ROLES OF SODIUM AND INTEGRATION HOST FACTOR IN THE VIRULENCE AND DEVELOPMENTAL CYCLE OF *LEGIONELLA PNEUMOPHILA*

by

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ABSTRACT

Legionella pneumophila is a facultative, intracellular parasite of freshwater protozoa that displays a developmental cycle where it alternates between an intracellularly replicating form, and an environmentally resistant, infectious form, termed the mature intracellular form (MIF). Virulent *L. pneumophila* strains are unable to replicate on BCYE medium that contains physiological concentrations of sodium chloride. Mutations in a number of post-exponential phase regulators, as well as a number of virulence factors, including the Dot/Icm secretion system, alleviate the sodium mediated growth repression. Previous studies revealed expression of the major outer membrane protein, OmpS, is decreased upon exposure to sodium chloride in the growth media during late exponential phase. An unidentified regulatory protein, termed OmpT, whose binding to the *ompS* promoter is abolished upon sodium chloride challenge, potentially mediates this decrease in expression. The first goal of this study was to identify proteins that bound to this DNA fragment, and to determine the effect of sodium challenge on global protein expression. The results of this study revealed that the sodium sensitive DNA binding protein is present in the *L. pneumophila* isogenic Lp02 strain, and recognizes multiple sequence elements based on mobility shift experiments using truncated *ompS* promoter fragments. When late exponential phase bacteria were exposed to equimolar amounts of sodium or potassium chloride, they exhibited the same changes in protein expression profiles. The *ompS* promoter contains multiple integration host factor (IHF) binding sites, and purified IHF subunits are capable of retarding the *ompS* amplicon in mobility shift experiments. Exponentially grown IHF deletion mutants display the same mobility shift profile as wild type, indicating IHF is not OmpT, but IHF is required for full retardation of the *ompS* amplicon in stationary phase. Western blot revealed that IHF is developmentally regulated, with increased expression in stationary phase, and maximal concentration occurring in MIFs. *L. pneumophila* strains that over expressed IHF, and to a lesser extent the IHF deletion mutant, were resistant to sodium chloride in the growth media. IHF mutants display several protein profile differences as determined by two-dimensional gel electrophoresis, when compared to wild type. IHF is not required for expression of the DotA, DotD or IcmT proteins, nor is it required for attachment, invasion or intracellular multiplication in HeLa cells, or infectivity in L929 cells, although alterations in the *Legionella*-containing vacuoles were observed. IHF mutants are defective for MIF morphogenesis, as they do not form thickened cell envelopes or cytoplasmic, multilaminated membranes. Poly-β-hydroxybutyrate synthesis is decreased in the over expressing strain, and the IHF mutant strains, including the complemented strains. In addition to the ultrastructural defects, several proteins were differentially regulated in the mature IHF mutant strains when compared to wild type MIFs. The mature IHF mutants showed slight decreases in the developmental marker MagA production, and some alterations in resistance to detergent and alkalinity, which are previously defined MIF characteristics. This work demonstrates that IHF specifically regulates several genes, and is required for complete MIF morphogenesis, but is dispensible for virulence in the epithelial cell lines used in this study.

List of abbreviations and symbols used

2D-SDS PAGE – Two-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis

16S rRNA – 16S ribosomal ribonucleic acid

AE – Attaching and effacing

Amp - Ampicilin

AP - Alkaline phosphatase

APS – Ammonium persulfate

AT rich – Adenine-thymidine rich

ATP – Adenosine triphosphate

BCIP – 5-Bromo-4-chloro-3-indolyl phosphate

BCYE – Buffered Charcoal Yeast Extract

BCYEα – Buffered Charcoal Yeast Extract supplemented with α-ketoglutaric acid

bp – Base pair

BSA – Bovine serum albumin

BYEα - Buffered yeast extract supplemented with α-ketoglutaric acid

CAH - Casein acid hydrolysate

Cm - Chloramphenicol

cAMP – Cyclic adenosine monophophate

CoA – Coenzyme A

CDC – Centers for Disease Control and Prevention

C.F.U. – Colony forming unit

CHAPS - 3-[(3-Cholamidopropyl)dimethylammonio]propanesulfonic acid

Comp - Lp02Δ*ihfAB*/pMMB206::*ihfAB*

CYE – Charcoal Yeast Extract

ddH_2O – Distilled deionized H_2O

DEPC – Diethylpyrocarbonate

DKO – Lp02Δ*ihfAB*

DMF – Dimethylformamide

DMSO - Dimethylsulfoxide

DNA – Deoxyribonucleic acid

DNaseI – Deoxyribonuclease I

dNTP – deoxynucleoside triphosphate

DTH – Delayed type hypersensitivity

DTT - dithiothreitol

EDTA - ethylenediaminetetraacetic acid

EPEC – Enteropathogenic *E. coli*

ELISA - Enzyme-linked immunosorbent assay

EMSA – Electrophoretic mobility shift assay

FBS – Fetal bovine serum

F - Faraday

F-G agar – Feeley-Gorman agar

FKBP - FK506-binding proteins

Gent - Gentamicin

GFP – Green fluorescent protein

HEPES - 1-Piperazineethane sulfonic acid

IgM – Immunoglobulin M

IHF – Integration host factor

kDa - kilodalton

IL-2 – Interleukin-2

IPTG - isopropyl-beta-D-thiogalactopyranoside

Kan - Kanamycin

KV – Kilovolts

L+C – Lp02/pMMB206::*ihfAB*

L+V – Lp02/pMMB206

LB – Luria Bertani

Lcr - Low calcium response

LDS – Lithium dodecyl sulfate

LEE – Locus for enterocyte effacing

LEF-1 - lymphoid enhancer-binding factor

LLAP – *Legionella*-like amoebal pathogen

Lrp - Leucine-responsive regulatory protein

Mbp – Mega base pair

MEM – Minimal essential media

Met - Metronidazole

MIF – Mature Intracellular Form

Mil – Macrophage infectivity locus

MIP – Macrophage infectivity potentiator

M-MLV – Moloney Murine Leukemia Virus

MOMP – Major outer membrane protein

MOPS - 3-(N-Morpholino) propanesulfonic acid

MW – Molecular weight

NBT – Nitro blue tetrazolium

NCS – Newborn calf serum

NEB – New England biolabs

OD – Optical density

PAS – PER (period clock protein), ARNT (vertebrate aryl hydrocarbon receptor nuclear Translocator), SIM (single-minded protein)

PBS – Phosphate buffered saline

PCR – Polymerase chain reaction

PHB – Poly-β-hydroxybutyrate

pI – isoelectric point

ppGpp – Guanosine 5'-diphosphate,3'-diphosphate

PMSF - Phenylmethylsulphonylfluoride

RER – Rough endoplasmic reticulum

RIP - repetitive IHF-binding palindromic

RNA – Ribonucleic acid

RNase – Ribonuclease

RT-PCR – Reverse transcriptase polymerase chain reaction

SCV - *Salmonella*-containing vacuole

SDS – Sodium dodecyl sulfate

SDS PAGE – Sodium dodecyl sulfate polyacrylamide gel electrophoresis

SMH – Supplemented Mueller-Hinton

SPI – *Salmonella* pathogenicity island

TAE – Tris-acetate EDTA

Taq – *Thermus aquaticus* DNA polymerase

TBE – Tris-borate EDTA

TE – Tris-EDTA

TEMED - N,N,N',N'-Tetramethylethylenediamine

TFB – Transformation buffer

Tris-HCl - 2-Amino-2-(hydroxymethyl)-1,3-propanediol, hydrochloride

tRNA – transfer ribonucleic acid

TTBS – Tris Tween buffered saline

Tween 20 - polyoxyethylene sorbitan monolaurate

UV – Ultraviolet

Vect - Lp02Δ*ihfAB*/pMMB206

X-gal - 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside

Yop - yersinial outer membrane proteins

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CHAPTER 1: LITERATURE REVIEW

1.1 Introduction

In 1976, an atypical pneumonia of unknown origin infected over 180 people, killing 29 at a Legionnaires' conventition in Philadelphia Pennsylvania. Multiple attempts to isolate the etiologic agent of the disease had failed, and it was only after nearly 6 months of research that the bacterium responsible for this disease was identified as *Legionella pneumophila* (McDade *et al.*, 1977). Numerous outbreaks of Legionnaires' disease have since occurred. In the 30 years of since the original Philadelphia outbreak, research has yielded a vast amount of knowledge into the etiology and pathogenesis of Legionnaires' disease.

1.2 The family Legionellaceae

There are now at least 49 described species of *Legionella*, as well as 70 subgroups within these species (Cordevant *et al.*, 2003) as determined by automated ribotyping. *L. pneumophila* is the predominant cause of Legionellosis in humans, accounting for ~ 90% of cases, with serogroup 1 accounting for 84.2% of *L. pneumophila* pneumonia (Yu *et al.*, 2002). The majority of remaining cases are caused by several species, predominantly *L. longbeachae* (3.9%) and *L. bozemanii* (2.4%). *Legionella* infections can also be caused by at least 16 other species of *Legionella* as determined by analysis of transfer DNA intergenic spacer length polymorphisms (De Gheldre *et al.*, 2001). *Legionella* infections

result from exposure to contaminated aerosols generated by a wide variety of mechanical sources from domestic hot water plumbing fixtures (Wadowsky *et al.* 1982) to hospital air conditioning cooling towers (Johnston *et al.*, 1987).

The legionellae are ubiquitous in natural aquatic systems, and survive in a wide range of harsh climates (Fliermans *et al.* 1981). Shortly after its discovery, *L. pneumophila* was found to be pathogenic for species of soil and water amoebae *Acanthamoebae* and *Naegleria* (Rowbotham, 1980). It was also suggested in this work that the *Legionella*-infected amoebae may represent the form responsible for human infection. It has been determined that the ability of *Legionella* species to grow within amoeba offered a survival benefit in harsh climates, as growth in *Acanthamoeba polyphaga* allowed for survival upon exposure to 50 mg/L free chlorine (Kilvington and Price, 1990). Since the initial description of the relationship between *Legionella* sp and amoebae, several species have been shown to be permissive for *Legionella* sp growth, including *Hartmanella vermiformis* (Fields *et al.*, 1993) and *Dictyostelium discoideum* (Hagele *et al.*, 2000).

1.3 *Legionella*-like amoebal pathogens (LLAP)

Legionella-like amoebal pathogens (LLAP) (Birtles et al, 1996) were first identified as a specific group within the family *Legionellaceae*, and share > 99% sequence identity to the previously described *L. lytica* LLAP. LLAPs are obligate intracellular pathogens of amoeba. The role of LLAPs in human infection has been assessed. In Nova Scotia, many

members of the LLAP group have been implicated in human infections, albeit infrequently, based on seropositive tests from individuals with community-acquired pneumonia (Marrie *et al.*, 2001). While some LLAPs could be cocultured with other pneumonia-causing agents, some patients were found to have been infected only with LLAPs (Marrie *et al.*, 2001).

1.4 Taxonomy

After comparing the DNA relatedness of the Legionnaires' disease bacterium *L. pneumophila* to environmental isolates, it was proposed that the legionellae be placed in a new family, the *Legionellaceae* (Brenner *et al.*, 1979). Primary investigations into the relatedness of the *Rickettsia*, *Coxiella* and *Legionella* species was done by analyzing fatty acid composition (Tzianabos *et al.*, 1981). These studies analyzed the lipid profiles by gas liquid chromatography, and suggested that *Coxiella* and *Legionella* shared evolutionary relatedness. These predictions were substantiated by phylogenetic analysis as determined by the 16S rRNA sequence relatedness (Weisburg *et al.*, 1989). The results of the 16S rRNA analysis demonstrated that *Rickettsia* and *Coxiella* do show distinct evolutionary relatedness with *Legionella*. More recently, research identified several homologous genes in both species, including the heat shock protein gene, *htpB* (Sampson *et al.*, 1990) and the macrophage infectivity potentiator (MIP) (Mo *et al.*, 1995). Furthermore, when sequences of the *dot/icm* virulence gene clusters of *Legionella* were compared to available sequences from the *Coxiella burnetti* genome, significant protein sequence identity in 3 genes, *icmT* (47%), *icmS* (50%) and *icmK* (68%) was identified

(Segal and Shuman, 1999). It is now recognized that the bacteria share considerable homology in these regions, and they are two of the small number of bacteria that use Type IVb secretion systems for pathogenesis (Segal *et al.*, 2005). The completed genome sequence made available in 2004 revealed that *Legionella pneumophila* shares ~ 42% of its genome (3.4 Mbp) with the *Coxiella burnetti* genome (1.9 Mbp) (Chien *et al.*, 2004).

1.5 Diseases caused by *Legionella*

The major disease caused by infection with *L. pneumophila* is an atypical pneumonia generally characterized by high fever, chills and non-productive cough (CDC fact sheet on *L. pneumophila*, 2005). There are an estimated 8,000-10,000 cases per year in the United States, with 10 – 15% being fatal. Incubation periods are generally between 2-10 days before the onset of symptoms. Several other symptoms have also been described during *Legionella* based pneumonias including diarrhea, abdominal pain and neurological difficulties (Yu *et al.*, 1982).

Pontiac fever is another disease caused by *Legionella* species. The disease is named after the original outbreak at an automotive plant in Pontiac, Michigan in 1968. 10 years after the original outbreak, serology demonstrated the causative agent to be idenitical to *L. pneumophila* the cause of Legionnaires' disease (Glick *et al.*, 1978). Pontiac fever is a febrile illness generally characterized by fever, malaise and headache. Infection usually lasts 2-5 days, with a high contact rate and low mortality rate (Glick *et al.*, 1978).

Other *Legionella* infections are less common and include pericarditis, surgical site infections and intraocular infections (Brown, 2004).

1.6 Diagnosis of legionellosis

Radiographic presentation of Legionnaires' disease is somewhat variable. While lower lobe infiltrate is common, all lobes of the lungs can become consolidated during infection, along with some pleural efusions (Kirby *et al.*, 1979). Infections with *Legionella* strains other than *L. pneumophila*, including *L. micdadei* and *L. bozemanii* show similar radiographic findings (Muder *et al.*, 1987). The variability in radiographic presentations makes it difficult to diagnose the disease on radiography alone. While sputum culture is capable of differentiating between pathogenic *Legionella* strains, the urine antigen test, which tests for the presence of *Legionella* specific polysaccharides, is the most reliable diagnostic test available (Murdoch, 2003). Recent data suggests that serological testing for *Legionella* antigens using ELISA may offer a complementary role to urine antigen detection (Rojas *et al.*, 2005). ELISA for anti-*Legionella* IgM antibodies positively identified 72.3% of infected patients compared to 53.3% for the urine analysis samples in a controlled study of previously diagnosed patients (Rojas *et al.*, 2005).

1.7 Metabolic requirements of *Legionella*

Early attempts to culture *Legionella* on laboratory media used for other fastidious bacterial species (i.e. Mueller-Hinton agar) were unsuccessful, and it was only after the

discovery of Charcoal-Yeast Extract agar, a modification of F-G agar that routine laboratory culture was made possible (Feeley *et al.*, 1979). It was later demonstrated that the addition of charcoal aided in the degradation of reactive oxygen species generated in the medium during aerobic growth (Hoffman *et al.*, 1983). Keto acids serve multiple roles in the growth of *Legionella* on laboratory media, including oxygen radical scavengers (Pine *et al.*, 1986), and as a primary carbon source (Tesh *et al.*, 1983). As well, *Legionella* has an absolute requirement for several amino acids including methionine, valine, and a partial requirement for L-cysteine (Tesh and Miller, 1981). Radiolabeling experiments demonstrated that glycerol and glucose were poorly incorporated into growing *Legionella* cells, while pyruvate and glutamate were more substantially incorporated. Glutamate was incorporated eight times more than glucose (Tesh *et al.*, 1983). It has been suggested that the lack of glucose utilization is due to a lack of glucose transporters (Keen and Hoffman, 1984 and Chien *et al.*, 2004).

1.8 Virulence factors

1.8.1 Phosphatases

The primary characterization of the *L. pneumophila* acid phosphatase was done in 1981 (Thorpe and Miller, 1981), and subsequent work by Nolte *et al.* demonstrated the presence of both acid and alkaline phosphatase enzymes (Nolte *et al.*, 1982). Analysis of the *L. micdadei* acid phosphatase demonstrated that the enzyme was capable of blocking superoxide production by neutrophils, suggesting that there was a role for the

phosphatases in virulence (Saha *et al.*, 1985). To determine whether the two phosphatases played a role in intracellular multiplication of *L. pneumophila*, random MudII4041 transposon mutagenesis was used to create mutants in both the alkaline and acid phosphatases (Kim *et al.*, 1994). Neither of the mutant strains was significantly impaired for intracellular growth in U937 cells, demonstrating that intracellular multiplication is not dependant on either of the phosphatases.

