine-hydrolyzing] OS=*Legionella pneumophila* subsp. pneumophila (strain Philadelphia 1 / ATCC 33152 / DSM 7513)	catalytic activity; nucleotide binding	cytoplasm	metabolic process	0.313
A5IDK4	Adenosylhomocysteinase OS=*Legionella pneumophila* (strain Corby)	catalytic activity	cytoplasm	metabolic process; transport	0.314
D5T9B1	UDP-N-acetylmuramoyl-tripeptide--D-alanyl-D-alanine ligase OS=*Legionella pneumophila* serogroup 1 (strain 2300/99 Alcoy)	catalytic activity; nucleotide binding	cytoplasm	cell division; cell organization and biogenesis; metabolic process; regulation of biological process	0.318
I7HRM3	S-adenosylmethionine synthase OS=*Legionella pneumophila* subsp. pneumophila	ATP binding, magnesium ion binding, methionine adenosyltransferase activity	cytoplasm	S-adenosylmethionine biosynthetic process, methanogenesis, one-carbon metabolic process	0.315
G8UVX1	Putative uncharacterized protein OS=*Legionella pneumophila* subsp. pneumophila ATCC 43290				0.317
E1XZ45	Putative uncharacterized protein OS=*Legionella pneumophila*	catalytic activity; metal ion binding; nucleotide binding		metabolic process	0.321
A5IGK8	2-amino-3-ketobutyrate coenzyme A ligase OS=*Legionella pneumophila* (strain Corby)	catalytic activity, glycine C-acetyltransferase activity, ligase activity, pyridoxal phosphate binding		metabolic process, biosynthetic process	0.322
G8USN9	Signal recognition particle protein OS=*Legionella pneumophila* subsp. pneumophila ATCC 43290	catalytic activity; nucleotide binding; RNA binding	cytoplasm	transport	0.323
E1Y247	3-oxoacyl-[acyl-carrier-protein] synthase 3 OS=*Legionella pneumophila*	catalytic activity, Acyltransferase, Transferase	cytoplasm; membrane	metabolic process, fatty acid biosynthetic process	0.323
J7FLS8	Major outer membrane protein (Fragment) OS=*Legionella pneumophila*				0.324
E1XZ79	Putative uncharacterized protein OS=*Legionella pneumophila*	catalytic activity		metabolic process	0.325

Accession	Description	Molecular Function	Cellular Component	Biological Process	fold change MIF-VBNCCs/ culturable MIFs
A5IAK9	Threonine--tRNA ligase OS=*Legionella pneumophila* (strain Corby)	catalytic activity; metal ion binding; nucleotide binding	cytoplasm	metabolic process	0.326
E1XWR4	Putative uncharacterized protein OS=*Legionella pneumophila*	catalytic activity		metabolic process	0.326
A5IF17	NADPH-dependent FMN reductase domain protein OS=*Legionella pneumophila* (strain Corby)				0.328
A5IAW9	Metallopeptidase PepO, peptidase, M13 family OS=*Legionella pneumophila* (strain Corby)	catalytic activity		metabolic process	0.329
Q0ZHF4	TrpS (Fragment) OS=*Legionella pneumophila*	catalytic activity; nucleotide binding	cytoplasm	metabolic process	0.330
I7I374	Uncharacterized protein OS=*Legionella pneumophila* subsp. pneumophila	Ligase, propionyl-CoA carboxylase activity			0.340
Q5ZVS1	Chaperone protein HtpG OS=*Legionella pneumophila* subsp. pneumophila (strain Philadelphia 1 / ATCC 33152 / DSM 7513)	nucleotide binding; protein binding	cytoplasm	metabolic process; response to stimulus	0.354
A5IGL5	Proline--tRNA ligase OS=*Legionella pneumophila* (strain Corby)	catalytic activity; nucleotide binding	cytoplasm	metabolic process	0.356
I7HR12	Putative Enhanced entry protein EnhA OS=*Legionella pneumophila* subsp. pneumophila	transferase activity			0.356
Q5ZWZ6	Isoleucine--tRNA ligase OS=*Legionella pneumophila* subsp. pneumophila (strain Philadelphia 1 / ATCC 33152 / DSM 7513)	catalytic activity; metal ion binding; nucleotide binding	cytoplasm	metabolic process; regulation of biological process	0.357
E1XZW2	Putative uncharacterized protein OS=*Legionella pneumophila*	catalytic activity; metal ion binding		metabolic process	0.368
A5IC95	Thiolase OS=*Legionella pneumophila* (strain Corby)	catalytic activity		metabolic process	0.369
A5I9Q3	Putative uncharacterized protein OS=*Legionella pneumophila* (strain Corby)	catalytic activity		metabolic process	0.371
A5IHD2	Acetyl CoA carboxylase, biotin carboxylase subunit OS=*Legionella pneumophila* (strain Corby)	catalytic activity; metal ion binding; nucleotide binding		metabolic process	0.379
E1Y208	Putative uncharacterized protein OS=*Legionella pneumophila*	catalytic activity		metabolic process	0.384
I7I3V0	Serine-protein kinase, PrkA type OS=*Legionella pneumophila* subsp. pneumophila	protein kinase activity		protein phosphorylation	0.391
Q5X6N0	Peptide chain release factor 3 OS=*Legionella pneumophila* (strain Paris)	catalytic activity; nucleotide binding; RNA binding	cytoplasm	cell organization and biogenesis; metabolic process	0.392
A5IGY8	Tyrosine--tRNA ligase OS=*Legionella pneumophila* (strain Corby)	catalytic activity; nucleotide binding; RNA binding	cytoplasm	metabolic process	0.393
A5III4	ATP synthase gamma chain OS=*Legionella pneumophila* (strain Corby)	catalytic activity; transporter activity	membrane	metabolic process; transport	0.394
I7I670	Thioredoxin OS=*Legionella pneumophila* subsp. pneumophila	electron carrier activity, protein disulfide oxidoreductase activity		cell redox homeostasis, glycerol ether metabolic process	0.396

Accession	Description	Molecular Function	Cellular Component	Biological Process	fold change MIF-VBNCCs/ culturable MIFs
E1XZW4	Oligopeptidase A OS=*Legionella pneumophila*	catalytic activity		metabolic process	0.400
Q5ZV92	Nucleoside diphosphate kinase OS=*Legionella pneumophila* subsp. pneumophila (strain Philadelphia 1 / ATCC 33152 / DSM 7513)	catalytic activity; metal ion binding; nucleotide binding	cytoplasm	metabolic process	0.402
Q5ZRC3	UPF0422 protein lpg2959 OS=*Legionella pneumophila* subsp. pneumophila (strain Philadelphia 1 / ATCC 33152 / DSM 7513)				0.402
A5ICY6	Acyl CoA carboxylase subunit alpha subunit OS=*Legionella pneumophila* (strain Corby)	catalytic activity; metal ion binding; nucleotide binding		metabolic process	0.403
Q6QG85	Aspartate-semialdehyde dehydrogenase (Fragment) OS=*Legionella pneumophila*	catalytic activity; nucleotide binding; protein binding	cytoplasm	metabolic process	0.406
I7HWA2	SecYEG protein translocase auxillary subunit OS=*Legionella pneumophila* subsp. pneumophila	Transport and binding proteins		Transport and binding proteins	0.406
I7HKU1	Glutamine synthetase OS=*Legionella pneumophila* subsp. pneumophila	Ligase	cytoplasm	glutamine biosynthetic process, nitrogen fixation	0.412
E1Y2K9	Putative uncharacterized protein OS=*Legionella pneumophila*	catalytic activity		metabolic process	0.416
A5IG89	Agglutination protein OS=*Legionella pneumophila* (strain Corby)	transporter activity	membrane	transport	0.420
B4XLN3	NADP(+)-dependent malate dehydrogenase OS=*Legionella pneumophila*	catalytic activity; metal ion binding; nucleotide binding		metabolic process	0.421
G8UVS7	Cytochrome c OS=*Legionella pneumophila* subsp. pneumophila ATCC 43290	metal ion binding, heme binding, iron ion binding, electron carrier activity			0.422
Q5ZVZ6	DNA-binding response regulator OS=*Legionella pneumophila* subsp. pneumophila (strain Philadelphia 1 / ATCC 33152 / DSM 7513)	DNA binding; signal transducer activity		cell communication; metabolic process; regulation of biological process; response to stimulus, Two-component regulatory system, regulation of transcription	0.428
A5I9R0	Ribonuclease R OS=*Legionella pneumophila* (strain Corby)	catalytic activity; RNA binding	cytoplasm	metabolic process‹ RNA metabolic process	0.429
I7HSC7	Putative ABC-type Taurine transport system, ATPase component OS=*Legionella pneumophila* subsp. pneumophila	ATP binding		ATP catabolic process‹ putative transporter	0.430
G8UR61	Alpha helix protein OS=*Legionella pneumophila* subsp. pneumophila ATCC 43290				0.431
A5I9V7	Phosphoglycerate kinase OS=*Legionella pneumophila* (strain Corby)	catalytic activity; nucleotide binding, ATP binding‹ Kinase‹ transferase	cytoplasm	metabolic process‹ glycolysis	0.436
E1Y108	Putative uncharacterized protein OS=*Legionella pneumophila*	catalytic activity		metabolic process	0.437
A5IE95	Ribonuclease E OS=*Legionella pneumophila* (strain Corby)	catalytic activity; RNA binding, ribonuclease activity		metabolic process, RNA processing	0.438

Accession	Description	Molecular Function	Cellular Component	Biological Process	fold change MIF-VBNCCs/ culturable MIFs
Q5WT83	Translation initiation factor IF-3 OS=*Legionella pneumophila* (strain Lens)	RNA binding, translation initiation factor activity	cytoplasm	metabolic process, Protein biosynthesis	0.439
I7I2W4	Acyl carrier protein OS=*Legionella pneumophila* subsp. pneumophila		cytoplasm	Fatty acid biosynthesis	0.442
A5ICV6	Alanine--tRNA ligase OS=*Legionella pneumophila* (strain Corby)	catalytic activity; metal ion binding; nucleotide binding; RNA binding, ATP binding	cytoplasm	metabolic process, Protein biosynthesis	0.446
Q5ZXS3	HlyD family secretion protein OS=*Legionella pneumophila* subsp. pneumophila (strain Philadelphia 1 / ATCC 33152 / DSM 7513)	Protein fate / hydrolases / secretion	membrane	transport, transmembrane transport	0.453
A5IHC4	Phenol hydroxylase OS=*Legionella pneumophila* (strain Corby)	catalytic activity, Oxidoreductase		metabolic process	0.458
A5ICK9	Elongation factor Ts OS=*Legionella pneumophila* (strain Corby)	protein binding; RNA binding, Elongation factor	cytoplasm	metabolic process, Protein biosynthesis	0.459
G8UR93	Carboxyphosphoenolpyruvate phosphonomutase OS=*Legionella pneumophila* subsp. pneumophila ATCC 43290	catalytic activity		metabolic process, propionate catabolic process, Carbohydrate Metabolism	0.459
E1XZB3	Putative uncharacterized protein OS=*Legionella pneumophila*				0.462
A5I9P2	Aspartate aminotransferase A OS=*Legionella pneumophila* (strain Corby)	catalytic activity, Aminotransferase		metabolic process, biosynthetic process	0.463
G8UUP8	LidA OS=*Legionella pneumophila* subsp. pneumophila ATCC 43290	nucleotide binding; protein binding, Toxin production			0.463
G8UYC0	Lon protease OS=*Legionella pneumophila* subsp. pneumophila ATCC 43290	catalytic activity; nucleotide binding, ATP binding	cytoplasm	metabolic process, proteolysis	0.464
E1XXY0	Translation initiation factor IF-2 OS=*Legionella pneumophila*	catalytic activity; nucleotide binding; RNA binding, GTP binding, translation initiation factor activity	cytoplasm	metabolic process, Protein biosynthesis, GTP catabolic process	0.468
Q9AGM8	Type II protein secretion LspD (Fragment) OS=*Legionella pneumophila*		membrane	transport, protein secretion	0.470
A5ID12	Enoyl-[acyl-carrier-protein] reductase [NADH] OS=*Legionella pneumophila* (strain Corby)	catalytic activity; nucleotide binding, oxidoreductase		metabolic process, fatty acid biosynthetic process	0.472
G8UXN7	Putative uncharacterized protein OS=*Legionella pneumophila* subsp. pneumophila ATCC 43290				0.473
A5IH90	Outer membrane protein assembly factor BamA OS=*Legionella pneumophila* (strain Corby)		membrane	protein insertion into membrane	0.475
Q5X763	10 kDa chaperonin OS=*Legionella pneumophila* (strain Paris)	nucleotide binding‹ Chaperone, ATP binding	cytoplasm	metabolic process; response to stimulus‹ protein folding‹ response to stress	0.476
E1XZ92	Putative uncharacterized protein OS=*Legionella pneumophila*	catalytic activity		metabolic process	0.480

Accession	Description	Molecular Function	Cellular Component	Biological Process	fold change MIF-VBNCCs/ culturable MIFs
A5IGC4	Acetyl-coenzyme A carboxylase carboxyl transferase subunit alpha OS=*Legionella pneumophila* (strain Corby)	catalytic activity; nucleotide binding, ATP binding	cytoplasm	metabolic process, fatty acid biosynthetic process	0.480
A5IEE9	2,4-dienoyl-CoA reductase FadH1 OS=*Legionella pneumophila* (strain Corby)	catalytic activity; nucleotide binding, FMN binding, oxidoreductase activity		metabolic process	0.486
Q5X1N9	50S ribosomal protein L21 OS=*Legionella pneumophila* (strain Paris)	RNA binding; structural molecule activity	cytoplasm; ribosome	metabolic process, translation	0.486
A5ICY5	Enoyl CoA hydratase/isomerase (Crotonase) OS=*Legionella pneumophila* (strain Corby)	catalytic activity, isomerase		metabolic process	0.496
I7I4T8	Putative oxidoreductase, Zn-dependent and NAD(P)-binding OS=*Legionella pneumophila* subsp. pneumophila				0.497
B8R5J1	60 kDa chaperonin 7 OS=*Legionella pneumophila*	nucleotide binding; protein binding, ATP binding, chaperone	cytoplasm	metabolic process; response to stimulus, protein refolding, response to stress	0.497
E1Y0Z9	Bifunctional purine biosynthesis protein PurH OS=*Legionella pneumophila*	catalytic activity, transferase, hydrolase		metabolic process, Purine biosynthesis	0.498
Q5X4X1	30S ribosomal protein S18 OS=*Legionella pneumophila* (strain Paris)	RNA binding; structural molecule activity	cytoplasm; ribosome	metabolic process, translation	0.499

APPENDIX III

Proteins with unchanged level in MIF-VBNCCs as compared to the culturable MIFs according to shotgun proteomic screen

Accession	Description	Molecular Function	Cellular Component	Biological Process	fold change MIF-VBNCCs/ culturable MIFs
A5IHZ6	Virulence regulator BipA OS=*Legionella pneumophila* (strain Corby)	catalytic activity; nucleotide binding, GTP binding		metabolic process, GTP catabolic process	0.623
D5TA86	Valine--tRNA ligase OS=*Legionella pneumophila* serogroup 1 (strain 2300/99 Alcoy)	catalytic activity; nucleotide binding, ATP binding, Aminoacyl-tRNA synthetase, Ligase	cytoplasm; membrane	metabolic process; regulation of biological process, Protein biosynthesis, regulation of translational fidelity, valyl-tRNA aminoacylation	0.732
G8URY6	Uroporphyrinogen III methylase OS=*Legionella pneumophila* subsp. pneumophila ATCC 43290	catalytic activity, Methyltransferase, Transferase		metabolic process, protein folding, Metabolism of Cofactors and Vitamins	0.849
A5IE48	Uracil phosphoribosyltransferase OS=*Legionella pneumophila* (strain Corby)	catalytic activity; nucleotide binding, GTP-binding, Glycosyltransferase, Transferase		metabolic process	0.768
A5IFX7	Universal stress protein A (UspA) OS=*Legionella pneumophila* (strain Corby)			response to stimulus, stress response	1.724
I7I0Y8	Uncharacterized protein OS=*Legionella pneumophila* subsp. pneumophila				0.815
I7HN29	Uncharacterized protein OS=*Legionella pneumophila* subsp. pneumophila				0.821
I7I578	Uncharacterized protein OS=*Legionella pneumophila* subsp. pneumophila				1.119
E1XWV4	Trigger factor OS=*Legionella pneumophila*	catalytic activity, peptidyl-prolyl cis-trans isomerase activity, chaperone	cytoplasm	cell division; metabolic process; transport, cell cycle, protein folding, protein transport	0.509
I7I1C1	Transporter OS=*Legionella pneumophila* subsp. pneumophila	ATP binding, nucleoside-triphosphatase activity		transport	0.574
G8UTE7	Transmembrane Tfp pilus assembly protein FimV (Twitching motility) OS=*Legionella pneumophila* subsp. pneumophila ATCC 43290		membrane	Chemotaxis / twitching motility / cell division, Metabolism of Complex Lipids	1.205
I7HMA6	Transcription termination factor OS=*Legionella pneumophila* subsp. pneumophila	ATP binding, RNA binding, RNA-dependent ATPase activity, helicase activity		Transcription, Transcription regulation, Transcription termination	0.663
A5IE25	TPR repeat protein, protein-protein interaction OS=*Legionella pneumophila* (strain Corby)	protein binding			0.865
G8UXT8	Thermostable carboxypeptidase 1 OS=*Legionella pneumophila* subsp. pneumophila ATCC 43290	catalytic activity,Carboxypeptidase, Hydrolase, Protease		metabolic process, proteolysis	1.050
I7HS93	Superoxide dismutase OS=*Legionella pneumophila* subsp. pneumophila	metal ion binding, superoxide dismutase activity, Oxidoreductase		Detoxification, Adaptations to atypical conditions	0.700