1.8.2 Phospholipase C

The study of enzymatic activity of *L. pneumophila* done by Thorpe and Miller also revealed the presence of phospholipase C activity (Thorpe and Miller, 1981). To further assess the role of phospholipase C in the pathophysiology of legionellosis, particularly tissue necrosis, various *Legionella* species were grown on media containing erythrocytes from various animals (Baine, 1985). All species tested were capable of lysing erythrocytes from animals whose cells contain high proportions of phosphatidylcholine. Purification of the phospholipase using anion exchange chromatography confirmed the ability of the protein to hydrolyse phosphatidylcholine, yet the purified protein was not haemolytic, indicating other factors are responsible for the observed erythrocyte lysis (Baine, 1988). It has been suggested that the phospholipase activity may disrupt cell membranes of immune system cells and lung tissue, and facilitate the progression of the disease. Similar to the phosphatase mutants, deletions in the gene encoding the phospholipase C protein (*plcA*) had no impact on infection of host cells (Aragon *et al.*, 2002). Also included in this analysis were chromosomal deletion mutants of two lipases,

LipA and LipB. Neither of these two lipases was required for intracellular growth, leading the authors to speculate that perhaps the cumulative role of these proteins is what is responsible for the tissue damage characteristic of the disease.

1.8.3 Zinc metalloprotease

The *L. pneumophila* 38-kDa zinc-metalloprotease has been extensively characterized (Keen and Hoffman, 1989). Mutant strains were non-haemolytic on blood agar, while otherwise serologically and biochemically identical to the parental strain. Expression of recombinant metalloprotease conferred haemolytic and cytotoxic activity in *E. coli*, further confirming the functions of this gene product (Quinn and Tompkins, 1989). The metalloprotease is exported to the periplasm before undergoing cleavage to produce the mature enzyme (Moffat *et al.*, 1994). Similarly to the phosphatase and phospholipase proteins discussed above, mutations in the metalloprotease (*mspA*) gene did not affect the intracellular growth of *L. pneumophila* in HL-60 cells (Szeto and Shuman, 1990). Again, like the other secreted enzymes, the MspA protein appears to play a role in the immune system alterations typical of the disease. The metalloprotease has been shown to inhibit neutrophil chemotactic response, while having no effect on monocyte chemotaxis, or on oxidative burst (Rechnitzer and Kharazmi, 1992). MspA is not detected upon initial infection of macrophages, but is detected after 24 hours, located in the phagosomes and distributed through the macrophage (Rechnitzer *et al.*, 1992). The role of MspA in immune system disruption was further elucidated when it was discovered that the protease could cleave CD4 molecules from the surface of T-cells, and cleave IL-2 (Mintz

et al., 1993). In further support of the notion that the MspA protein is involved in the pneumonia aspect of infection, mutations in the gene encoding MspA, *proA*, did attenuate virulence of mutant strains when compared to wild type in a guinea pig infection model (Moffat *et al.*, 1994b).

1.8.4 MIP

The macrophage infectivity potentiator, was first described as a 24-kDa surface expressed protein that was involved in infection of macrophages (Cianciotto *et al.*, 1989). It was determined that approximately 80-fold more mutant cells were required to initiate infection of macrophages when compared to wild type. The gene responsible for this protein was named *mip*, which encodes a highly basic (pI 9.8) protein (Engleberg *et al.*, 1989). Mutations in MIP have also been shown to affect the ability of *L. pneumophila* to infect and cause disease in animal models. MIP mutant strains not only required larger inocula to cause infection in guinea pigs, but also showed slower disease progression and fewer illnesses (Cianciotto *et al.*, 1990). The MIP protein was later shown to exhibit peptidyl-prolyl-cis/trans isomerase activity, an enzyme capable of refolding proteins, and bears significant homology to eukaryotic isomerases such as the FK506-binding proteins (FKBPs) (Fischer *et al.*, 1992). MIP is also required for maximal intracellular multiplication in *Hartmannella* amoebae and *Tetrahymena* ciliates, suggesting the possibility that *L. pneumophila* utilizes the same mechanism for infection of both human cells and protozoa (Cianciotto *et al.*, 1992). MIP has also been shown to be partially

required for replication in alveolar epithelial cells, and thus may also contribute to tissue damage during *L. pneumophila* infection (Cianciotto *et al.*, 1995).

Structural analysis revealed that the MIP protein exists in solution as a homodimer, with contact regions predicted to occur in the N-terminal ends of the monomers (Schmidt *et al.*, 1995). Recent data have, however, begun to separate the relationship between dimer formation and PPIase activity. N-terminal deletion mutants created in the MIP protein revealed that the truncated product retained isomerase activity, yet lost the ability to dimerize, and more significantly, was attenuated in a guinea pig model (Kohler *et al.*, 2003). Thus this work demonstrates that the isomerase activity of MIP is not the major function of MIP involved in infectivity. Immunolocalization studies have been used to determine the cellular location of MIP in extracellularly grown bacteria, as well as intracellularly grown bacteria and their host cells. During extracellular growth on BCYE, MIP is exposed on the cell surface. While intracellular bacteria also express the protein on their cell surface, MIP has been found on host cell membranes (Helbig *et al.*, 2001). Analysis of the expression of MIP RNA during intracellular growth in monocytes has demonstrated that immediately following infection, expression of MIP is temporarily repressed, but is at full expression levels by 24 hours (Wieland *et al.*, 2002).

1.8.5 Macrophage infectivity locus (mil)

Random transposon mutagenesis was used to isolate 32 mutant strains that had varying degrees of defects in replication and intracellular survival in macrophages, but little defect in protozoa (Gao *et al.*, 1998). The resulting mutants were determined not to

be altered in their response to sodium, and were classified into 6 groups based on the severity of their defects. Five of the 6 groups of mutants had no defect in intracellular multiplication in protozoan cells, suggesting the possibility that there may be specific loci that are responsible for fine-tuning infection in mamalian host cells. Similarly, transposon mutagenesis was used to isolate mutants defective for intracellular multiplication in both macrophages and protozoa, generally termed *pmi* mutants (Gao *et al.*, 1997). Further examination of a large set of macrophage-defective mutants, including many *pmi* mutants, also determined that the mutants defective for replication in macrophages were not necessarily defective for growth in alveolar epithelial cells, (Gao *et al.*, 1998b). As a result, mutant strains defective for intracellular survival only in macrophages were still capable of replicating in the lungs of A/J mice. Thus there appear to be loci specifically required for survival in phagocytes. To dissect the mechanism by which the *mil* defects affect intracellular multiplication in macrophages, one of the *mil* genes, GB111 was mutated and analyzed for intracellular growth defects (Harb and Abu Kwaik, 2000). Protein sequence analysis revealed homology to transport proteins, and laser scanning confocal microscopy demonstrated that the majority of phagosomes containing mutant cells co-localized with late endosomal marker (LAMP-1), suggesting an inability to avoid lysosomal fusion. Detailed analysis of *pmiA*, one of the genes identified by transposon mutagenesis, suggested that it was a transmembrane protein, and potentially homologous to proteins associated with type IV secretion systems in other bacteria (Miyake *et al.*, 2005). Interestingly, although the mutations in *pmiA* inhibit the pore forming activity attributed to the *dot/icm* system, the strains harbouring these mutations remain sensitive to sodium.

1.8.6 Legiolysin

Legiolysin was first described as a novel haemolysin (Rdest *et al.*, 1991), but later shown to be involved in production of brown pigment and yellow green fluorescence under UV light during stationary phase (Wintermeyer *et al.*, 1991). Legiolysin is a 38.9-kDa protein that bears homology to proteins involved in aromatic amino acid metabolism (Wintermeyer *et al.*, 1994). Mutagenesis of the *lly* gene encoding legiolysin revealed that while strains were still haemolytic and showed no defects in intracellular growth in macrophages or *A. castellanii*, they lost the ability to produce pigment and were non-fluorescent. The role of pigment production in *L. pneumophila* has been shown to offer protection from long-term exposure to light (Steinert *et al.*, 1995). *lly* mutants grown in media from wild type supernatants were protected from light based killing, whereas mutants grown without wild type supernatant were killed. Thus, the role of legiolysin appears to be in protecting *L. pneumophila* from the harmful effects of light.

1.8.7 Hsp60

Hsp60 is a protein of 548 amino acids is encoded by the *htpB* gene, which lies in an operon with the *htpA* gene (Hoffman *et al.*, 1989 and Hoffman *et al.*, 1990). The *Legionella pneumophila* Hsp60 shares 85% similarity to the Hsp60s from *E. coli* and *C. burnetti*. The significance of this protein in infection became apparent during studies of guinea pig immune responses to Hsp60 and OmpS (Weeratna *et al.* 1994). Animals that survived lethal challenges with *L. pneumophila* showed significant lymphocyte

proliferation responses and delayed –type hypersensitivity reactions to purified Hsp60. Further studies on the immune response to *L. pneumophila* Hsp60 revealed purified Hsp60 was able to induce interleukin-1 secretion in macrophage cultures (Retzlaff *et al.*, 1994).

The initial relationship between Hsp60 and virulence was determined using L929 cells and human monocytes (Fernandez *et al.*, 1996). Expression of Hsp60 increased initially upon internalization of virulent *L. pneumophila* in both cell lines, whereas adherent and internalized avirulent strains showed no increase in expression. Immunolocalization studies subsequently revealed that Hsp60 is surface localized in *L. pneumophila*, and also present in the periplasm (Garduno *et al.*, 1998). The role of Hsp60 in virulence was further clarified by the discovery that while polyclonal antibodies against OmpS inhibited association of both virulent and avirulent bacteria with HeLa cells, antibodies directed against Hsp60 inhibited only virulent bacteria (Garduno *et al.*, 1998b). A number of competition experiments with purified Hsp60 revealed it specifically contributes to the invasion of HeLa cells by wild type *L. pneumophila* (Garduno *et al.*, 1998b). It has thus been suggested that avirulent, salt-tolerant mutants have lost the ability to express Hsp60 on the bacterial cell surface, which results in their inability to invade HeLa cells.

The roles of Hsp60 as an immune system stimulant and virulence factor have been described in other species as well. The *H. pylori* Hsp60 has been shown to induce inflammatory responses in human gastric epithelial cells (Takenaka *et al.*, 2004).

Antibodies directed against the Hsp60 of *Clostridium difficile* have also been shown to partially inhibit adherence to host cells, and that the Hsp60 protein localizes with both the cytoplasmic and membrane fractions (Hennequin *et al.*, 2001).

1.8.8 OmpS

The major outer membrane protein (MOMP) of *L. pneumophila* was first described in 1985 (Butler *et al.*, 1985). Outer membranes were isolated by treating *L. pneumophila* cells with lysozyme to create spheroplasts, followed by density gradient centrifugation. Outer membrane fractions were then run on polyacrylamide gels and a single 28-kDa band was identified. Anti-MOMP antibodies were used to demonstrate the localization of MOMP on the bacterial cell surface. Purification of the protein and reconstitution into planar lipid membranes demonstrated that MOMP is a porin, with a channel conductance of 100 picoSiemens and a greater selectivity for cations rather then anions (Gabay *et al.*, 1985). Biochemical analysis of the protein revealed that it was complexed by intramolecular disulfide bonds (Butler *et al.*, 1985). Further characterization revealed that MOMP exists as a homotrimer, with two 28.5-kDa monomers, and a 31-kDa monomer which is covalently linked to the peptidoglycan through diaminopimelic acid, and serves as an anchor for the protein in the outer membrane (Butler and Hoffman, 1990 and Hoffman *et al.*, 1992b). The nucleotide sequence of *ompS*, the gene encoding MOMP, and subsequent protein sequence revealed that MOMP was expressed as a peptide with a 21 amino acid leader sequence that was cleaved to form the mature protein (Hoffman *et al.*, 1992). It was also determined that the promoter was not active in *E. coli*, and

furthermore, expression of the polypeptide from the inducible lac promoter was unsuccessful, possibly due to toxicity.

The major outer membrane protein has also been shown to be a significant antigen. Guinea pigs challenged with lethal intraperitoneal doses of *L. pneumophila* displayed strong delayed-type hypersensitivity (DTH) reactions, and strong lymphocyte proliferation responses to sublethal challenges of bacteria. The strong DTH response led to vaccination studies using purified MOMP, where it was shown that guinea pigs vaccinated with MOMP can survive lethal challenges with *L. pneumophila* (Weeratna *et al.*, 1994). These data suggest that MOMP plays an important role in the development of cellular immunity.

The role of MOMP in binding to complement and cellular uptake has also been examined. Purified MOMP has been shown to bind directly to the complement component C1q independent of antibody (Mintz *et al.*, 1995). Thus MOMP is capable of activating the classical complement pathway directly by binding component C1q. Additionally, a role for MOMP in cellular uptake and binding complement component C3 has been described (Bellinger-Kawahara and Horwitz, 1990). It was concluded that C3 opsonized MOMP containing liposomes adhered to monocytes, and were effectively phagocytosed. It was thus suggested that the major outer membrane protein might aid the uptake of *L. pneumophila* by monocytes. The physiological relevance of this work has however been brought into question, as complement levels in the lung are low, and monoclonal antibodies against C3 receptors were unable to block phagocytosis in a

variety of cell lines, including HeLa cells (Weissgerber *et al.*, 2003). Thus, while C3 can serve as an opsonin in vitro, the low levels of complement in the lung and the fact that anti-complement receptor antibodies do not block phagocytosis demonstrate a substantially diminished role for C3 in *L. pneumophila* uptake.

1.8.9 Type II secretion

L. pneumophila encodes a type II secretion system, partially encoded by the *pilBCD* operon (Liles *et al.*, 1998). Expression of these genes was most prominent during growth at 30°C when compared to 37°C, correlating with increased piliation. The third gene in the operon, *pilD*, is predicted to be a pre-pilin peptidase responsible for the cleavage of leader sequences from secreted proteins (Liles *et al.*, 1999). Mutations in this gene resulted in impaired intracellular growth in *H. vermiformis* and human macrophages. Additionally, mutants lacked three proteins normally found in the supernatant, and also lacked haemolytic activity, suggesting that the metalloprotease may not be secreted. Additional analysis also revealed a defect in the secretion of phospholipase A, acid phosphatase, lipase and an RNase in the mutant strain (Aragon *et al.*, 2000). Furthermore, mutations in the structural components of the type II secretion system and the pseudopilins only slightly inhibited intracellular growth, suggesting that there was some other *pilD* dependent mechanism required for intracellular survival (Rossier and Cianciotto, 2001).

1.8.10 Dot/Icm

The *dot/icm* genes, required for intracellular multiplication of *L. pneumophila*, were discovered simultaneously by two groups (Berger and Isberg, 1993 and Marra *et al.*, 1992). Avirulent mutants were unable to replicate in a variety of host cells, and are sodium tolerant (i.e. capable of growth on laboratory media containing sodium chloride). Several components of the Dot/Icm system, including IcmE and DotB have been implicated in conjugation of the broad host range plasmid RSF1010 (Segal and Shuman, 1998). Furthermore, *L. pneumophila* cells containing the RSF1010 plasmid were substantially inhibited in intracellular multiplication, suggesting that components of the conjugation system of the plasmid may inhibit the interaction of the Dot/Icm system and its natural substrate (Segal and Shuman, 1998). Components of a putative type IV secretion system, termed *lvh*, can functionally substitute for mutations in some *dot/icm* mutations that affect transfer of RSF1010 (Segal *et al.*, 1999). In 1998, it was reported that while many of the 19 known *dot/icm* genes did not have known homologues, four genes (*dotB, L, M,* and *G*) had some identity (~ 25% at the terminal ends) to components of conjugal transfer apparati (Vogel *et al.*, 1998). It was later reported that based on sequence identity, 14 of the *dot/icm* genes were homologous (>30% identity) to the ColIb-P9 IncI plasmid of *Shigella sonnei* (Segal and Shuman, 1999). One of the components of the *dot/icm* system, *icmT*, has been shown to be required for pore formation and escape from host cells (Molmoret *et al.*, 2002).

Despite several years of research, the effector proteins of this putative secretion system remained unknown. Several proteins have now been identified, through a variety of complex molecular analyses, that are secreted by the Dot/Icm system, including RalF (Nagai *et al.*, 2002), LidA (Conover *et al.*, 2003), SidA-H and SdeC (Luo and Isberg, 2004), SdeA (Bardill *et al.*, 2005) and YlfA (Campodonico *et al.*, 2005). None of the identified secreted proteins have been shown to be absolutely required for intracellular multiplication. LidA mutants were slightly inhibited intracellularly, but also showed altered viability on bacteriological media (Conover *et al.*, 2003). Similarly, SdeA mutants showed minor defects in intracellular multiplication (Bardill *et al.*, 2005). A few of the Dot proteins have been shown to be essential for viability on bacteriological media. In Lp02, mutations in the *dotL*, *dotM* and *dotN* genes, in the presence of an otherwise functional Dot/Icm system are non-viable, presumably as a result of the formation of a toxic membrane complex (Buscher *et al.*, 2005). In the *L. pneumophila* strain JR32, mutations in these genes are not lethal, but instead result in hypersensitivity to sodium chloride in the growth medium, demonstrating variation between the two strains.

Expression of the *dot/icm* genes is at least partially regulated by several proteins including the ppGpp synthetase RelA (*icmP*), RpoS (*icmP and icmR*) (Zusman *et al.*, 2002) and the GacA/S homologue LetA (*icmP*, *icmR* and *icmT*) (Gal Mor and Segal, 2003b). The CpxA/R system has been shown to regulate *icmR, V* and *W* genes (Gal-Mor and Segal, 2003). The CpxAR system, involved in regulating genes required for dealing with extracytoplasmic stress, are not required for intracellular growth in amoeba or human macrophages (Gal-Mor and Segal, 2003). Because only some of the *dot/icm* genes

have been shown to be regulated, it has been proposed that there are other regulatory proteins, possibly including the vegetative sigma factor, that are required for expression of the *dot/icm* genes (Gal-Mor *et al.*, 2002). Despite these examples, there is little information regarding the regulation of the majority of the *dot/icm* genes (Segal and Shuman, 1998).

1.9 Intracellular growth of bacteria

There are several bacteria that have evolved to spend at least some of their life cycle intracellularly. Not unexpectedly, the bacteria have also evolved a variety of mechanisms to accomplish this. In this section, the mechanism of uptake and avoidance of the host cell's endocytic pathway by several bacteria, including *Coxiella, Salmonella* and *Legionella* will be discussed.

1.9.1 *Coxiella*

Coxiella burnetii, the causative agent of Q fever, is evolutionarily related to *L. pneumophila* species, and displays a similar intracellular life cycle. The bacterium causes transient cytoskeletal reorganization through alterations of the F-actin, resulting in membrane protrusion (Meconi *et al.*, 2001). In macrophages, the integrin associated protein and the CR3 integrin are involved in uptake of virulent *Coxiella* (Capo *et al.*, 2003). While avirulent bacteria are phagocytosed and destroyed through a CR3 dependent process, virulent bacteria interact with the integrin associated protein, which

results in inhibition of activation of the CR3 protein, thus avoiding destruction. The phagocytosis of virulent *Coxiella* stimulates protein tyrosine kinases, and activates F-actin cytoskeletal rearrangements (Meconi *et al.*, 2001). The pseudopodal formation resulting from F-actin rearrangement segregate the CR3 receptor from the bacteria/host cell interaction site, thus allowing the bacteria to procede with infection (Capo *et al.*, 2003).

While *Coxiella* reside in acidic vacuoles, the acidity of the vacuoles has been shown to be independent of bacteria. In addition, vacuoles containing *Coxiella* do not fuse with lysosomes, as shown by the lack of cathepsin D, but acquire late endosomal markers (Ghigo *et al.*, 2002). After 48 hours of infection, the *Coxiella* vacuole accumulates monodansylcadaverine and displays protein LC3, which are both autophagic markers (Beron *et al.*, 2002). Additionally, the GTPase Rab7, a late endosome marker involved in regulating endosome trafficking, also localizes to the *Coxiella*-containing phagosome. The induction of autophagy by nutrient depletion has been shown to increase the number and size of replicative vacuoles (Gutierrez *et al.*, 2005). Additionally, overexpression of the autophagy markers LC3 and Rab24 also increased development of replicative vacuoles, furthering the notion that *Coxiella* utilizes the autophagy pathway for establishment of a replicative vacuole.

Thus, *Coxiella*, like *Legionella*, remains in the phagosomal compartment, but inhibits the fusion of degradative lysosomes.

1.9.2 *Salmonella*

Salmonella typhimurium, a bacterium capable of causing a variety of infections, is also capable of intracellular multiplication. Whereas treatment of the bacteria with UV radiation or the protein synthesis inhibitor Streptomycin results in increased phagosome-lysosome fusion, live *Salmonella* inhibits this fusion through a mechanism requiring protein synthesis (Ishibashi and Arai, 1990). After acidification of the phagosome in macrophages, the expression of PhoP, a component of PhoP/Q two-component regulatory system, is up-regulated ~ 66 fold, and this increase is inhibited by neutralization of the phagosome with ammonium chloride (Alpuche Aranda *et al.*, 1992). *Salmonella typhimurium* is internalized via macropinocytosis, and resides within spacious phagosomes (Alpuche-Aranda *et al.*, 1994). Strains containing constitutive PhoP mutations were defective for the formation of spacious phagosomes, suggesting one or more of the regulon components are involved in maintenance of the phagosome. Interestingly, the expression of listeriolysin in *Salmonella* alters its cellular localization, resulting in a portion of bacteria that reside in the cytosol, and not in phagosomes, exemplifying the different strategies employed by *Listeria* and *Salmonella* (Gentschev *et al.*, 1995).

The *Salmonella* phagosome has also been shown to fuse with lysosomes in primary macrophages, but the co-localization of Cathepsin L is thought to be due to methanol fixation of cells (Oh *et al.*, 1996). These *Salmonella* phagosomes acquire LAMP-1, cathepsin L, and were also shown to merge with lysosomes containing fluorescent

markers. In contrast, work done with macrophage cell lines, which do not undergo apoptosis to the extent of primary cells during *Salmonella* infection, do not fuse with lysosomes (Rathman *et al.*, 1997). While phagosomes acquire lysosomal glycoproteins and acid phosphatase, they fail to acquire mannose 6-phosphate receptor and Cathepsin L, and thus diverge from the degradative pathway. One of the proteins responsible for escape from lysosomal degradation is the SpiC protein, encoded in the SPI-2 pathogenicity island (Uchiya *et al.*, 1999). Mutations in this protein resulted in decreased intracellular survival, and purified protein was able to inhibit phagosome-lysosome fusion. *Salmonella* containing phagosomes retain Rab5, and also deplete Rab7 and 9, ensuring evasion of the lysosomal pathway (Hashim *et al.*, 2000). In addition, *Salmonella* excludes one of the NADPH oxidase complex components, flavocytochrome b(558), in an SPI-2 dependent manner, thus protecting the bacteria from reactive oxygen species (Gallois *et al.*, 2001). Modulation of the size of the *Salmonella*-containing vacuole (SCV) is mediated in part by the SopB gene product (Hernandez *et al.*, 2004). SopB mutants are unable to form the spacious phagosomes seen in wild type infection, which is due to decreased levels of phosphatidylinositol-3-phosphate in the membrane of the SCV.

Thus, while *Salmonella* does not escape the phagosomes of its host cell, it causes the phagosome to diverge from the endocytic pathway to create its intracellular niche.

1.9.3 *Legionella*

Legionella multiplies in a phagosome that associates early with mitochondria, and subsequently becomes surrounded by ribosomes and does not fuse with primary or secondary lysosomes (Horwitz, 1983). The bacteria also become surrounded by rough endoplasmic reticulum. The phagosome not only associates with the RER, but the phagosomal membrane, after fusing with RER vesicles, developes into a membrane similar to RER (Tilney *et al.*, 2001). During infection by *L. pneumophila*, monocytes express normal levels of major histocompatibility complex, but phagogomes exclude the molecule (Clemens and Horwitz, 1993).

Virulent *Legionella* do not acquire the small GTPase Rab5, involved in endosome fusion, whereas avirulent mutants do (Clemens *et al.*, 2000). The events that lead to evasion of the lysomal pathway are determined early during infection (Wiater *et al.*, 1998). Within thirty minutes of infection, phagosomes containing several different Dot/Icm mutants fuse with lysosomes, while wild type cells remain in unfused phagosomes. The expression of one of these proteins, DotA, is required prior to engagement of the host cell (Roy *et al.*, 1998). DotA mutant phagosomes acquire the endosomal marker LAMP-1 and Rab7 as early as five minutes after uptake. In concordance with the notion that the phagosome biogenesis events occur early during infection, avirulent DotA mutants can be rescued by co-infection with wild type bacteria when both are added simultaneously, but not separately (Coers *et al.*, 1999). In addition, co-infection of DotA mutants with Lp02, a thymidine auxotroph, into macrophages deprived of thymide also permitted growth of the *dotA* mutant, indicating that the rescue of avirulent bacteria is not due to replication of wild type cells. Certain Dot/Icm mutants

are capable of intracellular multiplication, but display altered phagosome morphology (Coers *et al.*, 2000). The IcmR mutants, that are somewhat able to resist lysosomal fusion, are capable of forming small phagosomes that permit limited intracellular growth. While a functional Dot/Icm system is required for cleavage of caspase 3, activation of the caspase did not rescue DotA mutants (Molmoret *et al.*, 2004b). Activation of caspase 3 does, however, result in cleavage of the Rab5 effector Rababptin-5, and this activation was required for halting the progress of the phagosome in the lysosomal pathway. Despite activation of caspase-3 in infected cells, these cells delay apoptosis until the final stages of bacterial replication (Abu-Zant *et al.*, 2005).

The signalling events upon attachment to the protozoa *Hartmannella vermiformis* have been studied using *L. micdadei* (Abu Kwaik *et al.*, 1998). Multiple host cell proteins, including the Gal/GalNAc-inhibitable lectin are dephosphorylated upon attachment, yet result in a phagosome that is not surrounded by RER. In contrast, *L. pneumophila* attachment to the lectin results in RER association with the phagosome, indicating differences between the two strains. While the Dot/Icm components have also been implicated in pore forming ability in macrophage membranes, this process is not essential for abrogation of phagosome-lysosome fusion, as some mutants (IcmW) are capable of pore formation, but not avoidance of lysosomal fusion (Zuckman *et al.*, 1999). Other proteins translocated by the Dot/Icm system, the Vip proteins, have been implicated in vesicle maturation, as mutants are defective in lysosomal protein trafficking through different mechanisms (Shohdy *et al.*, 2005). The VipA protein affects carboxypeptidase Y trafficking, while VipD affects multivesicular body formation.

Recently it has been demonstrated that in the later stages of bacterial replication in macrophages, the *Legionella*-containing phagosomes acquire the endosomal marker LAMP-1 and becomes acidified (Sturgill-Koszycki and Swanson, 2000). Inhibition of vacuole maturation inhibits bacterial multiplication, and proper vacuole maturation is required for production of acid resistant bacteria. The importance of phagosome acidification has been questioned, as *L. pneumophila* has been shown to multiply in phagosomes of human monocytes regardless of phagosomal pH (Wieland *et al.*, 2004). Although the extent of phagosomal acidification in this model was substantially less than that shown previously (Sturgill-Koszycki and Swanson, 2000), it is similar to pH ranges shown to support growth of *L. pneumophila* (Pine *et al.*, 1979).

While the maturation process of the *Legionella*-containing vacuole resembles the process of autophagy, mutations in some of the autophagy genes of *Dictyostelium discoideum* did not inhibit maturation of the phagosome (Otto *et al.*, 2004). In macrophages however, phagosomes acquire autophagy enzymes Atg7 and Atg8 in response to 10-30 kDa Dot/Icm released protein species (Amer and Swanson, 2005). The maturation of phagosomes in naip5 A/J macrophages is delayed when compared to the non-permissive C57BL/6 J macrophages, and it is suggested that naip5, an inhibitor of caspase 3 may be responsible for this arrestation.

It has also been demonstrated that *L. pneumophila* is capable of replication in the host cell cytosol (Molmoret *et al.*, 2004). Independent of the hydrolytic enzymes secreted

by the type II secretion system, and during the later stages of *L. pneumophila* infection in macrophages and amoebae, the bacteria continue to replicate in the cytoplasm.

Thus, *L. pneumophila* requires the Dot/Icm system for the formation of a replicative vacuole that evades the lysosomal pathway during replication.

1.10 Developmental cycles of bacteria

Developmental cycles have been described for several bacteria, and two paradigms will discussed here to facilitate the discussion of the *Legionella* developmental cycle, *Chlamydia* and *Azotobacter*.

1.10.1 *Chlamydia*

Chlamydia species undergo intracellular differentiation from environmentally resilient, infectious elementary bodies to actively dividing reticulate bodies that are readily distinguishable by electron microscopy (Tamura *et al.*, 1971). After uptake of *Chlamydia* into host cells, the bacteria reside in a vacuole that does not fuse with lysosomes, or become acidified (Heinzen *et al.*, 1996). The elementary bodies quickly become associated with the Golgi apparatus, and remain associated with them throughout the developmental cycle (Rockey *et al.*, 1996). Electron and fluorescence microscopy revealed that *Chlamydia psittaci* resides in multiple, separate vacuoles located in the perinuclear region.

Microscopic and chemical examination of the inner membranes from both forms suggested differences in chemical composition. The reticulate bodies resemble prototypical gram-negative bacteria, and possess a poorly defined inner membrane, compared to a spherical, rigid appearance seen in the elementary bodies (Popov *et al.*, 1978). The differences in membrane structure correlate with infectivity, as elementary body cell envelopes are capable of proper trafficking in host cells (Eissenberg *et al.*, 1983). Not surprisingly, the differences in membrane structure correlate with differences in protein composition. The membranes of elementary bodies contain a number of cysteine-rich proteins that are not solubilized by SDS in the absence of reducing agents, while the reticulate bodies contain scarce amounts of these proteins (Hatch *et al.*, 1984). Studies using reticulate bodies purified from host cells revealed that they are able to synthesize proteins that closely resemble the cysteine rich proteins, while the elementary bodies do not synthesize proteins (Hatch *et al.*, 1985).

The role of cysteine in the developmental cycle has been characterized and shown to be required for proper intracellular differentiation (Allan *et al.*, 1985). The omission of cysteine from the growth media of host cells resulted in severely delayed development into reticulate bodies. Re-addition of cysteine restored proper differentiation of intracellular bacteria into reticulate bodies. In addition, cAMP also inhibits differentiation of reticulate bodies (Kaul and Wenman, 1986). A cAMP receptor, associated with reticulate bodies may be involved in the inhibition of differentiation, and withdrawl of cAMP at any point during infection restored the development into infectious elementary

bodies. Subsequent studies revealed that cAMP-mediated inhibition affects transcription of the MOMP gene (Kaul *et al.*, 1990).

Protein synthesis has been implicated in the reduction of the major outer membrane protein (MOMP) to monomers within one hour of internalization, a process required for proper intracellular growth (Hatch *et al.*, 1986). MOMP monomers became cross-linked upon exposure to the extracellular environment, while the cysteine-rich proteins were not altered. Thus, the final differentiation of *Chlamydia* occurs after intracellular growth, and is dependent on exposure to the extracellular environment. The cysteine-rich proteins are also developmentally regulated, with synthesis occurring late in the infection cycle (Sardinia *et al.*, 1988).

Stage specific DNA binding proteins have also been identified. By probing SDS PAGE separated lysates from elementary bodies with chlamydial DNA, three DNA binding proteins have been identified that are not present in reticulate bodies (Wagar and Stephens, 1988). One such protein, the HU homologue Hc1, has been identified and is expressed late in the developmental cycle (Hackstadt *et al.*, 1991). The Hc1 protein, when expressed in *E. coli* results in chromosome condensation, similar to that seen in *Chlamydia* (Barry *et al.*, 1992). Additionally, high-level expression of Hc1 results in a general decrease in transcription and translation in *E. coli* (Barry *et al.*, 1993). Lower levels of expression resulted in decreased expression of specific genes, including the OmpC and OmpF proteins, most likely due to a net relaxation in chromosomal DNA. Similarly, a second binding protein, termed KARP, also a homologue of eukaryotic DNA

binding proteins has also been identified (Perara *et al.*, 1992). Protein profiles of intracellular bacteria harvested at several time points revealed that, as hypothesized, multiple proteins are differentially synthesized, some unique to each phase (Plaunt and Hatch, 1988).