Accession	Description	Molecular Function	Cellular Component	Biological Process	fold change MIF-VBNCCs/ culturable MIFs
G8USU3	Succinyl-CoA ligase [ADP-forming] subunit beta OS=*Legionella pneumophila* subsp. pneumophila ATCC 43290	catalytic activity; metal ion binding; nucleotide binding, ATP binding, magnesium ion binding, manganese ion binding, ligase activity		metabolic process,tricarboxylic acid cycle	1.235
G8USU4	Succinyl-CoA ligase [ADP-forming] subunit alpha OS=*Legionella pneumophila* subsp. pneumophila ATCC 43290	catalytic activity; nucleotide binding, ATP binding, cofactor binding		metabolic process, Carbohydrate Metabolism, Energy Metabolism	0.710
G8USU0	Succinate dehydrogenase iron-sulfur protein subunit B OS=*Legionella pneumophila* subsp. pneumophila ATCC 43290	catalytic activity, electron carrier activity, iron-sulfur cluster binding, oxidoreductase activity		metabolic process, tricarboxylic acid cycle	0.635
G8UST9	Succinate dehydrogenase flavoprotein subunit A OS=*Legionella pneumophila* subsp. pneumophila ATCC 43290	catalytic activity		metabolic process, tricarboxylic acid cycle, electron transport chain	0.552
D5T7X6	Small subunit ribosomal protein S11 OS=*Legionella pneumophila* serogroup 1 (strain 2300/99 Alcoy)	RNA binding; structural molecule activity	cytoplasm; ribosome	metabolic process, translation	0.618
A5IG71	Signal peptide protein, toluene tolerance protein Ttg2D OS=*Legionella pneumophila* (strain Corby)				0.904
A5IGI2	Serine hydroxymethyltransferase OS=*Legionella pneumophila* (strain Corby)	catalytic activity‹ Pyridoxal phosphate‹ Transferase	cytoplasm	metabolic process‹ Amino-acid biosynthesis‹ One-carbon metabolism	0.782
I7I3B0	SecYEG protein translocase auxillary subunit OS=*Legionella pneumophila* subsp. pneumophila	P-P-bond-hydrolysis-driven protein transmembrane transporter activity	cytoplasm; membrane	intracellular protein transmembrane transport, protein targeting, protein transport by the Sec complex	1.004
G8UX56	SdbC OS=*Legionella pneumophila* subsp. pneumophila ATCC 43290				0.920
Q48805	SbpA OS=*Legionella pneumophila*	catalytic activity			0.919
A5IHB8	RNA polymerase sigma-54 factor OS=*Legionella pneumophila* (strain Corby)	catalytic activity; DNA binding, DNA-directed RNA polymerase activity, sigma factor activity, Transferase		metabolic process; regulation of biological process, Transcription, Transcription regulation	0.827
A5IEG4	RNA polymerase sigma factor OS=*Legionella pneumophila* (strain Corby)	DNA binding, sigma factor activity		metabolic process; regulation of biological process, Transcription, Transcription regulation	0.565
A5ICK7	Ribosome-recycling factor OS=*Legionella pneumophila* (strain Corby)		cytoplasm	metabolic process	0.889
I7I2E1	Ribosome-associated, sigma 54 modulation protein OS=*Legionella pneumophila* subsp. pneumophila			Transcription	1.227
A5IFZ9	Ribosomal RNA small subunit methyltransferase H OS=*Legionella pneumophila* (strain Corby)	catalytic activity, Methyltransferase	cytoplasm	metabolic process, rRNA processing	0.667
G8USL7	Ribosomal protein S6 modification protein OS=*Legionella pneumophila* subsp. pneumophila ATCC 43290	catalytic activity; metal ion binding; nucleotide binding, ATP binding		metabolic process, Amino Acid Metabolism, Translation	0.709

Accession	Description	Molecular Function	Cellular Component	Biological Process	fold change MIF-VBNCCs/ culturable MIFs
Q5X7X4	Ribosomal protein S6 modification protein OS=*Legionella pneumophila* (strain Paris)	catalytic activity; metal ion binding; nucleotide binding, ATP binding		metabolic process, Protein biosynthesis,cellular protein modification process, translation	1.699
I7HQU1	Ribose-phosphate pyrophosphokinase OS=*Legionella pneumophila* subsp. pneumophila	ATP binding, kinase activity, magnesium ion binding	cytoplasm	nucleoside metabolic process, nucleotide biosynthetic process	0.572
I7I593	Pyruvate dehydrogenase, dihydrolipoyltransacetylase component E2 OS=*Legionella pneumophila* subsp. pneumophila	Acyltransferase, Transferase		glycolysis	1.029
E1Y2F8	Pyruvate dehydrogenase E1 component OS=*Legionella pneumophila*	catalytic activity, Oxidoreductase		metabolic process, Carbohydrate Metabolism, Amino Acid Metabolism	0.806
A5IG37	Pyridine nucleotide transhydrogenase, alpha subunit OS=*Legionella pneumophila* (strain Corby)	catalytic activity		metabolic process, proton transport	0.858
A5IB08	Putative uncharacterized protein paiA OS=*Legionella pneumophila* (strain Corby)	catalytic activity			0.858
G8UXA1	Putative uncharacterized protein OS=*Legionella pneumophila* subsp. pneumophila ATCC 43290				1.546
G8UY57	Putative uncharacterized protein OS=*Legionella pneumophila* subsp. pneumophila ATCC 43290				0.769
G8UTC8	Putative uncharacterized protein OS=*Legionella pneumophila* subsp. pneumophila ATCC 43290				0.520
G8USB7	Putative uncharacterized protein OS=*Legionella pneumophila* subsp. pneumophila ATCC 43290				1.292
E1Y3H6	Putative uncharacterized protein OS=*Legionella pneumophila*			metabolic process	1.790
E1Y348	Putative uncharacterized protein OS=*Legionella pneumophila*	catalytic activity; nucleotide binding		metabolic process	1.013
E1XZ78	Putative uncharacterized protein OS=*Legionella pneumophila*	catalytic activity		metabolic process	0.574
E1XWS6	Putative uncharacterized protein OS=*Legionella pneumophila*				1.127
E1XWI6	Putative uncharacterized protein OS=*Legionella pneumophila*	catalytic activity		metabolic process	0.572
E1Y1A4	Putative uncharacterized protein OS=*Legionella pneumophila*	transporter activity	membrane	transport	0.927
E1Y0J5	Putative uncharacterized protein OS=*Legionella pneumophila*	catalytic activity		metabolic process	0.964
A5IE80	Putative uncharacterized protein OS=*Legionella pneumophila* (strain Corby)				0.728
G8UW56	Putative signal peptide protein OS=*Legionella pneumophila* subsp. pneumophila ATCC 43290				1.297
Q5ZWQ9	Putative outer membrane lipoprotein OS=*Legionella pneumophila* subsp. pneumophila (strain Philadelphia 1 / ATCC 33152 / DSM 7513)		membrane		1.422

Accession	Description	Molecular Function	Cellular Component	Biological Process	fold change MIF-VBNCCs/ culturable MIFs
G8USV0	Putative membrane protein YdgA-like protein OS=*Legionella pneumophila* subsp. pneumophila ATCC 43290		membrane		0.886
A5IDK9	Protein GrpE OS=*Legionella pneumophila* (strain Corby)	enzyme regulator activity; nucleotide binding; protein binding	cytoplasm	metabolic process; response to stimulus, protein folding,response to stress	0.577
I7HLS1	Protein export chaperone OS=*Legionella pneumophila* subsp. pneumophila	Chaperone	cytoplasm	protein folding, protein transport	0.730
A5ICJ2	Proline dehydrogenase/delta-1-pyrroline-5-carboxylate dehydrogenase = bifunctional PutA protein OS=*Legionella pneumophila* (strain Corby)	catalytic activity, Oxidoreductase		metabolic process; regulation of biological process, amino acid biosynthetic pathway	0.749
A5IHU3	Polyribonucleotide nucleotidyltransferase OS=*Legionella pneumophila* (strain Corby)	catalytic activity; RNA binding, Transferase	cytoplasm	metabolic process, RNA processing	0.507
G8UYG8	Polyketide synthase, type I OS=*Legionella pneumophila* subsp. pneumophila ATCC 43290	catalytic activity; nucleotide binding, phosphopantetheine binding, transferase activity		metabolic process	0.872
Q5WW20	Phosphoribosylaminoimidazole-succinocarboxamide synthase OS=*Legionella pneumophila* (strain Lens)	catalytic activity; nucleotide binding, ATP binding		metabolic process, 'de novo' IMP biosynthetic process	0.814
G8UXA5	Phospho-2-dehydro-3-deoxyheptonate aldolase OS=*Legionella pneumophila* subsp. pneumophila ATCC 43290	catalytic activity,Transferase		metabolic process, Amino-acid biosynthesis, chorismate biosynthetic process	0.968
P26493	Peptidoglycan-associated lipoprotein OS=*Legionella pneumophila*	OmpA family. Pal subfamily	membrane		0.828
Q5ZXE0	Outer membrane protein MIP OS=*Legionella pneumophila* subsp. pneumophila (strain Philadelphia 1 / ATCC 33152 / DSM 7513)	catalytic activity, peptidyl-prolyl cis-trans isomerase activity	membrane	metabolic process, Virulence, protein folding, protein peptidyl-prolyl isomerization	1.337
G8USV5	Outer membrane lipoprotein LolB OS=*Legionella pneumophila* subsp. pneumophila ATCC 43290		membrane	transport	1.194
Q5X1B0	NADH-quinone oxidoreductase subunit D OS=*Legionella pneumophila* (strain Paris)	catalytic activity; nucleotide binding, NAD binding	membrane	metabolic process; transport	0.563
G8US26	NADH dehydrogenase I, G subunit OS=*Legionella pneumophila* subsp. pneumophila ATCC 43290	catalytic activity; metal ion binding‹NADH dehydrogenase (ubiquinone) activity electron carrier activity‹ iron-sulfur cluster binding	membrane	metabolic process‹ ATP synthesis coupled electron transport	1.129
G8US28	NADH dehydrogenase I, E subunit OS=*Legionella pneumophila* subsp. pneumophila ATCC 43290	catalytic activity; metal ion binding; nucleotide binding, oxidoreductase activity, 2 iron, 2 sulfur cluster binding		metabolic process, Metabolism of Cofactors and Vitamins, Energy Metabolism	0.948
E1XXZ0	NADH dehydrogenase I chain F OS=*Legionella pneumophila*	catalytic activity; metal ion binding; nucleotide binding		metabolic process	0.677

Accession	Description	Molecular Function	Cellular Component	Biological Process	fold change MIF-VBNCCs/ culturable MIFs
D5TB19	NAD(P) transhydrogenase subunit beta OS=*Legionella pneumophila* serogroup 1 (strain 2300/99 Alcoy)	catalytic activity; nucleotide binding, Oxidoreductase, NADP binding	membrane	metabolic process	0.642
A5IC79	NAD-glutamate dehydrogenase OS=*Legionella pneumophila* (strain Corby)	catalytic activity; nucleotide binding, glutamate dehydrogenase (NAD+) activity		metabolic process	1.574
A5IHU8	N utilization substance protein A OS=*Legionella pneumophila* (strain Corby)	DNA binding; nucleotide binding; RNA binding		cell organization and biogenesis; metabolic process; regulation of biological process; response to stimulus, DNA repair, regulation of DNA-dependent transcription, termination	0.762
Q5ZXL1	Multidrug resistance protein OS=*Legionella pneumophila* subsp. pneumophila (strain Philadelphia 1 / ATCC 33152 / DSM 7513)	transporter activity	membrane	transport	1.528
A5II31	MoxR protein (ATPase) methanol dehydrogenase regulatory protein OS=*Legionella pneumophila* (strain Corby)	catalytic activity; DNA binding; nucleotide binding, ATP binding		metabolic process, ATP catabolic process	0.521
A5IE61	Metallo-beta-lactamase superfamily protein OS=*Legionella pneumophila* (strain Corby)	catalytic activity, hydrolase activity			1.431
I7I2N4	Membrane-associated, 16S rRNA-binding GTPase OS=*Legionella pneumophila* subsp. pneumophila	GTP binding, RNA-binding	cytoplasm; membrane	GTP catabolic process, Ribosome biogenesis	0.534
A5IIK4	Membrane protein insertase YidC OS=*Legionella pneumophila* (strain Corby)	Chaperone	membrane	cell organization and biogenesis; transport, protein insertion into membrane, protein transport	0.662
A5IHB0	Membrane protease subunit HflC OS=*Legionella pneumophila* (strain Corby)	catalytic activity, peptidase activity	membrane	metabolic process, proteolysis, regulation of peptidase activity	0.595
A5IF30	Malonate decarboxylase alpha subunit OS=*Legionella pneumophila* (strain Corby)	catalytic activity, CoA-transferase activity		metabolic process	1.335
A5IEF4	Malate dehydrogenase OS=*Legionella pneumophila* (strain Corby)	catalytic activity; nucleotide binding		metabolic process, Tricarboxylic acid cycle, cellular carbohydrate metabolic process	1.440
E5D656	Macrophage infectivity potentiator surface protein (Fragment) OS=*Legionella pneumophila*	catalytic activity, peptidyl-prolyl cis-trans isomerase activity	membrane	metabolic process, protein folding, protein peptidyl-prolyl isomerization	0.700
A5IHK9	LemA protein OS=*Legionella pneumophila* (strain Corby)		membrane		1.079
E1XVB7	Isocitrate dehydrogenase [NADP] OS=*Legionella pneumophila*	catalytic activity; metal ion binding; nucleotide binding, NAD binding, magnesium ion binding, Oxidoreductase		metabolic process, glyoxylate cycle, tricarboxylic acid cycle	0.816
Q5X1H9	Integration host factor subunit alpha OS=*Legionella pneumophila* (strain Paris)	DNA binding		metabolic process; regulation of biological process, DNA recombination, Transcription, Transcription regulation, Translation regulation	1.909
I7HTJ5	Inositol monophosphatase OS=*Legionella pneumophila* subsp. pneumophila	Hydrolase		inositol phosphate dephosphorylation, Transcription , Protein interactions	0.735

Accession	Description	Molecular Function	Cellular Component	Biological Process	fold change MIF-VBNCCs/ culturable MIFs
E1XWF8	Inosine-5'-monophosphate dehydrogenase OS=*Legionella pneumophila*	catalytic activity; metal ion binding; protein binding		metabolic process, GMP biosynthesis , Purine biosynthesis	0.662
A5IG82	Indole-3-glycerol phosphate synthase OS=*Legionella pneumophila* (strain Corby)	catalytic activity, Decarboxylase, Lyase		metabolic process, Amino-acid biosynthesis	1.430
Q48801	IcmX OS=*Legionella pneumophila*	IcmX		intracellular multiplication	0.699
A5IHE3	IcmE (DotG) OS=*Legionella pneumophila* (strain Corby)	IcmE (DotG)	cytoplasm; membrane	metabolic process	1.027
G8URT5	Hydrolase of the alpha/beta superfamily OS=*Legionella pneumophila* subsp. pneumophila ATCC 43290	catalytic activity, hydrolase activity,Protein fate / hydrolases / secretion		metabolic process	1.290
G8URK2	HflK protein OS=*Legionella pneumophila* subsp. pneumophila ATCC 43290		membrane		0.893
I7HWK1	Glyoxalase/bleomycin resistance protein/dioxygenase OS=*Legionella pneumophila* subsp. pneumophila	Dioxygenase, Oxidoreductase		bleomycin resistance protein	1.011
I7I187	Glutathionine S-transferase OS=*Legionella pneumophila* subsp. pneumophila	glutathione transferase activity			1.454
A5IC29	Glutamate-1-semialdehyde 2,1-aminomutase OS=*Legionella pneumophila* (strain Corby)	catalytic activity, transaminase activity	cytoplasm	metabolic process, Porphyrin biosynthesis	0.563
Q5ZRE5	Fumarate hydratase class II OS=*Legionella pneumophila* subsp. pneumophila (strain Philadelphia 1 / ATCC 33152 / DSM 7513)	catalytic activity	cytoplasm	metabolic process	0.941
I7HS97	F0 sector of membrane-bound ATP synthase, subunit b OS=*Legionella pneumophila* subsp. pneumophila	hydrolase activity, ATPase activity	membrane	ATP synthesis, Hydrogen ion transport	0.579
A5IEB7	Esterase of the alpha-beta hydrolase superfamily OS=*Legionella pneumophila* (strain Corby)	catalytic activity, hydrolase activity		metabolic process, lipid metabolic process	1.532
G8UZA4	Enhanced entry protein EnhC OS=*Legionella pneumophila* subsp. pneumophila ATCC 43290	protein binding		Toxin production / other pathogen functions	0.817
A5IBH9	Enhanced entry protein EnhA OS=*Legionella pneumophila* (strain Corby)	catalytic activity, transferase activity			1.329
E1XWI7	Endopeptidase Clp ATP-binding chain B OS=*Legionella pneumophila*	catalytic activity; nucleotide binding, ATP-binding, Hydrolase, Protease, Serine protease	cytoplasm	metabolic process; response to stimulus, proteolysis	1.193
Q5WZL4	Elongation factor Tu OS=*Legionella pneumophila* (strain Lens)	catalytic activity; nucleotide binding; RNA binding, GTP binding	cytoplasm	metabolic process, Protein biosynthesis, GTP catabolic process	0.562
Q5X862	Elongation factor G OS=*Legionella pneumophila* (strain Paris)	catalytic activity; nucleotide binding; RNA binding, GTP binding, GTPase activity, translation elongation factor activity	cytoplasm	metabolic process, Protein biosynthesis	0.624