Regulation of several stage-specific genes is dependent on the major sigma factor, sigma 66, including two genes of unknown function *ltuA* and *B*, and *hctA* (Fahr *et al.*, 1995). Expression of these genes is substantially up-regulated upon addition of chlamydial sigma 66 to in vitro expression assays. In comparison, regulation of *hctB* was unaffected by addition of the sigma factor. Sigma 66 (RpoD) is constitutively expressed throughout the growth cycle, while two alternate sigma factors are developmentally regulated: RpsD is up-regulated 4 hours after infection, and RpoN is increased after 30 hours (Mathews *et al.*, 1999). In addition, the putative sigma factors RsbW, RsvB1, RsbV2 and RsbU have also been identified, and they are all, with the exception of the constitutively expressed RsbV2 factor, upregulated during logarithmic growth (Douglas and Hatch, 2000). Another putative regulator, EUO, which is most prominent during intracellular replication, has been shown to bind the AT-rich regions in the promoter of the cysteine-rich proteins, but the effect of this binding in vivo is not known (Zhang *et al.*, 1998). Finally, microarray technology revealed that approximately 22% of the genes in the *C. trachomatis* genome may be developmentally regulated (Nicholson *et al.*, 2003). Thus, *Chlamydia* species contain a number of putative and documented regulators that are growth phase regulated and thought to regulate the genes necessary for progression through the developmental cycle.

1.10.2 *Azotobacter*

Detailed electron microscopic examination of the encystment process in *Azotobacter vinelandii* has been performed (Wyss *et al.*, 1961). Within 36-48 hours on appropriate media, the cell body begins to condense, and is accompanied by the formation of a "bark-like exine" structure surrounding the cell. Coinciding with this, the space between the outer material and the cell body becomes filled with an electron permeable structure, termed intine. Membranes become clearly defined, and the formation of inclusions occurs by 5 days (Wyss *et al.*, 1961). The cyst like form is resilient to a number of harsh stimuli, including UV and gamma radiation, desiccation and sonic resistance (Socolofsky and Wyss, 1962).

The formation of poly-β-hydroxybutyric acid (PHB) also occurs concurrently with the differentiation into the cyst form, reaching a maximum of 35% of the dry weight of the cell, and decreasing as the culture ages (Stevenson and Socolofsky, 1966). The *phbBAC* operon encodes the biosynthetic pathway required for PHB synthesis. The expression of these genes is regulated by an AraC family activator named PhbR, which binds one of the two promoters located in the upstream region of *phbB*, while the other is regulated by the stationary phase sigma factor (Peralta-Gil *et al.*, 2002).

Alginate, an exopolysaccharide produced by several bacteria, including *P. aeruginosa* and *A. vinelandii*, and has been implicated in cyst formation, and formation of the intine and exine layers in the latter (Campos *et al.*, 1996). The AlgD protein,

necessary for the production alginate is essential to cyst formation. Expression of the *algD* is partially dependent on the sigmaE sigma factor, which in turn is sequestered to the inner membrane through an anti-sigma factor MucA. The regulation of alginate production is controlled by the alternative sigma factor E, encoded by the *algU* gene (Moreno *et al.*, 1998). Mutations in the *algU* protein, or over expression of the MucABCD genes encoding the anti-AlgU sigma factors, resulted in a defect in encystment, and down-regulation of AlgD expression. Similarly, the response regulator AlgR is also necessary for production of alginate in an AlgD independent manner, suggesting multiple levels of regulation may exist in the expression of alginate (Nunez *et al.*, 1999).

A GacS homologue has been identified in *A. vinelandii* that is responsible for full alginate production, and for the production of PHB (Casteneda *et al.*, 2000). The particular signal sensed by the GacS homologue is unknown. GacA, the cognate response regulator of GacS, regulates expression of *algD* and of the stationary phase sigma factor *rpoS* (Casteneda *et al.*, 2001) and the latter, in turn, also regulates the *algD* gene. The regulation of cell wall biosynthesis by the *ampDE* operon, encoding a membrane protein of unknown function (AmpE) and a N-acetyl-anhydromuramyl-L-alanine amidase (AmpD), has also been shown to regulate alginate biosynthesis and encystment (Nunez *et al.*, 2000).

Thus a complex network exists in *A. vinelandii* that regulate not only the encystment process, but also the production of alginate, an exopolysaccharide produced during encystment.

1.10.3 *Legionella*

Preliminary ultra-structural analyses of intracellularly grown *L. pneumophila* demonstrated morphological differences when compared to bacteria grown on bacteriological media (Rodgers, 1979). Similar findings were obtained with the causative agent of Pittsburgh pneumonia agent, *L. micdadei* (Gress *et al.*, 1980). A thickened cell envelope, characterized by a thick electron dense layer in the periplasmic space was found in many of the intracellularly grown bacteria, and found less frequently in bacteria grown on bacteriological media. Similar pleomorphisms of intracellular *Legionella* sp have been described following examination of infected guinea pig tissue (Katz *et al.*, 1984). The intracellular bacteria were typically short bacilli, but coccoid and coccobacillary forms were also present. Additional evidence of the morphogenesis of *L. pneumophila* during intracellular multiplication was obtained using *Acanthamoeba castellanii* (Cirillo *et al.*, 1994). Amoebae grown bacteria, while also being more invasive for epithelial cells, showed dramatic differences in ultrastructure when grown intracellularly. The amoebae grown bacteria had a thicker cell wall, a denser cytoplasm and multiple electron-transparent vesicles, when compared to agar grown bacteria although no phenotypes were connected with this morphogenesis (Cirillo *et al.*, 1994).

The differentiation of intracellular *L. pneumophila* has subsequently been studied in detail, and the terminally differentiated form has been termed the Mature Intracellular Form (MIF) (Garduno *et al.*, 2002). During intracellular replication, *L. pneumophila* cells lack the ability to retain the Gimenez stain; however, after being intracellular for 48-72 hours, the bacteria acquire the ability to retain it. By comparison, stationary phase bacteria, while having the ability to retain the Gimenez stain, do not stain as brightly as MIFs, suggesting differences in membrane composition. Electron microscopy revealed that while stationary phase bacteria generally showed typical Gram-negative cell wall ultrastructure, MIFs displayed definite differences. These include an abundance of poly-β-hydroxybutyrate (PHB) cytoplasmic inclusions, a thickened cell wall, and multilaminated intracytoplasmic membranes. The differences in ultrastructure also correlate with increased resistance to environmental stimuli, including resistance to rifampin and gentamicin, alkaline pH and detergents. Definitive characterization of the ultrastructural changes experienced by *L. pneumophila* during its progression through the intracellular growth cycle in HeLa cells was accomplished by Faulkner and Garduno (2002). While bacteria grown on plates showed little ultrastructural change during the early stages in intracellular replication, within four to six hours, MIFs differentiated into the typical Gram-negative form seen during replication. In the late stages of replication the bacteria begin to regain the ability to retain Gimenez stain, and finally, in the post replicative phase they become MIFs, completing the cycle. The MIF like morphology has also been demonstrated during *L. pneumophila* infection of *Hartmanella vermiformis*, demonstrating that this cycle also occurs in amoebae (Greub and Raoult, 2003).

Several virulence traits are regulated in response to nutrient starvation, which is characterized by the accumulation of the alarmone ppGpp (Hammer and Swanson, 1999). These include motility, sodium sensitivity, cytotoxicity, infectivity, and collectively these traits make up what is termed the transmission phenotype. The stationary phase sigma factor RpoS is not required for intracellular growth in HL-60 and macrophage cells, but it is required for growth in *A. castellanii* (Hales and Shuman, 1999). In these studies, where RpoS was disrupted by a transposon, the mutant strain did not display growth phase resistance to stress, including osmotic, oxidative and acid tolerance. Construction of a null-mutant strain confirmed that resistance to the above stresses was RpoS independent (Bachman and Swanson, 2001). Mutations in RpoS did, however, alter the response to sodium and reduce expression of flagellin, but were not required for contact-dependent cytotoxicity. The mutants were able to persist in phagosomes that, like DotA mutants, co-localized with LAMP-1, but were partially defective for intracellular multiplication.

The RpoS mediated effects on flagella production are dependent on ppGpp synthesis (Bachman and Swanson, 2001). The RelA protein, a ppGpp synthase that induces expression of the RpoS in response to nutrient limiting conditions, is required for pigment production and flagellin expression, but is dispensible for intracellular growth (Zusman *et al.*, 2002). Strains induced to express RelA during exponential phase exit the exponential phase and express the transmission traits (Hammer *et al.*, 2002). More recent evidence suggests that RpoS may also inhibit the transmission phase during exponential growth (Bachman and Swanson, 2004). A model has been proposed that suggests ppGpp

mediated up-regulation of RpoS, along with LetA/S mediated repression of CsrA (see below) are required for induction of the transmission phenotype.

Screens for mutants that did not properly express the flagellin genes identified a number of other regulators, including the flagellar sigma factor FliA, a two-component regulatory system termed LetA/S, and an associated protein termed LetE (Hammer *et al.*, 2002). While having no effect on intracellular multiplication in macrophages, LetA/S mutants were unable to express the transmission traits previously identified. LetA has been shown to be required for intracellular multiplication in *A. castellanii* (Gal-Mor and Segal, 2003).

Repression of the stationary phase phenotypes is mediated by the conserved RNA binding protein CsrA, and this repression is relieved by the LetA/S system (Molofsky and Swanson, 2003). CsrA repression must be alleviated in order for infection to procede properly, and CsrA activity must be restored for intracellular multiplication. CsrA mutant strains show increased expression of RpoS and LetE proteins, and also demonstrated delayed growth rates and lower levels of pigment production (Forsbach-Birk *et al.*, 2004). Another protein, Hfq, which is regulated by RpoS and LetA, stabilizes CsrA mRNA, and mutant strains show decreased growth rates and pigmentation defects (McNealy *et al.*, 2005).

In summary, *L. pneumophila* undergoes differentiation in the later stages of infection to a resilient, hyperinfectious form. During in vitro growth, a complex regulatory

cascade, in response to nutrient conditions, induces the expression of genes that confer resilience to environmental stimuli, motility and cytotoxicity.

1.11 Integration Host Factor

1.11.1 History

Integration Host Factor (IHF) was initially purified as the bacterial host factor necessary for recombination of the λ bacteriophage (Nash and Robertson, 1981). Integration of λ bacteriophage requires two phage-encoded proteins, Int and Xis that are capable, in the presence of *E. coli* extracts, of promoting integration of phage DNA in vitro (Pastrana and Brammar, 1979). Crude cell lysates of *E. coli* were subjected to several chromatographic purifications, and the host factor activity was attributed to a heterodimer, composed of two proteins of 9.5 and 11-kDa (Nash and Robertson, 1981). Subsequent investigations revealed that in addition to being required for λ phage recombination, mutants in either of the subunits also demonstrate defects in isoleucine biosynthesis, prompting the authors to speculate that IHF regulated one of the enzymes in the isoleucine biosynthetic pathway (Friedman *et al.*, 1984). These predictions were confirmed for the isoleucine pathway, and also in valine biosynthesis, due to decreased levels of acetohydroxy acid synthetase I (Friden *et al.*, 1984). The decrease in enzymatic activity was attributed to lowered levels of Ilv-specific mRNA, suggesting that IHF may be a positive regulator of gene expression. Integration host factor has also been shown

not only to be involved in integration of λ phage, but also in the expression of cII and O genes, as in vitro expression studies demonstrated decreased levels of cII and O protein in IHF mutants (Peacock *et al.*, 1984). IHF has also been implicated in regulation of the early promoter of bacteriophage Mu, and of two of the repressor gene promoters, as a positive regulator (Goosen *et al.*, 1984). The genes for each subunit were cloned and sequenced soon after IHF was shown to be responsible for the integration of several bacteriophages, and their gene expression. The DNA sequence of the α subunit of IHF revealed homology to the DNA binding protein HU (Miller, 1984), and the gene was first identified, based on mutagenesis and restoration of Mu replication, as a gene lying downstream of the *pheST* operon (Mechulam *et al.*, 1985). The gene encoding the β subunit of IHF (*himD*, *hip*) was initially isolated by exonucleolytic digestion of DNA fragments capable of complementing an IhfB mutant (Flamm and Weisberg, 1985). Comparison of the deduced nucleotide sequence revealed that this subunit, along with the α subunit, belong to the type II family of prokaryotic DNA binding proteins, also known as the histone-like proteins. The IHF genes are conserved among bacteria, and can also functionally complement across species, as was demonstrated by the ability of the *himA* and *hip* gene products of *Serratia marcescens* to substitute for the *E. coli* IHF subunits in vitro (Haluzi *et al.*, 1991).

The DNA sequence that is recognized by the IHF complex was originally determined using a small subset of promoters. Nuclease protection assays revealed a putative recognition sequence of 5'-T(C/T)AA(A/G)TTGaTA(A/G)TT(C/T)AACtA-3' (Craig and Nash, 1984). This sequence was later confirmed as the site required for replication of the

pSC101 plasmid, located between the DnaA and RepA binding sites (Gamas *et al.*, 1986). Further mutational analysis revealed specific conserved nucleotides required in the consensus site defining it as 5'-(A/G)N(A/G)AANNNNTTGAT(A/T) (Gardner and Nash, 1986). It was speculated that the proximity of these essential binding sites (DnaA, RepA and IHF) indicated these proteins might function as a complex necessary for efficient plasmid replication. The currently accepted IHF recognition sequence is now defined as 5'-WATCAANNNNTTR-3', where W = A or T, and R = A or G (Hales *et al.*, 1994).

The precise effect of IHF binding to specific targets in DNA was first described in 1987 by using oligonucleotides that contained consensus binding sites located at different positions on the DNA fragment in gel retardation assays (Prentki *et al.*, 1987). These synthetic oligonucleotides demonstrated altered electrophoretic mobility of the fragments when incubated with IHF. Fragments containing centered IHF binding sites displayed less mobility than DNA fragments containing sites located on the ends, demonstrating that IHF serves to bend the DNA molecule at this point (Prentki *et al.*, 1987). Furthermore, the presence of multiple IHF binding sites within the same molecule resulted in minimal increases in mobility, demonstrating only minor co-operativity of binding. The binding of IHF to target DNA is also dependent on the extent of supercoiling of the DNA, as IHF is required for Int binding to negatively supercoiled DNA in order to promote lambda integration (Richet *et al.*, 1986). Further research determined that not only is IHF required for bending, but purified Int is not capable of bending DNA, as judged by electrophoretic mobility shift (Robertson and Nash, 1988).

The importance of bending DNA in the activity of IHF has been documented. Inversion of the consensus sequence in the *ilvPG* promoter (thus the sequence is moved to the opposite strand, and is in the opposite direction), which has been previously shown to be regulated in part by IHF, had no influence on protein expression levels (Parekh and Hatfield, 1996). Furthermore, the IHF consensus could be replaced with the consensus sequence of a non-prokaryotic DNA bending protein, the lymphoid enhancer-binding factor (LEF-1), and using in vitro expression assays, purified LEF results in similar levels of expression of the *ilvPG* operon. Thus, the role of IHF as an activator is to position the DNA into a conformation that is accessible by RNA polymerase, and activity is not due to a specific IHF-RNA polymerase interaction.

The interaction of the IHF complex with the target sequence occurs mainly through its contacts with the minor groove of the double helix (Yang and Nash, 1989), and a minor interaction with the major groove as determined by base analogue substitutions in the target sequence (Wang *et al.*, 1995). The crystal structure of IHF with DNA containing the consensus sequence has been determined. The bend in the DNA molecule of approximately 160 degrees results mainly from intercalation of the target sequence with proline residues in the middle of both IHF subunits, and the interaction in the crystal structure occurs exclusively in the minor groove (Rice *et al.*, 1996). In addition, the N-terminal arms of each subunit associate tightly with the minor groove, and act like clamps to stabilize the structure. Mutational analysis of the C-terminal region of the IhfB gene has shown that this is the region necessary for sequence specific binding (Mengeritsky *et al.*, 1993). Specifically, mutations in conserved amino acids 87, 89 and 90, all located in

the third α-helix, resulted in dramatic decreases in binding efficiency. In addition, a mutation in the C-terminal domain, R87G, allowed IHF to bind DNA containing two mismatches in the consensus sequence, while the same mismatches abolished binding of wild type IHF protein. Despite the difference in specificity, both wild type and mutant IHF molecules were still able to bend DNA, as judged by electrophoresis (Mengeritsky *et al.*, 1993). DNA binding domain swapping between IHF subunits has allowed for heterodimers of IHF, with both of the monomers containing a DNA binding domain from IhfA (Zulianello *et al.*, 1994). These heterodimers showed similar binding efficiencies to wild type heterodimers, whereas the opposite domain swapping (heterodimers containing only β binding domains) yielded little binding affinity. Thus, the specificity of binding to the consensus sequence appears to reside mainly in the β monomer binding domain. While the sequence specificity resides in the C-terminal domain of the β subunit, the C-terminal domain of the α subunit appears to determine binding strength (Zulianello *et al.*, 1995). While IHF functions in many bacteria as a heterodimer, purified subunits have also been shown to form homodimers capable of specifically binding DNA in vitro (Zulianello *et al.*, 1994). In *E. coli*, it has also been suggested that at least IhfB subunits may form functional heterodimers in vivo, based on the ability of IhfA mutants to maintain proper levels of *ori*p15A (Hiszczynska-Sawicka and Kur, 1997).