Accession	Description	Molecular Function	Cellular Component	Biological Process	fold change MIF-VBNCCs/ culturable MIFs
E1XZ34	Dot/Icm T4SS effector OS=*Legionella pneumophila*	Dot/Icm T4SS effector			1.629
Q5WZL8	DNA-directed RNA polymerase subunit beta' OS=*Legionella pneumophila* (strain Lens)	catalytic activity; DNA binding, DNA-directed RNA polymerase activity		metabolic process, Transcription	0.594
A5IHS1	DNA-directed RNA polymerase subunit beta OS=*Legionella pneumophila* (strain Corby)	catalytic activity; DNA binding		metabolic process, Transcription	0.647
Q5ZYL8	DNA-directed RNA polymerase subunit alpha OS=*Legionella pneumophila* subsp. pneumophila (strain Philadelphia 1 / ATCC 33152 / DSM 7513)	catalytic activity; DNA binding; protein binding		metabolic process, Transcription	0.530
A5IAX8	DNA topoisomerase OS=*Legionella pneumophila* (strain Corby)	catalytic activity; DNA binding; nucleotide binding, ATP binding, DNA topoisomerase type I activity	chromosome	metabolic process, DNA topological change	1.616
I7HKX7	Dihydrolipoyl dehydrogenase OS=*Legionella pneumophila* subsp. pneumophila	Oxidoreductase‹ flavin adenine dinucleotide binding	cytoplasm	cell redox homeostasis	1.168
G8USU2	Dihydrolipoamide succinyltransferase OS=*Legionella pneumophila* subsp. pneumophila ATCC 43290	catalytic activity, Acyltransferase, Transferase		metabolic process, tricarboxylic acid cycle	1.356
A5IAR1	Dienelactone hydrolase family protein OS=*Legionella pneumophila* (strain Corby)	catalytic activity, hydrolase activity			0.525
E1Y3P4	Defect in organelle trafficking protein DotB OS=*Legionella pneumophila*	catalytic activity; nucleotide binding, ATP binding		transport	0.746
Q93A14	Defect in organelle trafficking protein (Fragment) OS=*Legionella pneumophila*	dotA			1.078
G8UT42	Cytochrome c type biogenesis protein CycH OS=*Legionella pneumophila* subsp. pneumophila ATCC 43290		membrane		0.777
D5TCQ9	Cysteine--tRNA ligase OS=*Legionella pneumophila* serogroup 1 (strain 2300/99 Alcoy)	catalytic activity; nucleotide binding, metal ion binding, ATP binding	cytoplasm	metabolic process, protein biosynthesis	0.673
I7HQS4	Component of the Dot/Icm secretion system OS=*Legionella pneumophila* subsp. pneumophila	IcmB/DotO			1.456
G8UY36	Citrate synthase OS=*Legionella pneumophila* subsp. pneumophila ATCC 43290	catalytic activity, Transferase	cytoplasm	metabolic process, cellular carbohydrate metabolic process, tricarboxylic acid cycle	0.552
G8UWH7	Citrate synthase OS=*Legionella pneumophila* subsp. pneumophila ATCC 43290	catalytic activity	cytoplasm	metabolic process, cellular carbohydrate metabolic process, tricarboxylic acid cycle	0.685
Q5ZTY3	Chaperone protein DnaK OS=*Legionella pneumophila* subsp. pneumophila (strain Philadelphia 1 / ATCC 33152 / DSM 7513)	nucleotide binding; protein binding, ATP binding, chaperone		cell organization and biogenesis; metabolic process; response to stimulus, protein folding, response to stress	0.508
Q5ZZ17	Catalase-peroxidase 2 OS=*Legionella pneumophila* subsp. pneumophila (strain Philadelphia 1 / ATCC 33152 / DSM 7513)	antioxidant activity; catalytic activity; metal ion binding, heme binding		metabolic process; response to stimulus, hydrogen peroxide catabolic process	0.544

Accession	Description	Molecular Function	Cellular Component	Biological Process	fold change MIF-VBNCCs/ culturable MIFs
Q5ZSX7	Catalase-peroxidase 1 OS=*Legionella pneumophila* subsp. pneumophila (strain Philadelphia 1 / ATCC 33152 / DSM 7513)	antioxidant activity; catalytic activity; metal ion binding, heme binding		metabolic process; response to stimulus, hydrogen peroxide catabolic process	1.473
G8UX75	Carbonic anhydrase Mig5 OS=*Legionella pneumophila* subsp. pneumophila ATCC 43290	catalytic activity; metal ion binding, zinc ion binding		Detoxification / adaptation	1.055
A5IAV1	Carbamoyl-phosphate synthase large chain OS=*Legionella pneumophila* (strain Corby)	catalytic activity; metal ion binding; nucleotide binding, ATP binding, magnesium ion binding, manganese ion binding		metabolic process, Amino-acid biosynthesis, 'de novo' UMP biosynthetic process	0.840
A5IAA3	Benzoylformate decarboxylase OS=*Legionella pneumophila* (strain Corby)	catalytic activity; metal ion binding		metabolic process	1.861
E1Y127	ATP-dependent protease ATPase subunit HslU 2 OS=*Legionella pneumophila*	catalytic activity; nucleotide binding; protein binding, ATP binding, peptidase activity, Chaperone	cytoplasm	metabolic process; regulation of biological process, ATP catabolic process	0.538
Q5ZUE0	ATP-dependent Clp protease ATP-binding subunit ClpX OS=*Legionella pneumophila* subsp. pneumophila (strain Philadelphia 1 / ATCC 33152 / DSM 7513)	catalytic activity; metal ion binding; nucleotide binding; protein binding, ATP binding, zinc ion binding, nucleoside-triphosphatase activity		metabolic process; response to stimulus, protein folding	0.785
A5IGV9	ATP transporter, ABC binding component, ATP-binding protein OS=*Legionella pneumophila* (strain Corby)	catalytic activity; nucleotide binding, ATP binding		metabolic process; transport, ATP catabolic process	0.562
Q5X0P3	ATP synthase subunit beta OS=*Legionella pneumophila* (strain Paris)	catalytic activity; nucleotide binding; transporter activity, ATP binding	membrane	metabolic process; transport, ATP synthesis, hydrogen ion transport	0.667
Q5WSG6	ATP synthase subunit alpha OS=*Legionella pneumophila* (strain Lens)	catalytic activity; nucleotide binding; transporter activity, ATP binding	membrane	metabolic process; transport, ATP synthesis, Hydrogen ion transport	0.560
A5IE87	Asparagine--tRNA ligase OS=*Legionella pneumophila* (strain Corby)	catalytic activity; nucleotide binding, ATP binding, ligase	cytoplasm	metabolic process, Protein biosynthesis	0.501
G8UUR1	AMP-binding protein OS=*Legionella pneumophila* subsp. pneumophila ATCC 43290	catalytic activity		metabolic process, Lipid Metabolic process	1.502
Q5X188	Aminopeptidase N OS= (strain Paris)	catalytic activity; metal ion binding, zinc ion binding		metabolic process, proteolysis	0.581
I7HP54	Alkyl hydroperoxide reductase OS=*Legionella pneumophila* subsp. pneumophila	antioxidant activity			0.762
A5ID10	Alkaline phosphatase OS=*Legionella pneumophila* (strain Corby)	catalytic activity		metabolic process	1.393
Q5ZSQ2	Adenosine monophosphate-protein hydrolase SidD OS=*Legionella pneumophila* subsp. pneumophila (strain Philadelphia 1 / ATCC 33152 / DSM 7513)	catalytic activity; metal ion binding; protein binding, Hydrolase, Rab GTPase binding	extracellular, cytolasm	metabolic process; regulation of biological process, Virulence, regulation of Rab GTPase activity	1.061

Accession	Description	Molecular Function	Cellular Component	Biological Process	fold change MIF-VBNCCs/ culturable MIFs
G8UY88	Acyl CoA C-acetyltransferase OS=*Legionella pneumophila* subsp. pneumophila ATCC 43290	catalytic activity, transferase activity		metabolic process, Lipid Metabolism, Amino Acid Metabolism, Carbohydrate Metabolism, Biodegradation of Xenobiotics	1.067
E1XWC8	Aconitate hydratase OS=*Legionella pneumophila*			metabolic process	1.817
G8URI1	Acetyl CoA carboxylase, biotin carboxyl carrier protein OS=*Legionella pneumophila* subsp. pneumophila ATCC 43290	catalytic activity, acetyl-CoA carboxylase activity	cytoplasm	metabolic process, fatty acid biosynthetic process	1.667
A5IC87	50S ribosomal protein L9 OS=*Legionella pneumophila* (strain Corby)	RNA binding; structural molecule activity	cytoplasm; ribosome	metabolic process, translation	1.055
Q5X867	50S ribosomal protein L7/L12 OS=*Legionella pneumophila* (strain Paris)	structural molecule activity	cytoplasm; ribosome	metabolic process, translation	0.927
Q5X844	50S ribosomal protein L6 OS=*Legionella pneumophila* (strain Paris)	RNA binding; structural molecule activity	cytoplasm; ribosome	metabolic process, translation	1.042
Q5X847	50S ribosomal protein L5 OS=*Legionella pneumophila* (strain Paris)	RNA binding; structural molecule activity	cytoplasm; ribosome	metabolic process, translation	0.777
A5IHR3	50S ribosomal protein L4 OS=*Legionella pneumophila* (strain Corby)	RNA binding; structural molecule activity	cytoplasm; ribosome	metabolic process, translation	0.796
Q5X5H4	50S ribosomal protein L32 OS=*Legionella pneumophila* (strain Paris)	structural molecule activity	cytoplasm; ribosome	metabolic process, translation	0.569
Q5X7A1	50S ribosomal protein L31 OS=*Legionella pneumophila* (strain Paris)	metal ion binding; RNA binding; structural molecule activity	cytoplasm; ribosome	metabolic process, translation	1.064
E1Y0L9	50S ribosomal protein L3 OS=*Legionella pneumophila*	RNA binding; structural molecule activity	cytoplasm; ribosome	metabolic process, translation	0.846
Q5X1P0	50S ribosomal protein L27 OS=*Legionella pneumophila* (strain Paris)	structural molecule activity	cytoplasm; ribosome	metabolic process, translation	0.882
A5IAS4	50S ribosomal protein L25 OS=*Legionella pneumophila* (strain Corby)	RNA binding; structural molecule activity	cytoplasm; ribosome	metabolic process, translation	0.722
A5IHQ3	50S ribosomal protein L24 OS=*Legionella pneumophila* (strain Corby)	RNA binding; structural molecule activity	cytoplasm; ribosome	metabolic process, translation	0.887
Q5X1H6	50S ribosomal protein L20 OS=*Legionella pneumophila* (strain Paris)	RNA binding; structural molecule activity	cytoplasm; ribosome	metabolic process, translation	0.885
Q5X856	50S ribosomal protein L2 OS=*Legionella pneumophila* (strain Paris)	catalytic activity; RNA binding; structural molecule activity, transferase activity	cytoplasm; ribosome	metabolic process, translation	0.778
I7HSJ3	50S ribosomal protein L19 OS=*Legionella pneumophila* subsp. pneumophila	structural molecule activity	cytoplasm; ribosome	metabolic process, translation	0.721
Q5X843	50S ribosomal protein L18 OS=*Legionella pneumophila* (strain Paris)	RNA binding; structural molecule activity	cytoplasm; ribosome	metabolic process, translation	0.830
A5IHP6	50S ribosomal protein L15 OS=*Legionella pneumophila* (strain Corby)	RNA binding; structural molecule activity	cytoplasm; ribosome	metabolic process, translation	0.892
Q5X1I1	50S ribosomal protein L13 OS=*Legionella pneumophila* (strain Paris)	structural molecule activity	cytoplasm; ribosome	metabolic process, translation	0.880