An architectural role for integration host factor also exists. Analysis of *E. coli* and *S. typhimurium* genomes revealed palindromic sequences of approximately 100 base pairs separated by IHF consensus sites, termed repetitive IHF-binding palindromic (RIP) sites (Oppenheim *et al.*, 1993). These RIP elements are evenly dispersed throughout their

respective genomes, and not necessarily in promoters, suggesting that IHF may be not only a regulator of gene expression, but may also play a role in condensation of the chromosome. In addition, analysis of the concentration of free IHF in stationary phase cells indicates IHF may also bind DNA non-specifically at higher concentrations (Murtin *et al.*, 1998). The implication of this finding is that high concentrations of IHF in stationary phase result in non-specific binding of the chromosome by IHF, and serves to model the DNA late in the growth cycle. In support of this finding, compaction of the *E. coli* nucleoid occurs concomitantly with the increase in IHF expression, and IHF has been shown to be a major component of the stationary phase nucleoid (Ali Azam *et al.*, 1999). By measuring the torsion in small DNA molecules, it was determined that IHF did induce compaction of DNA molecules (Ali *et al.*, 2001). In addition, IHF has also been shown to be universally distributed throughout the *E. coli* nucleoid (Azam *et al.*, 2000).

1.11.2 Roles of IHF in osmoregulation in *E. coli*

As mentioned above, integration host factor has been implicated in the regulation of enzymes responsible for isoleucine biosynthesis. Several other roles have also been documented. The OmpF protein of *E. coli* is responsible for osmoregulation under low osmolarity conditions. IHF mutants have been shown to continue production of OmpF under high osmolarity conditions, compared to wild type strains in which OmpF becomes undetectable (Tsui *et al.*, 1988). *LacZ* reporter fusions to the promoter of the *ompF* gene revealed that the increased expression of OmpF was due to increased transcription. Further analysis of this system revealed that IHF binds two regions within the *ompF*

promoter and gel electrophoresis demonstrated that it introduced a bend in the DNA (Ramani *et al.*, 1992). Using an in vitro transcription system, it was demonstrated that the inhibition of *ompF* transcription seen upon addition of IHF could be reversed by addition of the positive regulator OmpR, thus OmpR was capable of out competing IHF. The role of IHF binding in *ompC* expression appears to be the same, as IHF mutants display higher levels of OmpC than does the wild type (Huang *et al.*, 1990). Similarly, IHF has also been implicated in the negative regulation of the *ompB* gene, demonstrating the multifaceted role of IHF in osmoregulation in *E. coli* (Tsui *et al.*, 1991).

1.11.3 Developmental Regulation

The expression of IHF has been shown to be growth phase dependent. As determined by western blot, IHF levels increased from 0.5 to 1 ng of protein during exponential phase, and increased to between 5 and 6 ng of protein in stationary phase *E. coli* cells (Ditto *et al.*, 1994). Expression of both subunits is dependent on RelA and SpoT, as determined by promoter activity levels in the respective null mutants, suggesting that IHF is upregulated during the starvation response (Aviv *et al.*, 1994). Furthermore, expression of the *himA* gene also requires the stationary phase sigma factor RpoS, whereas expression of the *himD* gene is unchanged in *rpoS* mutants. In *E. coli*, IHF is also autoregulatory. Expression of both subunits is increased in each of the single deletion mutant backgrounds, with *himA* being expressed to the greatest level, suggesting that IHF is a negative regulator of its own gene expression (Bykowski and Sirko, 1998). Despite the differences in IHF expression, no subunit specific phenotypes were observed.

IHF is also involved in the regulation of glucose starvation response in *E. coli*, as judged by two-dimensional gel electrophoresis, and IHF has been directly implicated in transcriptional control of at least one of these genes, *uspA* (Nystrom, 1994). The genes in this regulon are upregulated in stationary phase, and are poorly induced in a *himA* mutant. Regulation of the *him* genes is not identical in all species, as *himA* and *himB* levels decrease upon entry into stationary phase in *N. gonorrhoeae* (Hill *et al.*, 1998). While the *himB* transcript was generally more highly expressed than that of *himA*, both transcripts were decreased upon entry into stationary phase.

The role of IHF in developmental regulation has been studied in other bacteria. In *Caulobacter crescentus*, expression of several genes involved in hook biogenesis is temporally regulated. While expression of these genes is dependent on sigma-54, a number of genes also require IHF for proper expression (Gober and Shapiro, 1990). IHF has also been implicated in the regulation of *C. crescentus* flagellar genes (Gober and Shapiro, 1992). This study demonstrated that the IHF binding sites and 3' enhancer sequence are required for transcription of the *flaNQ* operon, and implicates IHF in the controlled expression of genes required for differenciation into swarmer cells.

In *Chlamydia trachomatis*, the cysteine-rich proteins are expressed late in infection, and are encoded in an operon (*omcAB*), which is partially regulated by integration host factor (Zhong *et al.*, 2001). In vitro transcription assays demonstrated that IHF positively affected expression of the *omcAB* operon, leading the authors to speculate that IHF plays

a role in the stage-specific expression of late chlamydial genes, and may therefore be required for proper morphogenesis from the reticulate to the elementary bodies.

1.11.4 IHF and plasmids

A role in plasmid replication has also been identified for IHF. IHF binds the origin of replication of the R6K gamma plasmid and is essential for replication (Filutowicz and Appelt, 1988). In addition, maintenance of the pSC101 plasmid also involves IHF. Mutations in topoisomerase in an IHF mutant background, which resulted in less negative supercoiling of the plasmid DNA, allowed maintenance of the plasmid, suggesting that IHF is required for proper supercoiling of the pSC101 plasmid (Biek and Cohen, 1989). IHF has also been implicated in the partitioning system of P1 plasmid prophage, and required for maximal binding of the partitioning protein ParB (Funnell, 1988). IHF is also involved in the regulation of transfer genes in several plasmids, including the *traM* gene of plasmid R100, as it is required for inhibition of *traM* gene expression (Abo *et al.*, 1993). IHF can also regulate plasmid copy number, as mutants in either or both of the subunits resulted in decreased copy number of the pACYC184 and 177 plasmids in *E. coli* (Hiszczynska-Sawicka and Kur, 1997).

1.11.5 Roles of IHF in virulence

IHF plays a role in virulence gene expression in several pathogens. In enteropathogenic *E. coli* (EPEC) strains, the ability to promote enterocyte

attaching/effacing lesions (AE), is dependent on a type III secretion system for the characteristic cytoskeletal rearrangements observed in target cells (Jarvis *et al.*, 1995). The expression of several genes, including *escJ*, *escV*, *eae*, *tir* and the gene encoding intimin, were each dependent on a functional IHF protein (Friedberg *et al.*, 1999). The LEE1 region is directly regulated by IHF, which includes the Ler regulator. The Ler regulator subsequently regulates genes in the LEE2 and LEE3 regions, thus IHF both directly and indirectly regulates the genes in the LEE pathogenicity island, and is required for full virulence (Friedberg *et al.*, 1999). IHF is also involved in the expression of the K5 capsule gene cluster. Mutations in both integration host factor subunits resulted in five- fold lower expression of the KpsE protein, which is involved in transport of the polysaccharide through the membrane (Simpson *et al.*, 1996).

IHF is also required for virulence in *Brucella abortus*. In *B. abortus*, intracellular multiplication is dependent on a functional type IV secretion system that is regulated in part by IHF (Sieira *et al.*, 2004). When the promoter of the first gene in the operon, *virB*, had its IHF binding site removed, the resulting mutant strains were unable to replicate intracellularly, demonstrating the importance of IHF mediated regulation of the type IV system.

The regulation of the *Salmonella* plasmid virulence (spv) locus is dependent on the DNA binding protein SpvR, whose expression is regulated by IHF (Marshall *et al.*, 1999). Inactivation of IHF resulted in a decrease in SpvR expression, and subsequent reduction in the expression of the *spvABCD* genes. Transcriptional up-regulation of the

spv operon by SpvR is antagonized by the Leucine-responsive regulatory protein (Lrp), and thus the interplay between IHF and Lrp proteins determines expression levels of the *spv* operon.

In *Pseudomonas aeruginosa*, the production of alginate, a mucoid substance involved in biofilm formation and antibiotic resistance, is regulated in part by a two component regulatory system involving the regulator AlgB (Ma *et al.*, 1998). While high expression of AlgB requires the sigma factor AlgT, basal levels of expression in non-mucoid strains requires IHF (Wozniak and Ohman, 1993). The alginate biosynthetic gene *algD* is the first gene in the operon for alginate biosynthesis, and is also regulated by IHF (Wozniak, 1994). Further examination of the role IHF plays in regulation of AlgD production revealed that in IHF mutant strains, alginate production was decreased by 50%, thus demonstrating that while dispensable, IHF is required for full expression (Delic-Attree *et al.*, 1996).

As mentioned above, the *N. gonorrhoeae* IHF is down-regulated in stationary phase. Purified IHF was previously shown to bind the promoter of *pilE*, responsible for production of the type IV pili, by gel retardation assay (Hill *et al.*, 1997). IHF is responsible for expression from one of the *pilE* gene promoters ($pilEp_1$), as determined by reporter analysis (Fyfe and Davies, 1998). Reporter activity was three to four-fold lower in the IHF mutant backgrounds.

Finally, *Shigella flexneri* plasmid-encoded virulence genes are also regulated in part by IHF (Porter and Dorman, 1997). Transcription of invasion genes is specifically repressed upon entry into stationary phase in IHF mutant backgrounds. Further analysis revealed that both the *virF* and *virB* genes are also repressed in IHF mutants.

Thus, IHF has been implicated in the stage specific expression of several virulence genes and systems across a broad range of bacterial species.

1.11.6 Other DNA binding proteins related to IHF

The HU proteins of *E. coli* are small, heterodimeric, basic DNA binding proteins encoded by the *hupA* and *hupB* genes (Laine *et al.*, 1980). Sequence analysis of the proteins revealed that they are closely related to each other and to the IHF subunits (Drlica *et al.*, 1987). The HU protein is one of the most abundantly produced nucleoid proteins in exponential phase (Ali Azam *et al.*, 1999) and may participate in the replication initiation complex of the chromosome (Dixon and Kornberg, 1984). In this regard, *E. coli* strains containing mutations in both of the HU subunits displayed asymmetric cell division and were unable to support infection of the Mu bacteriophage (Huisman *et al.*, 1989). Furthermore, the HU protein has been shown to specifically bind to DNA molecules containing single stranded breaks, which are common during DNA replication (Castaing *et al.*, 1995). Mutations in the HU protein, which binds and bends DNA in a non-specific manner, are not complemented by over expression of IHF subunits, demonstrating separate roles for these DNA binding proteins (Boubrik *et al.*,

1991). As well, due to the non-specificity of the binding, HU can inhibit the binding of IHF to the *E. coli* oriC at high concentrations, presumably by randomly occupying the specific IHF binding site (Bonnefoy and Rouviere-Yaniv, 1992).

H-NS is a 15-kDa DNA binding protein that preferentially binds double stranded DNA (Friedrich *et al.*, 1988). H-NS forms dimers at low concentrations, mainly through hydrophobic interactions, and tetramers at higher concentrations (Falconi *et al.*, 1988). H-NS has also been shown to preferentially bind curved DNA molecules in electrophoretic assays (Yamada *et al.*, 1990). Mutants in H-NS display a pleotropic phenotype, attributed to changes in the topology of the chromosome, suggesting an architectural role for this protein (Hulton *et al.*, 1990). The over-expression of H-NS in *E. coli* is toxic, resulting in cell death. Microscopic analysis revealed that the nucleoids of cells overproducing H-NS were extremely dense and compacted, furthering the notion that the protein models the chromosomal structure (Spurio *et al.* 1992). H-NS is expressed throughout the growth cycle, most abundantly in exponential phase (Ali Azam *et al.*, 1999). Interestingly, the *L. pneumophila* genome does not contain homologues of the *E. coli* H-NS protein.

1.12 Role of ions in virulence gene expression

There are multiple examples of ions regulating gene expression in bacteria. While there are fewer examples of bacteria that regulate expression of their virulence genes in this manner, two systems have been extensively characterized, the *Yersinia* low calcium

response, and *Bordetella* BvgA/S systems. Specific examples in other bacteria will also be discussed below.

1.12.1 *Yersinia* low calcium response

The relationship between calcium and virulence has been intensively characterized, and the correlation between growth, morphology and virulence has been reported (Berche and Carter, 1982). When plated on media depleted for calcium at 37°C, wild type *Y. enterolytica* forms both fast growing avirulent colonies, and slow growing, highly virulent colonies. Mutants have been isolated that display a calcium blind phenotype, as they are affected for virulence gene expression regardless of the calcium concentration (Yother and Goguen, 1985). These mutations were localized to the pCD1 plasmid, and the genes for the low calcium response (lcr) were identified therein. The role of calcium in multiple gene regulation was determined in 1986 using reporter fusion assays, where 4 proteins, termed Yops (yersinial outer membrane proteins) were shown to be co-ordinately regulated upon exposure to calcium (Straley and Bowmer, 1986).

Initial characterizations of the role of calcium involved measuring the uptake of radiolabeled calcium by virulent *Yersinia* species (Perry and Brubaker, 1987). Virulent bacteria did not accumulate calcium due to simultaneous export, yet at sub-inhibitory concentrations, virulent bacteria bound higher amounts of calcium then did pCD1-less strains. Thus, while it was suggested that this finding indicated a specific calcium binding ligand, the ligand responsible was not determined. It has also been shown that sodium

may play a small role, and appears to be involved in arresting growth during calcium depletion (Brubaker, 2005).

The first regulator implicated in the calcium response was the *lcrH* protein, identified by transposon mutagenesis (Perry *et al.*, 1986). LcrH mutants were limited for growth in both ATP and calcium containing media, were less virulent then wild type, and showed altered expression of the YopJ protein, indicating a general role in expression of virulence determinants (Price and Straley, 1989). The role of LcrH is to act as a chaperone for the YopD protein, which regulates Yop translocation (Francis *et al.*, 2000). Thus, the virulence defects previously seen in LcrH mutants are due to defects in Yop secretion, while the LcrH mediated defects in calcium sensing remain unknown.

While mutations in LcrH resulted in both calcium and ATP sensing defects, the LcrR protein has been implicated solely in the calcium response. While expression of LcrR is not significantly affected by calcium, mutant strains were unable to suppress expression of virulence genes in the presence of the ion (Barve and Straley, 1990). These studies also demonstrated that the LcrR mutants failed to express the *lcrG* gene, the first in the *lcrGVH* operon. Subsequent research determined that it was the loss of LcrG that was responsible for the calcium blind phenotype seen in LcrR mutants, and that LcrG is produced regardless of calcium, but is secreted in response to calcium (Skryzpek and Straley, 1993). The LcrG mutants also continued to export V antigen and YopM in the presence of calcium, indicating LcrG is a negative regulator of transport.

While the mechanism by which calcium modulates gene expression is currently unknown, recent studies have implicated calcium-binding crystallins. A protein termed *Yersinia* crystalline has been identified that is related to a super family of proteins with diverse biological functions, collectively termed crystallins (Jobby and Sharma, 2005). The *Yersinia* crystallin has been shown to bind calcium with high and low affinity sites, and sequence analysis suggests it may be periplasmic. The authors suggest that the role of this protein may be to bind extra calcium as a stress response. It has been suggested that this protein may be involved in virulence based solely on comparative genomics, but this requires further investigation (Golubov *et al.*, 2003).

1.12.2 BvgAS locus

The changes in virulence factor production of *Bordetella pertussis* in response to magnesium in the growth media have been well characterized (Idigbe *et al.*, 1981). Control of virulence gene expression in *B. pertussis* is controlled primarily by the BvgAS two-component regulatory system (Scarlato *et al.*, 1990). The BvgAS system is composed of a transmembrane sensor protein, BvgS, and a cytoplasmic activator protein, BvgA. Analysis of the *bvgAS* promoter revealed the presence of 5 promoters, the P2 promoter being constitutively expressed, and the others being expressed in response to environmental stimuli such as magnesium sulfate and nicotinic acid (Scarlato *et al.*, 1990). This system, when transferred into *E. coli* is capable of regulating virulence gene reporter expression in response to environmental stimuli (Roy *et al.*, 1989). Exposure of *B. pertussis* to magnesium sulfate or nicotinic acid results in a Bvg mediated repression

of virulence genes (*ptx*, *cya* and *fha*) in minutes, while negative autoregulation occurs several hours later (Scarlato and Rappuoli, 1991). The bacteria therefore alternate between three phases depending on the growth conditions; expressing virulence genes (Bvg+), expressing only some virulence genes (Bvg^i), and expressing no virulence determinants (Bvg-) (Vergara-Irigaray *et al.*, 2005).

The mechanism by which BvgS transmits signals to BvgA in response to environmental stimuli has been extensively examined. Spontaneous mutations created in the linker region of BvgS, which connects the transmembrane domains with the cytoplasmic domain, resulted in strains that were non-responsive to magnesium sulfate (Miller *et al.*, 1992). These constitutive strains, termed Bvg^C produced high levels virulence factors despite the presence of magnesium sulfate in the media. Mutations in the transmembrane and C-terminal domains abolished BvgS mediated signalling. Signalling from BvgS occurs by an immediate autophosphorylation, followed by transfer of the phosphate to the BvgA protein, which is then able to activate gene expression (Uhl and Miller, 1994). The mechanism of BvgA phosphorylation is more complex than for the prototypical two-component regulators. Upon autophosphorylation of the transmitter domain, the phosphate is transferred to the receiver domain, and then to the C-terminal domain of the protein before the final phosphorylation transfer to BvgA (Uhl and Miller, 1996).

As was the case with *Yersinia*, the exact mechanism by which magnesium sulfate inhibits the activation of BvgA is unknown. Sequence analysis has revealed the presence

of PAS domains in the linker/sensor region of the BvgS protein, domains that can sense the presence of oxygen, redox potential, and light (Taylor and Zhulin, 1999). PAS proteins have also been found in voltage-sensitive ion channels. The incubation of purified BvgS with quinones has been shown to inhibit phosphorylation of BvgA, but mutations in the PAS domain only weakly inhibited phosphorylation, suggesting the quinone does not inhibit the signal (Bock and Gross, 2002). Thus, while it is tempting to speculate that these PAS domains are responsible for sensing magnesium sulfate levels, PAS domains have not been shown to sense magnesium or nicotinic acid, and therefore the mechanism by which this occurs remains unknown.

1.12.3 Sodium/virulence mechanisms

Sodium has been implicated in the virulence of several bacterial species. In *Pseudomonas aeruginosa*, ShaA, a sodium/proton antiporter, is required for sodium sensitivity and virulence (Kosono *et al.*, 2005). Sha mutants displayed decreased viability on media containing 0.3 M sodium chloride, but not potassium chloride, specifically during stationary phase. Additionally, the mutant strains were 50% less lethal in a mouse model, and bacterial counts from various organs were decreased. It is suggested that, similar to sodium antiporter of *B. subtilis*, which is required for sporulation, the activity of ShaA may affect the regulation of several transcriptional regulators required for virulence gene expression, including the alginate production anti-sigma factors (Kosono, *et al.*, 2004).

In *Vibrio cholerae*, the production of exopolysaccharide, involved in biofilm formation, is dependent on the sodium-driven motor protein Mot (Lauriano *et al.*, 2004). It has been previously shown that lack of flagella induced the production of exopolysaccharide, which is required for biofilm production (Watnick *et al.*, 2001). The mutations isolated in the Mot protein, as well a chemical inhibition of the sodium-driven motor, resulted in a decrease in exopolysaccharide production, and reduced amounts of cholera toxin and Tcp. The relationship between exopolysaccharide production and Mot function appears to be mediated through the response regulator VpsR. Thus, it is predicted that sodium driven Mot activity begins a regulatory cascade that, through VpsR, mediates exopolysaccharide production.

The effect of sodium chloride exposure on virulence factor production has also been studied in *Streptococcus pyogenes* (Nakamura *et al.*, 2004). Two-dimensional gel electrophoresis was used to monitor expression of virulence proteins upon exposure to 3% sodium chloride. The expression of Sic, SpeF and mitogenic factor 3 were all decreased upon exposure to sodium chloride. While general ion effects cannot be ruled out due to the exclusion of other cations in the experiment, this study does demonstrate an effect of sodium on virulence gene expression in these bacteria.

Finally, cytotoxin production in *Salmonella choleraesuis* is down regulated in response to 4% sodium chloride (Ho and Chou, 2001). Again, while the possibility that this change in expression is a general osmolarity occurance, sodium is capable of altering

gene expression. Repeating these experiments using other cations such as potassium and calcium would clarify whether this is a sodium specific effect.

1.12.4 Role of Sodium in Virulence of *Legionella*

The inhibitory role of sodium chloride in the growth of *Legionella* species has been known for some time. The addition of NaCl to Charcoal Yeast Extract (CYE) agar resulted in a decrease in colony forming units (c.f.u) from tissue inoculum, whereas there was no c.f.u. difference in stock cultures (Feeley *et al.*, 1979). Similar results were obtained by omitting NaCl from liquid media (Saito *et al.*, 1981). The influence of serial passage on media on virulence has been examined, and it was determined that repeated passage on Supplemented Mueller Hinton (SMH) agar (supplemented with ferric pyrophosphate and L-cysteine) resulted in decreased virulence in guinea pigs and embryonated eggs (Elliot and Johnson, 1982). Avirulent mutants can be selected for by as few as five serial passages on SMH agar (Catrenich and Johnson, 1988). While virulent bacteria are not capable of growth on media containing sodium chloride, they are not killed, and can remain viable and infectious following incubation on media containing sodium chloride.

In addition, the inability of tissue derived *L. pneumophila* to grow on media containing NaCl led investigators to study the effects of sodium on the growth of virulent versus avirulent strains (Catrenich and Johnson, 1989). By individually adding components of the virulent-inhibitory media SMH agar, specifically the Casein Acid

Hydrolysate (CAH), to potassium buffered CYE media, the growth of virulent bacteria was inhibited. Liquid chromatography determined that the inhibitory component was sodium chloride. *Legionella pneumophila* has been shown to require magnesium and potassium for optimal growth (Tesh and Miller, 1982), and it was suggested that increased amounts of sodium may affect intracellular levels of potassium and magnesium and thus may alter mechanisms required for virulence (Catrenich and Johnson, 1989).

By exploiting the sodium dependent conversion of virulent bacteria, mutants have been isolated that are unable to replicate in macrophages (Vogel *et al.*, 1996). Random transposon mutagenesis has been used to identify mutants that are unable to grow in macrophages, and are also resistant to sodium chloride (Sadosky *et al.*, 1993). Some of these mutants were found to contain mutations in the previously described *icm* genes. Interestingly, the addition of LiCl to the growth media was permissive for growth of avirulent mutants, whereas it was not for virulent strains. Thus, LiCl could also be used to distinguish virulent from avirulent bacteria, although the growth differences were not as pronounced (Sadosky *et al.*, 1993). In addition, some mutants that were complemented *in trans* regained the ability to kill macrophages while remaining sodium resistant, suggesting either incomplete complementation or plasmid effects in some mutants. One of the Dot proteins, DotL, has been implicated in increased sensitivity to sodium (Buscher *et al.*, 2005). In Lp02, the mutation in DotL is lethal, whereas in JR32, the mutation results in a hypersensivity to sodium, leading the authors to suggest that the DotL protein serves to regulate the activity of the Dot/Icm complex, and the decreased

viability results either due to the formation of toxic membrane complexes, or by forming an unregulated pore in the Dot/Icm system.

Several of the regulatory proteins that have been studied also have roles in sodium resistance. The stationary phase sigma factor (RpoS) has been implicated in sodium sensitivity (Bachman and Swanson, 2001). While mutations in RpoS result in sodium resistance, and despite the number of genes known to be regulated by this sigma factor, the mechanism by which it controls sodium sensitivity remains unknown. Mutations in the two component regulatory system LetA/S and the associated protein LetE are also known to cause sodium resistance (Hammer *et al.*, 2002). Over expression of the ppGpp synthase RelA can somewhat restore the sodium sensitivity phenotype, particularly in LetE mutants. As mentioned above, these regulators have been shown to alter the expression of some of the *dot/icm* genes, also involved in the sodium sensitivity response. Another regulator shown to influence sodium sensitivity is the CsrA RNA binding protein, which represses the transmission phenotype and is regulated by the LetA/S system (Molofsky and Swanson, 2003). Mutations in CsrA cause exponentially growing cells, which are normally resistant to sodium, to become sensitive. Another regulator, termed LpnR1 has been implicated in negative regulation of RpoS, yet the effect on sodium sensitivity remains unknown (Lebeau *et al.*, 2004).

Research Objectives

One of the first observations regarding the virulence of *Legionella pneumophila* was made by Catrenich and Johnson (1988), who demonstrated that avirulent mutants could be generated by repeated passage on media supplemented with 145 mM sodium chloride. Subsequent to this original observation, several genes have been identified that are associated with this sodium tolerant, avirulent phenotype, yet the relationship between sodium and virulence is not understood. Previous research in this lab, demonstrated that the promoter of the *ompS* gene, encoding the Major Outer Membrane Protein (MOMP), is bound by an unidentified protein termed OmpT that, upon exposure to sodium chloride, is no longer able to bind.

It is hypothesized that when vegetative bacteria go from a low sodium environment such as natural freshwater (where Na^+ concentrations are in the μM range) to environments with higher sodium content such as in host cells, where the concentration of sodium is expected to be higher, sodium sensing could act as a signal to induce the expression of genes required for intracellular multiplication. The aim of this study is to use the gene encoding the major outer membrane protein as a tool to elucidate the effector(s) responsible for the sodium mediated effects on virulence in *Legionella pneumophila*. The first step was to characterize the sodium mediated protein expression differences by looking globally at changes by using a proteomic approach. Secondly, regulatory elements in the *ompS* promoter region, and subsequently, proteins that bind this region of DNA will be identified both by bioinformatics and PCR based mutagenesis.

The second goal of this study was to characterize the protein identified above, Integration Host Factor. The role of Integration Host Factor will then be examined for its role in sodium sensitivity, and subsequently in virulence, as judged by the ability to multiply intracellularly in HeLa cells. In addition, the role of IHF in expression of known virulence genes, including *htpAB* and *dot/icm* genes will be analyzed. As a protein whose expression is increased post exponentially, the role of this protein is also analayzed for its function in the developmental cycle, as determined by electron microscopy, and by analyzing its effects on a variety of other MIF specific phenotypes.

CHAPTER 2: MATERIALS AND METHODS

2.1 Bacterial strains and Cloning Vectors

All bacterial strains used in this study are listed in table 1. *Legionella pneumophila* Philadelphia 1 (serogroup 1), the type strain for the genus, was obtained from the Center of Infectious Diseases, Centers for Disease Control, Atlanta GA, USA. Svir, the type strain, is a spontaneous streptomycin resistant derivative of *Legionella pneumophila* Philadelphia-1, provided by J. C. Feeley from the Centers for Disease Control and Prevention (Hoffman *et al.*, 1989). *Legionella pneumophila* Lp02, an isogenic restriction modification mutant, thymidine auxotroph, was obtained from Dr. Michelle Swanson at University of Michigan Medical School, Ann Arbor, MI, USA. The Lp02 strain is presumed to be a restriction modification mutant based introduction and subsequent curing of the pAM40 plasmid, and spontaneous thymine auxotrophs were generated by selection on trimethoprim (Berger and Isberg, 1993). *Lp02ΔdotB* deletion mutant JV918 was obtained from Dr. Joe Vogel. All bacterial frozen stocks were stored in nutrient broth supplemented with 10% dimethylsulfoxide (DMSO) at -70˚C.

All *L. pneumophila* strains were grown at 37˚C on Buffered Charcoal Yeast Extract (BCYEα) or in Buffered Yeast Extract (BYEα) broth (per liter of ddH_2O: 10 g Yeast extract, 1 g [2-(2-amino-2-oxoethyl)-amino] ethanesulfonic acid (ACES), 1 g α-ketoglutaric acid, and potassium hydroxide to a final pH of 6.6-6.7. For plates, 8.5 g agar and 1.5 g of activated charcoal were added prior to adjustment of pH with potassium

hydroxide. After autoclaving, media received 0.04 g/L of L-cysteine pH 6.6-6.7, 0.025% ferric pyrophosphate and streptomycin sulphate to a final concentration of 100 μg/ml (Str100). Where appropriate the following additional compounds were added to the indicated final concentrations: kanamycin 40 μg/ml (Kan40), gentamicin 10 μg/ml (Gent10), chloramphenicol 4 μg/ml (Cam4), metronidazole 20 μg/ml (Met20) and thymidine 100 μg/ml (Thy100). All broth cultures were incubated statically at 37°C for three hours before being incubated with aeration at 37°C at 100 rpm.

All *E. coli* strains were grown on Luria Bertani (LB) media (per liter of ddH_2O, 5 g Yeast extract, 10 g tryptone peptone, 10 g of sodium chloride, and for agar plates 16 g of agar). Where appropriate the following additional compounds were added to the indicated final concentrations: ampicilin 100 μg/ml (Amp100), kanamycin 40 μg/ml (Kan40), gentamicin 10 μg/ml (Gent10), chloramphenicol 20 μg/ml (Cam20) and metronidazole 20 μg/ml (Met20). Kanamycin and gentamicin were used in media for all passages and starting frozen stocks, and for enumeration, but were generally omitted for most experiments. For transformations, 40 μl of 20 mg/ml (in dimethylformamide (DMF)) 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) was spread on each plate. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 or 2 mM where indicated.

Table 1. List of all strains and plasmids used in this study.