Accession	Description	Molecular Function	Cellular Component	Biological Process	fold change MIF-VBNCCs/ culturable MIFs
Q5X870	50S ribosomal protein L11 OS=*Legionella pneumophila* (strain Paris)	RNA binding; structural molecule activity	cytoplasm; ribosome	metabolic process, translation	0.879
A5IHS3	50S ribosomal protein L10 OS=*Legionella pneumophila* (strain Corby)	structural molecule activity	cytoplasm; ribosome	metabolic process, translation, ribosome biogenesis	0.900
Q5X869	50S ribosomal protein L1 OS=*Legionella pneumophila* (strain Paris)	RNA binding; structural molecule activity	cytoplasm; ribosome	metabolic process; regulation of biological process, translation, regulation of translation	0.595
A5IHQ0	30S ribosomal protein S8 OS=*Legionella pneumophila* (strain Corby)	RNA binding; structural molecule activity	cytoplasm; ribosome	metabolic process, translation	0.580
Q5X863	30S ribosomal protein S7 OS=*Legionella pneumophila* (strain Paris)	RNA binding; structural molecule activity	cytoplasm; ribosome	metabolic process, translation	0.699
Q5X842	30S ribosomal protein S5 OS=*Legionella pneumophila* (strain Paris)	RNA binding; structural molecule activity	cytoplasm; ribosome	metabolic process, translation	1.076
A5IHP2	30S ribosomal protein S4 OS=*Legionella pneumophila* (strain Corby)	RNA binding; structural molecule activity	cytoplasm; ribosome	metabolic process, translation	0.668
Q5X853	30S ribosomal protein S3 OS=*Legionella pneumophila* (strain Paris)	RNA binding; structural molecule activity	cytoplasm; ribosome	metabolic process, translation	0.772
Q5X2T0	30S ribosomal protein S21 OS=*Legionella pneumophila* (strain Paris)	structural molecule activity	cytoplasm; ribosome	metabolic process, translation	0.992
Q5WVY7	30S ribosomal protein S2 OS=*Legionella pneumophila* (strain Lens)	structural molecule activity	cytoplasm; ribosome	metabolic process, translation	0.709
A5IHU4	30S ribosomal protein S15 OS=*Legionella pneumophila* (strain Corby)	RNA binding; structural molecule activity	cytoplasm; ribosome	metabolic process, translation	0.658
Q5X837	30S ribosomal protein S13 OS=*Legionella pneumophila* (strain Paris)	RNA binding; structural molecule activity	cytoplasm; ribosome	metabolic process, translation	0.724
Q5X864	30S ribosomal protein S12 OS=*Legionella pneumophila* (strain Paris)	RNA binding; structural molecule activity	cytoplasm; ribosome	metabolic process, translation	0.778
Q5X860	30S ribosomal protein S10 OS=*Legionella pneumophila* (strain Paris)	structural molecule activity	cytoplasm; ribosome	metabolic process, translation	0.797
I7I2W9	30S ribosomal protein S1 OS=*Legionella pneumophila* subsp. pneumophila	RNA binding, structural molecule activity	ribosome	translation	0.568
D5T6W4	3-oxoacyl-(Acyl carrier protein) synthase III OS=*Legionella pneumophila* serogroup 1 (strain 2300/99 Alcoy)	catalytic activity, Acyltransferase, Transferase	cytoplasm; membrane	metabolic process, fatty acid biosynthetic process	1.903
G8UUY1	3-hydroxyacyl CoA dehydrogenase oxidoreductase protein OS=*Legionella pneumophila* subsp. pneumophila ATCC 43290	catalytic activity; nucleotide binding, coenzyme binding, oxidoreductase		metabolic process, fatty acid metabolic process	0.509
G8UYA4	27 kDa outer membrane protein OS=*Legionella pneumophila* subsp. pneumophila ATCC 43290	catalytic activity	membrane		0.728
G8USU1	2-oxoglutarate dehydrogenase E1 component OS=*Legionella pneumophila* subsp. pneumophila ATCC 43290	catalytic activity, oxoglutarate dehydrogenase (succinyl-transferring) activity, thiamine pyrophosphate binding		metabolic process, tricarboxylic acid cycle	0.994

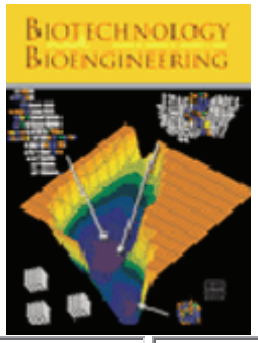
Accession	Description	Molecular Function	Cellular Component	Biological Process	fold change MIF-VBNCCs/ culturable MIFs
G8UVA1	16 kD immunogenic protein OS=*Legionella pneumophila* subsp. pneumophila ATCC 43290		periplasmic space		0.876

Appendix IV

false	true	true	false	false	true
true	0				

Copyright Clearance Center RightsLink®

Home | Account Info | Help



Title: Bacterial polyhydroxyalkanoates
Author: Sang Yup Lee
Publication: Biotechnology & Bioengineering
Publisher: John Wiley and Sons
Date: Mar 26, 2000

Logged in as:
Badii Al
Account #:
3000663085

LOGOUT

140	-5555	Wiley	BIT	26581	1
296	false		false	viewlicense	additionaldata
	4278034	/App/Includes/He	/App/ViewLicense	/App/Includes/Fo	1370273899736
0Q1ypDZx7t4R5J	unknown	unknown			

Order Completed

Thank you very much for your order.

This is a License Agreement between Badii Al-Bana ("You") and John Wiley and Sons ("John Wiley and Sons"). The license consists of your order details, the terms and conditions provided by John Wiley and Sons, and the payment terms and conditions.

Get the printable license.

License Number	3161411506983
License date	Jun 03, 2013
Licensed content publisher	John Wiley and Sons
Licensed content publication	Biotechnology & Bioengineering
Licensed content title	Bacterial polyhydroxyalkanoates
Licensed copyright line	Copyright © 1996 John Wiley & Sons, Inc.
Licensed content author	Sang Yup Lee
Licensed content date	Mar 26, 2000
Start page	1
End page	14
Type of use	Dissertation/Thesis
Requestor type	University/Academic
Format	Print and electronic
Portion	Figure/table
Number of figures/tables	1
Original Wiley figure/table number(s)	Figure 1
Will you be translating?	No
Total	0.00 USD

ORDER MORE... | CLOSE WINDOW

2013060_137027		f144a4e0-35d5-4

false	true	true	false	false	false
true	0				

Copyright Clearance Center RightsLink®

Home | Create Account | Help

AMERICAN SOCIETY FOR MICROBIOLOGY

Title: Ultrastructural Analysis of Differentiation in Legionella pneumophila

Author: Gary Faulkner, Rafael A. Garduño

Publication: Journal of Bacteriology

Publisher: American Society for Microbiology

Date: Dec 15, 2002

User ID

Password

Enable Auto Login

LOGIN

Forgot Password/User ID?

If you're a copyright.com user, you can login to RightsLink using your copyright.com credentials.

Already **a RightsLink user** or want to learn more?

Permissions Request

ASM authorizes an advanced degree candidate to republish the requested material in his/her doctoral thesis or dissertation. If your thesis, or dissertation, is to be published commercially, then you must reapply for permission.

BACK | CLOSE WINDOW

100	-5555	asm	jb	19561	1
296	false		false	denyredirect	
	3621	/App/Includes/He	/App/SimplePage	/App/Includes/Fo	1370264049988
ETAv6gRZXQUN	unknown	unknown			

false	true	true	false	false	true
true	0				

RightsLink®

Home | Account Info | Help

Title: Intracellular Growth of Legionella pneumophila Gives Rise to a Differentiated Form Dissimilar to Stationary-Phase Forms

Author: Rafael A. Garduño, Elizabeth Garduño, Margot Hiltz et al.

Publication: Infection and Immunity

Publisher: American Society for Microbiology

Date: Nov 1, 0001

Logged in as:

Badii Al

Account #:
3000663085

LOGOUT

Permissions Request

ASM authorizes an advanced degree candidate to republish the requested material in his/her doctoral thesis or dissertation. If your thesis, or dissertation, is to be published commercially, then you must reapply for permission.