Strain	Description	Reference or source
L. pneumophila		
Svir	Spontaneous Str^R isolate of Philadelphia-1 strain	Hoffman *et al.*, 1989
Lp02	Str^R, Thy^-, $HsdR^-$ derivative of Philadelphia-1 strain	Berger and Isberg, 1993
JV918	Lp02Δ*dotB* mutant strain	Sexton *et al.*, 2004
Lp02*ihfA*	Lp02 Str^R, Thy^-, $HsdR^-$ derivative of Philadelphia-1 strain *ihfA*::*gent*^{R} (single knock-out) mutant strain	This study
Lp02*ihfB*	Lp02 Str^R, Thy^-, $HsdR^-$ derivative of Philadelphia-1 strain *ihfB*::*kan*^{R} (single knock-out) mutant strain	This study
Lp02Δ*ihfAB*	Lp02 Str^R, Thy^-, $HsdR^-$ derivative of Philadelphia-1 strain *ihfA*::*gent ihfB*::*kan* (IHF double knock-out) mutant strain	This study
Lp02Δ*rpoS*	Lp02 Str^R, Thy^-, $HsdR^-$ derivative of Philadelphia-1 strain *rpoS*::*kan* mutant strain	Bachman and Swanson, 2001
Lp02/pMMB206	Lp02 containing plasmid pMMB206	This study
Lp02/pMMB206::*ihfAB*	Lp02 containing plasmid pMMB206::*ihfAihfB*	This study
Lp02Δ*ihfAB*/pMMB206	Lp02Δ*ihfAB* containing pMMB206	This study

Lp02Δ*ihfAB*/ pMMB206::*ihfAB*	Lp02Δ*ihfAB* containing pMMB206::*ihfAihfB*	This study
E. coli		
DH5α	F' *endA1 hsdR17* (r_k^- m_k^+) *supE44 thi-1 recA1 gyrA* (Nal^r) *relA1* Δ(*lacIZYA-argF*)*U169 deoR* (ϕ80d*lac*Δ(*lacZ*)M15), host cells for plasmid constructs	New England Biolabs
X1849/pPH1JI	F- *tonA*53 *dapD*8 *minA*1 *purE*41 *supE*42 Δ(*gal-uvrB*)47 λ- *minB*2 *his*-53 *nalA*25 *metC*65 *oms-1 T3*^{r} Δ(*bioH-asd*)*29 ilv*-277 *cycB*2 *cycA*1 *hsdR*2 containing plasmid pPH1JI (gentr)	Our lab
DH5α/pMALp2intB2	DH5α containing the pMAL vector (New England Biolabs) containg the p2intB2 gene; Amp^R	Hiltz, M.Sc. thesis, Dalhousie, University, 1999
Plasmids		
pBluescriptII KS(+)	Cloning vector; Amp^R	Stratagene
pUC18	Cloning vector; Amp^R	Invitrogen
pMMB206	Derivative of pRSF1010; P_{tac} promoter and IPTG-inducible *lacIQ* system; Amp^R, Cam^R	Michele Swanson
pBH6119	Promoterless green fluorescent protein (GFP) vector; Amp^R, thymidylate synthetase	Hammer and Swanson, 1999

pBORdxA	Dual suicide vector pBOC20 with *Bacillus subtilis sacB* and *Helicobacter pylori rdxA* (nitroreducase) as counter-selectable markers; Cam^R	Ann Karen C. Brassinga
pMMB206::*ihfAB*	pMMB206 containing coding sequences of *ihfA* and *ihfB*	This study
pBS::P1/P4 (*ihfA*)	pBS containing the P1/P2 and P3/P4 amplicons of *ihfA*	This study
pBS::P1/P4:: km^r (*ihfA*)	pBS::P1/P4 (*ihfA*) containing the kanamycin resistance marker km^r	This study
pBO*rdx*::P1/P4::$Gent^R$ (*ihfA*)	pBOCRdxA containing the P1/P4::$Gent^R$ of *ihfA*	This study
pBO*rdx*::P1/P4:: km^r (*ihfA*)	pBOCRdxA containing the P1/P4::km^r of *ihfA*	This Study
pUC18::P1/P4 (*ihfB*)	pUC18 containing the P1/P2 and P3/P4 amplicons of *ihfB*	This study
pUC18::P1/P4::km^r (*ihfB*)	pUC18::P1/P4 (*ihfB*) containing the kanamycin resistance marker km^r	This study
pBO*rdx*::P1/P4::km^r (*ihfB*)	pBO*rdx* containing P1/P4::km^r of *ihfB*	This study

Table 2. List of oligonucletides used in this study

Name	Oligonucleotide sequence (5' to 3' direction)
IHFα P1	ATGGAATTCCATCAGATTAATGAC
IHFα P2	AGAGGATCCTCTCATTACACCAAG
IHFα P3	CTGGGATCCTTGCTTAGTGCGTTC
IHFα P4	AGTGAGCTCAGATTGTGATTAACG
IHFβ P1	GCGAATTCGGGATGATTTCTCCATTTCAG
IHFβ P2	GCGGATCCCGTCGCGAGCCAAGTTCCCAC
IHFβ P3	GCGGATCCCTCACCTCGTTTTGTCGCGTA
IHFβ P4	GCAAGCTTTGGAAGGCTATACAGAGAGCA
IHFA comp F2	GCGGAATTCGAGACAACAAACTACTTTC
IHFαCOMPF	GCGGAATTCGAGACAACAAACTACTTTC
IHFαCOMPR	GCGGATCCAAGGAGCGTTCATGTATT
IHFβCOMPF	GCGAATTCCGCGACAAAACGAGGTGAGT
IHFβCOMPR	GCAAGCTTGGGTACACTACCACGC
IHFαCOMPF2	GCGGAATTCGAGACAACAAACTACTTTC
IHFβCOMPR2	GCGAATTCGGGTACACTACCACGC
IHFβ comp R2	GCGAATTCCGCGACAAAACGAGGTGAGT
IHFA2(βcompF)	GCAAGCTTGGGTACACTACCACGC
IHF no prom	GCGGATCCGCTATAATCAAGAAACTGG
RT IHFα Rev	ATGATCGTGAACGCACTAAGC

RT IHFα For	GGCTTAAAGGTAACAACCCTTTCT
RT IHFβ For	AACTTGGCTCGCGACGAATTGACT
RT IHFβ Rev	TCCGAACTCATTGAACACATCGCT
R6	GATTAGCGGATCCTAACAGAA
F6	ATCTTGAATTCCTCCACTGTA
Prom Rev	AGCAAGCTTTGTAAGATTTGATCAGTATCT
F9	TTAATAGTCATGCACTCCC
DotA For	GAGAATTCGTACTTTGCGGAGGCTTCAT
DotA Rev	GAGGATCCTCTCACCCAATAACCCTTGC
DotD For	GAGAATTCCTGTCTGGATGCTTTTGCAG
DotD Rev	GAGGATCCTCATTGTTCCAGCGCAACCA
IcmT For	GAGAATTCATCGGCCAGTGCCGAATTTT
IcmT Rev	GAGGATCCGCTGAAAATCCACCTGCCAT
Gent For	CTCGCGGCCGCTTGTTAGGTGGTGGCGGTACTT
Gent Rev	CTCGCGGCCGCTTGACATTAAGCCTGTTAGG

* Underlined nucleotides indicate restriction sites

2.2 Molecular Techniques

2.2.1 Restriction digests

Restriction digests were performed according to manufacturer's (New England Biolabs (NEB) MA, USA) instructions in 25 µl reactions. Approximately 1 µg of DNA was incubated with 10x reaction buffer and 1x BSA where indicated. Restriction digests were generally incubated at 37°C (unless otherwise indicated) for 1 hour. Digests done with 2 enzymes were allowed to digest for an additional 30 minutes. Reactions were stopped with loading buffer (see gel electrophoresis below) and then subjected to gel electrophoresis. When required, sticky ends resulting from endonuclease digestion were filled in using T4 DNA polymerase (NEB). Immediately following restriction digest, 1 µl of T4 DNA polymerase and 1 µl of 10 mM dNTPs were added, and the reaction incubated at room temperature for 30 minutes. The reaction was then stopped with loading buffer (see below) and DNA subjected to agarose gel electrophoresis.

2.2.2 Agarose gel electrophoresis

Agarose gel electrophoresis of DNA was done in 1% agarose, with 1x TAE (made from a 50x stock (per liter of ddH_2O; 242 g Tris base, 57.1 ml glacial acetic acid and 100 ml 0.5 M EDTA pH 8.0), and Ethidium Bromide (approximately 18 µl of a 0.1 mg/ml stock solution added to 50 ml of molten agarose). All samples were mixed with loading buffer (200 mM Tris-HCl, pH 7.5, 200 µM EDTA, pH 8.0, 20% glycerol and 0.25%

xylene cyanol) and gels were run in 1x TAE buffer. Gels were visualized using a transilluminator (Fotodyne, WI, USA) and bands excised using a clean scalpel blade.

2.2.3 Purification of DNA

Purification of DNA from agarose was done in two ways, depending on the size of the DNA fragment. For DNA less then 8 kilobases (kb) in length, Qiagen Gel extraction spin column kits (Qiagen, Ont. Canada) were used. Manufacturer's instructions were followed for the centrifugation protocol. Agarose was melted using the provided chaotropic salts at 37°C until completely dissolved. Ten microliters of 3 M sodium acetate, pH 5.0 was added to ensure proper pH before being applied to the column. DNA was eluted in 30 µl of elution buffer.

For DNA larger then 8 kb, UltraClean 15 DNA purification kit (Mo Bio, CA, USA) was used and manufacturer's instructions followed. Again, the provided chaotropic salts were used to melt the agarose, followed by addition of beads. Beads were centrifuged for 20 seconds instead of five, as in the instructions. DNA was eluted in two steps, using equal volumes of ddH_2O.

2.2.4 Ligations

Ligations were performed using T4 DNA ligase (NEB). Amounts of template DNA for ligation were estimated from agarose gels. Generally, equal amounts of DNA were

added to 1 µl of T4 DNA ligase enzyme, and 4 µl of 10x DNA ligase buffer, in 20 µl of ddH_2O. Ligations were conducted at room temperature overnight. For blunt end ligations, a ratio of 10 parts insert to one part vector was used.

2.2.5 Polymerase Chain Reaction (PCR)

Polymerase chain reactions were conducted with either Taq DNA polymerase (MBI fermentas, Ont, Canada), or with high fidelity *Pfu* turbo DNA polymerase (Stratagene CA, USA). Taq based PCR was done in 50 µl reactions (8 µl 1.25 mM dNTPs, 3 µl 25 mM $MgCl_2$, 5 µl of 10x reaction ammonium sulfate buffer (MBI fermentas), 400 ng of each primer, 1 µl of Taq, and approximately 0.1 µg of template). All RT-PCR, PCR reactions were also done using this protocol. For *Pfu* Turbo based PCR, the same proportions were used as above, except reactions contained 10x cloned *Pfu* Turbo DNA polymerase reaction buffer (containing 20 mM $MgCl_2$), and 1 µl of *Pfu* Turbo Hot Start polymerase. Generally, PCR conditions were as follows; 5 min 94°C, 30 cycles of amplification (94°C for 30 seconds, 55°C for 30 seconds and 72°C for 30 seconds) and a final extension at 72°C for 7 minutes. Specific alterations in this generic protocol are listed below where appropriate. All PCR reactions were carried out in a Gene Amp PCR system 2400 (Perkin Elmer, Norwalk, CT, USA). For whole cell PCR, bacterial cells were harvested using a pipette tip, and placed in 50 µl of ddH_2O, boiled for 10 minutes, and then centrifuged at 16,060 x *g* for five minutes at room temperature to remove cell debris. Two microliters of this solution was used in each PCR reaction. For hot start PCR,

reaction mixes were prepared without polymerase, heated to 94°C, prior to receiving 0.5 µl of Taq.

All DNA was stored at -20°C for long-term storage, or at 4°C if DNA was to be used within 24 hours.

2.3 Genomic DNA isolation

Genomic DNA was isolated from *L. pneumophila* by harvesting 2 loopfuls of overnight bacterial growth into 440 µl of TE (Tris-HCl and EDTA pH 8.0). Fifty microliters of proteinase K solution (10 mg/ml Proteinase K, reconstituted in 50 mM Tris-HCl pH 8.0, 1 mM $CaCl_2$) was added, followed by 10 µl of 10% sodium dodecyl sulfate (SDS). This sample was incubated at 37°C with rocking for 1-2 hours until clearing had occurred. The mixture was then sequentially extracted with equal volumes of phenol, phenol/chloroform (50/50) and chloroform for 30 minutes, each with rocking, with a 10 minute, 16,000 x *g*, 37°C centrifugation after each extraction step. The genomic DNA solution was then brought up to 1 ml with TE (pH 8.0), and precipitated 0.3 M sodium acetate, pH 5.0 and 1 volume of isopropanol. Precipitated DNA was spooled on a Pasteur pipette, washed with 1.5 ml of ice cold 70% ethanol, briefly air-dried, and redissolved in 0.5 ml of 8 mM NaOH. Genomic DNA was stored at 4°C until use.

2.4 Plasmid Preps: Alkaline lysis and commercial columns

E. coli cultures (50 ml) were grown overnight at 37°C with aeration (200 rpm). Bacterial cells were harvested at 4800 x *g* for 6 minutes at room temperature. Following harvesting, all procedures were done at room temperature. The pellet was resuspended in 2 ml of solution 1 (50 mM glucose, 25 mM Tris-HCl pH 8.0 and 10 mM EDTA) and left on ice for 5 minutes. Then 4 ml of freshly prepared solution 2 (0.2 N NaOH, 1% SDS) was added, and the solution rocked by hand until cleared, followed by incubation on ice for 5 minutes. Three milliliters of solution 3 (3 M sodium acetate pH 5) was added and rocked until precipitation had occurred. The solution was then incubated at –75°C until frozen, thawed on ice, and then centrifuged at ~ 4500 x *g* for 15 minutes at 4°C. Seven and a half milliliters of supernatant was removed, added to 4.5 ml of ice-cold isopropanol, mixed by inversion, and then incubated at -75°C until frozen. After thawing on ice, the solution was centrifuged at 4800 x *g* for 10 minutes at 4°C, and washed with 70% ethanol (4800 x *g*, 6 minutes, 4°C). The resulting pellet was resuspended in 1 ml of Tris-EDTA buffer (100 mM Tris-HCl pH 8.0, and 10 mM EDTA pH 8.0), divided into two 1.5 ml Eppendorf tubes, to which 1 µl of RNase (10 mg/ml) was added, followed by a 30-minute incubation at 37°C. The contents of each tube were then phenol-chloroform extracted in three stages (0.5 ml phenol, 0.25 ml phenol + 0.25 ml chloroform, 0.5 ml chloroform), shaken by hand, and centrifuged at 16,060 x *g*, 10 minutes, 4°C, after each step. The phenol-chloroform extracted material was then allowed to precipitate overnight at -75°C with 0.1 volumes of 3 M sodium acetate in 2 volumes of ice cold 95% ethanol. Following incubation, the tubes were centrifuged at 16,060 x *g*, 10 minutes, 4°C. The resulting pellet was washed four times with 70% ethanol (16,060 x *g*, 10 minutes, 4°C) to remove salts, and the pellet resuspended in 30 µl of sterile ddH_2O.

Plasmid preparations used for simple cloning procedures, screening and construction of reporters was done using Wizard Plus SV minipreps DNA Purification System (Promega). Protocol for purification by centrifugation was followed exactly according manufacturers instructions.

2.5 Determination of Bacterial Optical Density

All bacterial optical densities were determined using a Unico UV-2100 spectrophotometer. For plate grown bacteria, cells were harvested in 1 ml of ddH_2O, or appropriate media, and vortexed until evenly resuspended. Ten microlioters of this suspension were then removed and added to 990 µl of ddH_2O, or appropriate media (i.e 1/100 dilution). The diluted sample was then mixed by inversion, transferred to a quartz cuvette, and the optical density determined at 600 nm for sodium sensitivity experiments, or 620 nm for all other experiments. For broth cultures, 1 ml samples were removed, diluted 1/10 in appropriate media, and the optical density read at 620 nm. Any deviations from this protocol are listed below where appropriate.

2.6 *E. coli* competent cells

2.6.1 $RbCl_2$ competent cells

E. coli cultures (25 ml) were grown overnight at 37°C with aeration (200 rpm). Prewarmed LB (500 ml) broth was then inoculated 1:20 with the overnight culture, and

allowed to grow for two hours until an O.D. of ~ 0.6 was obtained. All harvests were performed at 3000 x *g* for 15 minutes at 4°C. Cells were then incubated for 5 minutes on ice. The pellet was then resuspended in 200 ml of TFB I solution (30 mM potassium acetate, 100 mM $RbCl_2$, 10 mM $CaCl_2 \cdot H_2O$ 50 mM $MnCl_2 4H_2O$ and 15% glycerol), incubated on ice for 5 minutes, and then harvested by centrifugation. The pelleted cells were then resuspended in 20 ml of TFB 2 solution (10 mM 3-(N-morpholino)propanesulfonic acid (MOPS), 75 mM $CaCl_2 \cdot H_2O$, 10 mM $RbCl_2$, 15% glycerol, adjusted to pH 6.3 with 1 M KOH). Cells were then aliquoted into 200 µl fractions, and stored at -75°C until use.

2.6.2 $CaCl_2$ competent cells

Similarly to $RbCl_2$ competent cells described above, 5 ml *E. coli* cultures were grown overnight at 37°C with aeration (200 rpm) and used to inoculate (1:100) 50 ml of LB broth. Cell suspensions with an O.D. of ~ 0.3 to 0.5 were harvested by centrifugation at 4800 x *g*, 6 minutes, 4°C, and the pellet was resuspended in 40 ml of 0.1 M $CaCl_2$, followed by a 20 minute incubation on ice. Cells were then harvested (4800 x *g*, 6 minutes, 4°C), resupended in 4 ml of 0.1 M $CaCl_2$ and then stored up to 4 days at 4°C.

2.7 *E. coli* transformation

Transformation of $RbCl_2$ and $CaCl_2$ competent cells was performed identically. Competent cells (200 µl) were placed on ice, and received 10 µl of ligation mix or

plasmid DNA. Cells were then placed at 37°C for 90 seconds, followed by 5 minutes on ice. The transformed cells were then added to 2 ml of prewarmed LB broth and incubated with aeration (100 rpm) for 1 hour at 37°C. Cells were then harvested (4800 x *g*, 6 minutes, 4°C) and resuspended in 111 µl of LB broth. 1, 10 and 100 µl of the transformants were then plated and spread on 3 LB agar plates with appropriate selection. Plates were then incubated at 37°C overnight. Occasionally, plates were further incubated at 4°C for 2-4 hours to enhance β-galactosidase activity. Colonies were replica plated to fresh LB agar plates with appropriate selection, incubated overnight at 37°C, and then stored at 4°C.