BACK | CLOSE WINDOW

100	-5555	asm	iai	19559	1
296	false		false	denyredirect	
	3621	/App/Includes/He	/App/SimplePage	/App/Includes/Fo	1370261835962
Gh36NXqAoTV7x	unknown	unknown			

JOHN WILEY AND SONS LICENSE
TERMS AND CONDITIONS

Jun 03, 2013

This is a License Agreement between Badii Al-Bana ("You") and John Wiley and Sons ("John Wiley and Sons") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by John Wiley and Sons, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

License Number	3161240557646
License date	Jun 03, 2013
Licensed content publisher	John Wiley and Sons
Licensed content publication	Molecular Microbiology
Licensed content title	Differentiate to thrive: lessons from the Legionella pneumophila life cycle
Licensed copyright line	Copyright © 2004, John Wiley and Sons
Licensed content author	Ari B. Molofsky,Michele S. Swanson
Licensed content date	Jun 4, 2004
Start page	29
End page	40
Type of use	Dissertation/Thesis
Requestor type	University/Academic
Format	Print and electronic
Portion	Figure/table
Number of figures/tables	1
Original Wiley figure/table number(s)	Figure 2
Will you be translating?	No
Total	0.00 USD

Terms and Conditions

TERMS AND CONDITIONS

This copyrighted material is owned by or exclusively licensed to John Wiley & Sons, Inc. or one of its group companies (each a "Wiley Company") or a society for whom a Wiley Company has exclusive publishing rights in relation to a particular journal (collectively "WILEY"). By clicking "accept" in connection with completing this licensing transaction, you agree that the following terms and conditions apply to this transaction (along with the billing and payment terms and conditions established by the Copyright Clearance Center Inc., ("CCC's Billing and Payment terms and conditions"), at the time that you opened your RightsLink account (these are available at any time at http://myaccount.copyright.com).

Terms and Conditions

LICENSORS AND WAIVED BY YOU.

6. WILEY shall have the right to terminate this Agreement immediately upon breach of this Agreement by you.

7. You shall indemnify, defend and hold harmless WILEY, its Licensors and their respective directors, officers, agents and employees, from and against any actual or threatened claims, demands, causes of action or proceedings arising from any breach of this Agreement by you.

8. IN NO EVENT SHALL WILEY OR ITS LICENSORS BE LIABLE TO YOU OR ANY OTHER PARTY OR ANY OTHER PERSON OR ENTITY FOR ANY SPECIAL, CONSEQUENTIAL, INCIDENTAL, INDIRECT, EXEMPLARY OR PUNITIVE DAMAGES, HOWEVER CAUSED, ARISING OUT OF OR IN CONNECTION WITH THE DOWNLOADING, PROVISIONING, VIEWING OR USE OF THE MATERIALS REGARDLESS OF THE FORM OF ACTION, WHETHER FOR BREACH OF CONTRACT, BREACH OF WARRANTY, TORT, NEGLIGENCE, INFRINGEMENT OR OTHERWISE (INCLUDING, WITHOUT LIMITATION, DAMAGES BASED ON LOSS OF PROFITS, DATA, FILES, USE, BUSINESS OPPORTUNITY OR CLAIMS OF THIRD PARTIES), AND WHETHER OR NOT THE PARTY HAS BEEN ADVISED OF THE POSSIBILITY OF SUCH DAMAGES. THIS LIMITATION SHALL APPLY NOTWITHSTANDING ANY FAILURE OF ESSENTIAL PURPOSE OF ANY LIMITED REMEDY PROVIDED HEREIN.

9. Should any provision of this Agreement be held by a court of competent jurisdiction to be illegal, invalid, or unenforceable, that provision shall be deemed amended to achieve as nearly as possible the same economic effect as the original provision, and the legality, validity and enforceability of the remaining provisions of this Agreement shall not be affected or impaired thereby.

10. The failure of either party to enforce any term or condition of this Agreement shall not constitute a waiver of either party's right to enforce each and every term and condition of this Agreement. No breach under this agreement shall be deemed waived or excused by either party unless such waiver or consent is in writing signed by the party granting such waiver or consent. The waiver by or consent of a party to a breach of any provision of this Agreement shall not operate or be construed as a waiver of or consent to any other or subsequent breach by such other party.

11. This Agreement may not be assigned (including by operation of law or otherwise) by you without WILEY's prior written consent.

12. Any fee required for this permission shall be non-refundable after thirty (30) days from receipt

13. These terms and conditions together with CCC's Billing and Payment terms and conditions (which are incorporated herein) form the entire agreement between you and

WILEY concerning this licensing transaction and (in the absence of fraud) supersedes all prior agreements and representations of the parties, oral or written. This Agreement may not be amended except in writing signed by both parties. This Agreement shall be binding upon and inure to the benefit of the parties' successors, legal representatives, and authorized assigns.

14. In the event of any conflict between your obligations established by these terms and conditions and those established by CCC's Billing and Payment terms and conditions, these terms and conditions shall prevail.

15. WILEY expressly reserves all rights not specifically granted in the combination of (i) the license details provided by you and accepted in the course of this licensing transaction, (ii) these terms and conditions and (iii) CCC's Billing and Payment terms and conditions.

16. This Agreement will be void if the Type of Use, Format, Circulation, or Requestor Type was misrepresented during the licensing process.

17. This Agreement shall be governed by and construed in accordance with the laws of the State of New York, USA, without regards to such state's conflict of law rules. Any legal action, suit or proceeding arising out of or relating to these Terms and Conditions or the breach thereof shall be instituted in a court of competent jurisdiction in New York County in the State of New York in the United States of America and each party hereby consents and submits to the personal jurisdiction of such court, waives any objection to venue in such court and consents to service of process by registered or certified mail, return receipt requested, at the last known address of such party.

Wiley Open Access Terms and Conditions

Wiley publishes Open Access articles in both its Wiley Open Access Journals program [http://www.wileyopenaccess.com/view/index.html] and as Online Open articles in its subscription journals. The majority of Wiley Open Access Journals have adopted the Creative Commons Attribution License (CC BY) which permits the unrestricted use, distribution, reproduction, adaptation and commercial exploitation of the article in any medium. No permission is required to use the article in this way provided that the article is properly cited and other license terms are observed. A small number of Wiley Open Access journals have retained the Creative Commons Attribution Non Commercial License (CC BY-NC), which permits use, distribution and reproduction in any medium, provided the original work is properly cited and is not used for commercial purposes.

Online Open articles - Authors selecting Online Open are, unless particular exceptions apply, offered a choice of Creative Commons licenses. They may therefore select from the CC BY, the CC BY-NC and the Attribution-NoDerivatives (CC BY-NC-ND). The CC BY-NC-ND is more restrictive than the CC BY-NC as it does not permit adaptations or modifications without rights holder consent.

Wiley Open Access articles are protected by copyright and are posted to repositories and

subject to a fee. Commercial purposes include:

- Copying or downloading of articles, or linking to such articles for further redistribution, sale or licensing;
- Copying, downloading or posting by a site or service that incorporates advertising with such content;
- The inclusion or incorporation of article content in other works or services (other than normal quotations with an appropriate citation) that is then available for sale or licensing, for a fee (for example, a compilation produced for marketing purposes, inclusion in a sales pack)
- Use of article content (other than normal quotations with appropriate citation) by for-profit organizations for promotional purposes
- Linking to article content in e-mails redistributed for promotional, marketing or educational purposes;
- Use for the purposes of monetary reward by means of sale, resale, license, loan, transfer or other form of commercial exploitation such as marketing products
- Print reprints of Wiley Open Access articles can be purchased from: corporatesales@wiley.com

  The modification or adaptation for any purpose of an article referencing the CC BY-NC-ND License requires consent which can be requested from RightsLink@wiley.com .

  Other Terms and Conditions:

  BY CLICKING ON THE "I AGREE..." BOX, YOU ACKNOWLEDGE THAT YOU HAVE READ AND FULLY UNDERSTAND EACH OF THE SECTIONS OF AND PROVISIONS SET FORTH IN THIS AGREEMENT AND THAT YOU ARE IN AGREEMENT WITH AND ARE WILLING TO ACCEPT ALL OF YOUR OBLIGATIONS AS SET FORTH IN THIS AGREEMENT.

v1.8

140	-5555	Wiley	MMI	27333	1
296			false	printablelicense	
	4278036	/App/Includes/Pri	/App/PrintableLic	/App/Includes/No	1370247285301
8Eoe1VoQRoO4S	unknown	unknown			

ELSEVIER LICENSE
TERMS AND CONDITIONS

Jun 03, 2013

This is a License Agreement between Badii Al-Bana ("You") and Elsevier ("Elsevier") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by Elsevier, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

Supplier	Elsevier Limited The Boulevard,Langford Lane Kidlington,Oxford,OX5 1GB,UK
Registered Company Number	1982084
Customer name	Badii Al-Bana
Customer address	1206-1094 Wellington street Halifax, NS B3H2Z9
License number	3161330652960
License date	Jun 03, 2013
Licensed content publisher	Elsevier
Licensed content publication	Journal of Bioscience and Bioengineering
Licensed content title	Fermentative production of (*R*)-(−)-3-hydroxybutyrate using 3-hydroxybutyrate dehydrogenase null mutant of*Ralstonia eutropha*and recombinant*Escherichia coli*
Licensed content author	Mari Shiraki,Takakazu Endo,Terumi Saito
Licensed content date	December 2006
Licensed content volume number	102
Licensed content issue number	6
Number of pages	6
Start Page	529
End Page	534
Type of Use	reuse in a thesis/dissertation
Portion	figures/tables/illustrations
Number of figures/tables/illustrations	1
Format	both print and electronic
Are you the author of this Elsevier article?	No
Will you be translating?	No
Order reference number	
Title of your thesis/dissertation	Characterization of The Viable but Non-Culturable Legionella pneumophila in Water and the Role of 3-Hydroxybutyrate Dehydrogenase in Its Formation
Expected completion date	Aug 2013
Estimated size (number of pages)	200
Elsevier VAT number	GB 494 6272 12
Permissions price	0.00 USD

VAT/Local Sales Tax	0.0 USD / 0.0 GBP
Total	0.00 USD

Terms and Conditions

INTRODUCTION

1. The publisher for this copyrighted material is Elsevier. By clicking "accept" in connection with completing this licensing transaction, you agree that the following terms and conditions apply to this transaction (along with the Billing and Payment terms and conditions established by Copyright Clearance Center, Inc. ("CCC"), at the time that you opened your Rightslink account and that are available at any time at http://myaccount.copyright.com).

GENERAL TERMS

2. Elsevier hereby grants you permission to reproduce the aforementioned material subject to the terms and conditions indicated.

3. Acknowledgement: If any part of the material to be used (for example, figures) has appeared in our publication with credit or acknowledgement to another source, permission must also be sought from that source. If such permission is not obtained then that material may not be included in your publication/copies. Suitable acknowledgement to the source must be made, either as a footnote or in a reference list at the end of your publication, as follows:

"Reprinted from Publication title, Vol /edition number, Author(s), Title of article / title of chapter, Pages No., Copyright (Year), with permission from Elsevier [OR APPLICABLE SOCIETY COPYRIGHT OWNER]." Also Lancet special credit - "Reprinted from The Lancet, Vol. number, Author(s), Title of article, Pages No., Copyright (Year), with permission from Elsevier."

4. Reproduction of this material is confined to the purpose and/or media for which permission is hereby given.

5. Altering/Modifying Material: Not Permitted. However figures and illustrations may be altered/adapted minimally to serve your work. Any other abbreviations, additions, deletions and/or any other alterations shall be made only with prior written authorization of Elsevier Ltd. (Please contact Elsevier at permissions@elsevier.com)

6. If the permission fee for the requested use of our material is waived in this instance, please be advised that your future requests for Elsevier materials may attract a fee.

7. Reservation of Rights: Publisher reserves all rights not specifically granted in the combination of (i) the license details provided by you and accepted in the course of this licensing transaction, (ii) these terms and conditions and (iii) CCC's Billing and Payment

terms and conditions.

8. License Contingent Upon Payment: While you may exercise the rights licensed immediately upon issuance of the license at the end of the licensing process for the transaction, provided that you have disclosed complete and accurate details of your proposed use, no license is finally effective unless and until full payment is received from you (either by publisher or by CCC) as provided in CCC's Billing and Payment terms and conditions. If full payment is not received on a timely basis, then any license preliminarily granted shall be deemed automatically revoked and shall be void as if never granted. Further, in the event that you breach any of these terms and conditions or any of CCC's Billing and Payment terms and conditions, the license is automatically revoked and shall be void as if never granted. Use of materials as described in a revoked license, as well as any use of the materials beyond the scope of an unrevoked license, may constitute copyright infringement and publisher reserves the right to take any and all action to protect its copyright in the materials.

9. Warranties: Publisher makes no representations or warranties with respect to the licensed material.

10. Indemnity: You hereby indemnify and agree to hold harmless publisher and CCC, and their respective officers, directors, employees and agents, from and against any and all claims arising out of your use of the licensed material other than as specifically authorized pursuant to this license.

11. No Transfer of License: This license is personal to you and may not be sublicensed, assigned, or transferred by you to any other person without publisher's written permission.

12. No Amendment Except in Writing: This license may not be amended except in a writing signed by both parties (or, in the case of publisher, by CCC on publisher's behalf).

13. Objection to Contrary Terms: Publisher hereby objects to any terms contained in any purchase order, acknowledgment, check endorsement or other writing prepared by you, which terms are inconsistent with these terms and conditions or CCC's Billing and Payment terms and conditions. These terms and conditions, together with CCC's Billing and Payment terms and conditions (which are incorporated herein), comprise the entire agreement between you and publisher (and CCC) concerning this licensing transaction. In the event of any conflict between your obligations established by these terms and conditions and those established by CCC's Billing and Payment terms and conditions, these terms and conditions shall control.

14. Revocation: Elsevier or Copyright Clearance Center may deny the permissions described in this License at their sole discretion, for any reason or no reason, with a full refund payable to you. Notice of such denial will be made using the contact information provided by you. Failure to receive such notice will not alter or invalidate the denial. In no event will Elsevier or Copyright Clearance Center be responsible or liable for any costs, expenses or damage incurred by you as a result of a denial of your permission

request, other than a refund of the amount(s) paid by you to Elsevier and/or Copyright Clearance Center for denied permissions.

LIMITED LICENSE

The following terms and conditions apply only to specific license types:

15. **Translation**: This permission is granted for non-exclusive world **English** rights only unless your license was granted for translation rights. If you licensed translation rights you may only translate this content into the languages you requested. A professional translator must perform all translations and reproduce the content word for word preserving the integrity of the article. If this license is to re-use 1 or 2 figures then permission is granted for non-exclusive world rights in all languages.

16. **Website**: The following terms and conditions apply to electronic reserve and author websites:
Electronic reserve: If licensed material is to be posted to website, the web site is to be password-protected and made available only to bona fide students registered on a relevant course if:
This license was made in connection with a course,
This permission is granted for 1 year only. You may obtain a license for future website posting,
All content posted to the web site must maintain the copyright information line on the bottom of each image,
A hyper-text must be included to the Homepage of the journal from which you are licensing at http://www.sciencedirect.com/science/journal/xxxxx or the Elsevier homepage for books at http://www.elsevier.com , and
Central Storage: This license does not include permission for a scanned version of the material to be stored in a central repository such as that provided by Heron/XanEdu.

17. **Author website** for journals with the following additional clauses:

All content posted to the web site must maintain the copyright information line on the bottom of each image, and the permission granted is limited to the personal version of your paper. You are not allowed to download and post the published electronic version of your article (whether PDF or HTML, proof or final version), nor may you scan the printed edition to create an electronic version. A hyper-text must be included to the Homepage of the journal from which you are licensing at http://www.sciencedirect.com/science/journal/xxxxx . As part of our normal production process, you will receive an e-mail notice when your article appears on Elsevier's online service ScienceDirect (www.sciencedirect.com). That e-mail will include the article's Digital Object Identifier (DOI). This number provides the electronic link to the published article and should be included in the posting of your personal version. We ask that you wait until you receive this e-mail and have the DOI to do any posting.

Central Storage: This license does not include permission for a scanned version of the

material to be stored in a central repository such as that provided by Heron/XanEdu.

18. **Author website** for books with the following additional clauses:
Authors are permitted to place a brief summary of their work online only.
A hyper-text must be included to the Elsevier homepage at http://www.elsevier.com . All content posted to the web site must maintain the copyright information line on the bottom of each image. You are not allowed to download and post the published electronic version of your chapter, nor may you scan the printed edition to create an electronic version.

Central Storage: This license does not include permission for a scanned version of the material to be stored in a central repository such as that provided by Heron/XanEdu.

19. **Website** (regular and for author): A hyper-text must be included to the Homepage of the journal from which you are licensing at http://www.sciencedirect.com/science/journal/xxxxx. or for books to the Elsevier homepage at http://www.elsevier.com

20. **Thesis/Dissertation**: If your license is for use in a thesis/dissertation your thesis may be submitted to your institution in either print or electronic form. Should your thesis be published commercially, please reapply for permission. These requirements include permission for the Library and Archives of Canada to supply single copies, on demand, of the complete thesis and include permission for UMI to supply single copies, on demand, of the complete thesis. Should your thesis be published commercially, please reapply for permission.

21. **Other Conditions**:

v1.6

If you would like to pay for this license now, please remit this license along with your payment made payable to "COPYRIGHT CLEARANCE CENTER" otherwise you will be invoiced within 48 hours of the license date. Payment should be in the form of a check or money order referencing your account number and this invoice number RLNK501034493.
Once you receive your invoice for this order, you may pay your invoice by credit card. Please follow instructions provided at that time.

Make Payment To:
Copyright Clearance Center
Dept 001
P.O. Box 843006
Boston, MA 02284-3006

For suggestions or comments regarding this order, contact RightsLink Customer Support: customercare@copyright.com or +1-877-622-5543 (toll free in the US) or +1-978-646-2777.

Gratis licenses (referencing $0 in the Total field) are free. Please retain this printable license for your reference. No payment is required.

70	-5555	ELS	1389-1723	12535	1
54			false	printablelicense	
	4278119	/App/Includes/Pri	/App/PrintableLic	/App/Includes/No	1370260991397
zIktT1Rg5EwRzvk	unknown	unknown			