2.8 Preparation of electrocompetent *Legionella pneumophila.*

All *L.pneumophila* strains were grown overnight at 37°C as heavy lawns on BCYEα media with appropriate selection. Bacterial lawns were harvested using disposable loops, and resuspended in 20 ml of sterile ddH_2O by vortexing. Cells were then harvested (3000 x *g*, 10 minutes, 4°C), and resuspended in 20 ml of cold 15% Glycerol. Cells were then harvested (3000 x *g*, 10 minutes, 4°C), and resuspended and harvested (3000 x *g*, 10 minutes, 4°C) twice in 10 ml 15% Glycerol. The resulting pellet was then resuspended in 100 µl of cold 15% glycerol and stored at -75°C in 40 µl aliquots.

2.9 Transformation of *Legionella pneumophila* cells.

Aliquots of electrocompetent cells were thawed on ice, and then mixed with ~ 10 μg of plasmid DNA. The cell/DNA mixture was then added to a 1 or 2 mM gapped electroporation cuvette, and placed on ice for 5 minutes. The cuvette was then electroporated at 2.1 kV, 200 Ω, 25 μF, and then the contents were immediately transferred to 1 ml of prewarmed incomplete BYE (no selection, ferric pyrophosphate or L-cysteine). This was then incubated with aeration (100 rpm) at 37°C for 1 hour. The transformation (100 μl) was then plated on BCYEα media with appropriate selection. Plates were then incubated for 4-6 days at 37°C, the resulting colonies were replica plated, and these plates incubated overnight at 37°C.

2.10 Construction of Δ*ihfA* and Δ*ihfB* allelic replacement constructs

The procedure used to make both constructs was essentially the same; any differences between the 2 constructs will be listed below. In addition, the basic procedure used to make these constructs is schematically represented in figure 1. ~ 500 base pairs of sequence 3' and 5' to the IHF coding sequence were PCR amplified from Lp02 genomic DNA using primers P1 and P2 (5') and P3 and P4 (3') specific for each gene (standard conditions for IHFA amplicons 45 sec extension, for IHFB amplicons P1/P2 (anneal 58°C) and P3/P4 (anneal 62°C), both 40 second extension times). P2 and P3 primers were engineered to contain BamHI restriction endonuclease sites. P1/P2 and P3/P4 amplicons were digested with BamHI, and ligated together. These ligations were then PCR amplified using P1 and P4 primers (IHFA (anneal 51°C), IHFB (anneal 58°C), both 1 minute extension), gel purified, and digested with appropriate restriction

endonucleases (IHFA = EcoRI and SacI, IHFB = EcoRI and HindIII). Also, cloning vectors pBluescript , and pUC18 were digested with appropriate endonucleases. Digested amplicons were ligated with appropriate cloning vectors to produce pBS::P1/P4 (*ihfA*) and pUC18::P1/P4 (*ihfB*). These ligations were then transformed into *E. coli* DH5α, and bacteria grown on LB agar containing ampicilin. The resulting colonies were then screened by PCR for the insert using the appropriate P1/P4 primer sets and conditions listed above. Positive clones were then grown in 5 ml LB broth cultures, and plasmids were isolated by rapid mini prep. Plasmids were then digested with BamHI, along with plasmid p34S, which contains the kanamycin resistance marker. Plasmids were also digested with appropriate endonucleases to confirm PCR screening. The resistance marker was then ligated into the P1/P4 plasmids, the resulting ligations transformed into DH5α, and transformants plated on LB agar containing ampicilin and kanamycin. The resulting clones were screened by PCR using the P1/P4 primers (same conditions as above, but 2 minute extension), and positive clones were grown up in 5 ml LB broth cultures, followed by rapid plasmid mini prep. For the *ihfA* construct, using the pBS::P1/P4::*km*^{r} (*ihfA*) plasmid as template, the allelic replacement construct was PCR amplified (*Pfu* Turbo) using the P1/P4 primer set, and cloned into the EcoRV site of the novel suicide vector pBO*rdx* to create pBO*rdx*::P1/P4::*km*^{r} (*ihfA*). To insert the *ihfB* construct into the pBO*rdx* vector, pUC18::P1/P4::*km*^{r} (*ihfB*) was digested with EcoRI and HindII, and the ends filled with T4 DNA polymerase. Similarly, pBO*rdx* was digested with BamHI and the ends filled with T4 DNA polymerase. Both products were then ligated together to form pBO*rdx*::P1/P4::*km*^{r} (*ihfB*) Ligations of both constructs were transformed into DH5α, and selected on LB agar containing kanamycin, and replica

plated on media containing kanamycin and chloramphenicol. To create the gentamicin resistant versions of these plasmids, pBO*rdx*::P1/P4::*km*^{r} (*ihfA*) and pBO*rdx*::P1/P4::*km*^{r} (*ihfB*) plasmids were digested with BamHI, the sticky ends filled, and ligated with a PCR amplified gentamicin resistance marker with the high fidelity *Pfu* turbo using boiled lysates of the *E. coli* strain χ1849 (58˚C anneal, 1 minute extension) and Gent For/Gent Rev primers. These transformants were selected on gentamicin, followed by replica plating on gentamicin and chloramphenicol. Screening of potential mutants was done using primer sets internal to the deleted coding sequence (for *ihfA* – RT IHFα For/Rev, for *ihfB* – RT IHFβ For/Rev), and using the P1/P4 primer sets specific to each gene (see above).

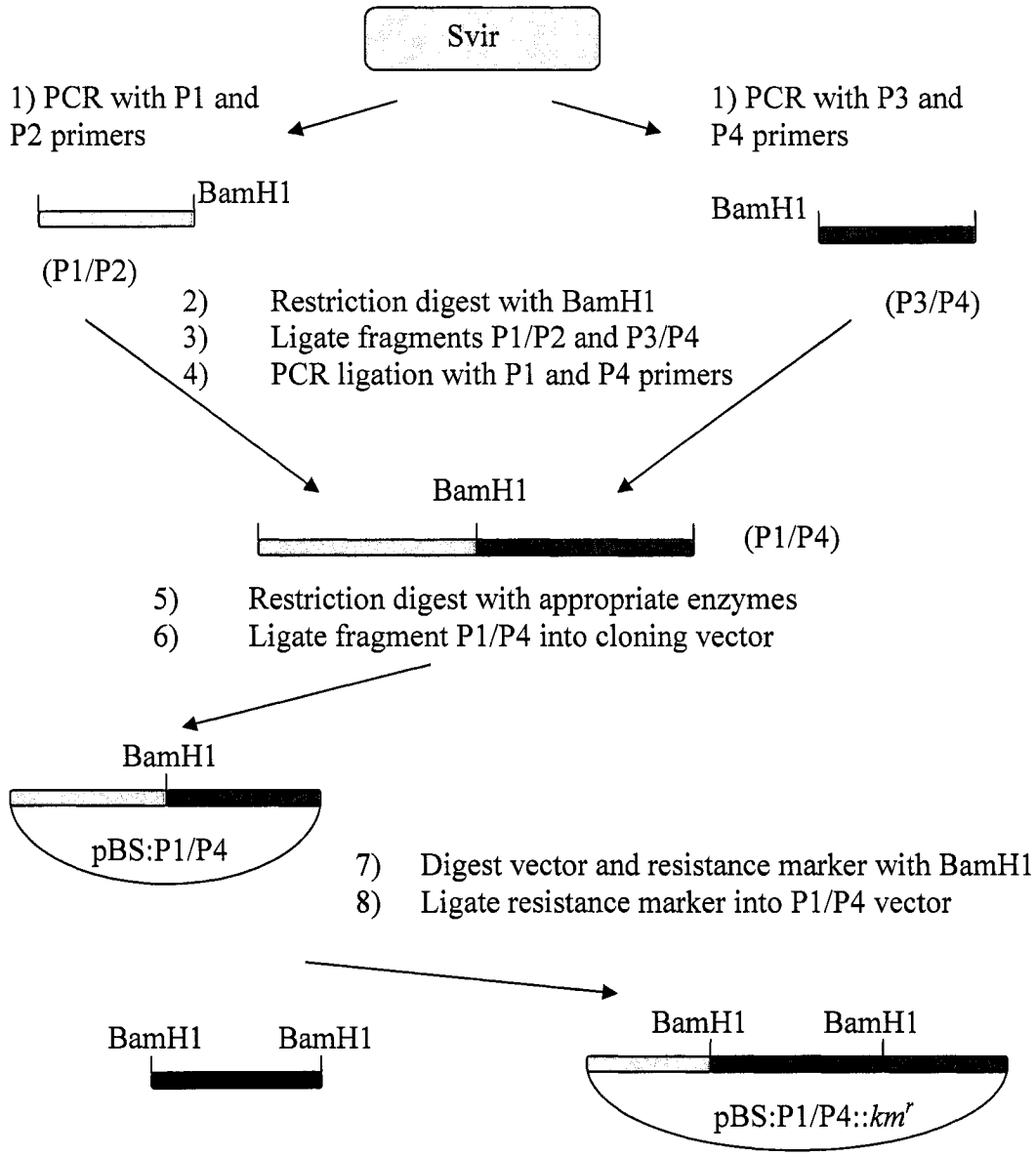


Figure 1. Generalized schematic representation of the procedure used to make the allelic replacement constructs used in this study. Listed above are primers (P1, P2, P3 and P4) used to PCR the P1/P2 upstream (green) and P3/P4 downstream (blue) regions of the *ihfA* and *ihfB* genes. Antibiotic resistance cassette shown in red. Tick marks on amplicons and in constructs represent positions of restriction sites, only BamHI (common to both allelic replacement constructs) is shown. In this diagram, pBS was chosen, although the *ihfB* construct was created in pUC18.

2.11 Construction of complementing constructs

In trans complementation constructs were created using the pMMB206, IPTG inducible vector. The coding sequence of the *ihfA* gene, along with ~ 700 base pairs of upstream sequence was PCR amplified using the primers IHFαCOMPR and IHFαCOMPF2 (1 minute extension, 30 cycles) using Lp02 genomic DNA. Similarly, the *ihfB* gene was PCR amplified using the IHFA2 and IHFβCOMPF2 (1 minute extension, 30 cycles). Both amplicons were digested with EcoRI and ligated together. The ligated product was then PCR amplified using IHFαCOMPR and IHFA2 (1:10 minute extension, 55°C anneal). The resulting amplicon was cloned into pMMB206 using BamHI and HindIII to produce pMMB206::*ihfAB* (operon) and transformed into DH5α. This construct was then used as template in PCR with primers IHF No Prom and IHFβCOMPF2 (1 minute extension), the resulting amplicon was cloned into pMMB206 with BamHI and HindIII to produce pMMB206::*ihfAB* and transformed into DH5α. The resulting plasmids, pMMB206::*ihfAB* and pMMB206::*ihfAB*, were then purified by alkaline lysis, and electroporated into Lp02 and into Lp02Δ*ihfAB*. Similarly, the vector pMMB206 was also electroporated into Lp02 and Lp02Δ*ihfAB*. These strains were all selected for on appropriate media containing chloramphenicol.

2.12 In vitro bacterial growth curves

Overnight plate growth of Lp02 and Lp02Δ*ihfAB* was separately resuspended in BYEα broth and the optical density of the suspension determined. Triplicate cultures of

both strains were started at an $O.D._{620}$ of 0.2, and incubated with aeration (100 rpm) at 37°C. For Lp02, media contained Str100, Thy100, while Lp02Δ*ihfAB* was grown in broth containing Str100, Thy100, Kan40 and Gent10. Samples (1 ml) were taken every 3 hours, and the optical density determined at 620 nm. Cultures were allowed to grow until stationary phase (27 hours). Following 27 hours, a sample of each culture was plated on BCYEα with appropriate selection to ensure there was no contamination.

2.13 RNA isolation

L. pneumophila was grown for BCYEα for 24 hours, or grown in BYEα broth with or without 2 mM IPTG. Approximately 10^8 bacterial cells were harvested (4500 x *g*, 6 minutes, room temp.), and lysed by repetitive pipetting in 1 ml of Trizol reagent, followed by incubation at room temperature for 5 minutes. Chloroform (0.2 ml) was then added, the sample shaken vigorously by hand for 15 seconds, followed by a 3-minute incubation at room temperature. The sample was then centrifuged at 12,000 x *g*, 15 minutes, 4°C, and the aqueous (upper) layer was removed to a new tube. 0.5 ml of ice-cold isopropanol was added, and the sample incubated at room temperature for 10 minutes. Following centrifugation (12,000 x *g*, 15 minutes, 4°C), a pellet was obtained, and the supernatant was removed. The pellet was washed with ice cold 70% ethanol and centrifuged at 7500 x *g*, 5 minutes at 4°C. The ethanol was removed, and the pellet allowed to air dry for 10 minutes. The pellet was subsequently allowed to dissolve in 30 µl of ddH_2O containing diethylpyrocarbonate (DEPC) at 55°C for 30 minutes.

2.14 DNaseI treatment of RNA

DNaseI (Sigma) was prepared as follows. DNaseI was diluted to a final concentration of 5 U per µl in DNaseI storage buffer (50% Glycerol, 20 mM sodium acetate pH 6.5, 5 mM $CaCl_2$ and 0.1 mM phenylmethylsulfonylfluoride (PMSF)). Crude RNA preparation (4 µl) was added to 4 µl of ddH_2O (DEPC), 1 µl of 5 U/µl DNaseI, and 1 µl of DNaseI reaction buffer (100 mM sodium acetate, pH 5.0, 5 mM $MgCl_2$). This reaction mixture was incubated at 37°C for 90 minutes, and the enzyme deactivated at 70°C for 10 minutes. To confirm the absence of DNA, 2 µl of this preparation was subjected to PCR with primers used in the RT-PCR reaction (see below).

2.15 Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

RT-PCR was done using M-MLV Reverse transcriptase. DNaseI treated RNA (2 µl) was added to 1 µl of random hexamers, 1 µl of 10 mM dNTPs and 9 µl of ddH_2O. This mixture was heated to 65°C for 5 minutes, followed by quick chill on ice. Next, 4 µl of 5x first strand buffer, 2 µl 0.1 M DTT, and 1 µl ddH_2O was added, and the mixture heated to 37°C for 2 minutes. Finally, 1 µl of M-MLV enzyme was added, and synthesis allowed to proceed for 50 minutes at 37°C, followed by inactivation at 70°C for 15 minutes. Two microliters of this reaction was used for PCR using specific primers for both *ihf* genes (IHFα RT for/rev and IHFβ RT for/rev (55°C anneal, 40 cycles, 35 sec extension).

2.16 Antibody purification

Anti- MagA antibody was purified from rabbit serum originally produced by Margot Hiltz (Master's thesis, Dalhousie University, 1999) from rabbits inoculated with purified MagA protein. To remove contaminating antibodies, serum was incubated with crude lysate from DH5α/pMALp2intB2, which does not contain the *mag*A gene. A 50 ml overnight broth culture of DH5α/pMALp2intB2, started from frozen stock, was grown at 37°C with aeration (200 rpm) and 25 ml used to inoculate 475 ml of LB broth. Cultures were grown at 37°C with aeration (200 rpm) until an optical density of ~0.9 was obtained, and then induced for three hours with 1 mM IPTG for 3 hours. Cells were then harvested (6,000 rpm, 6 minutes, room temperature) and frozen at -20°C overnight. The pellet was then thawed, and resuspended in 1 ml of buffer (20 mM Tris-HCl pH 7.4, 200 mM NaCl, 1 mM EDTA pH 8.0 with 770 mg/L DTT and 1 mM PMSF added fresh), sonicated in 30-second pulses and placed on ice in between pulses. Cells were then centrifuged to remove unbroken cells (4,800 x *g*, 10 minutes, room temperature), followed by high-speed centrifugation to remove cellular debris (50,000 rpm, 30 minutes, 4°C) using Optima Max ultracentrifuge (Beckman Coulter). The lysate was then placed on ice until beads were prepared.

Carboxylated microparticle beads were obtained from Polysciences Inc. and manufacturer's instructions were followed exactly for their preparation. Separate sets of beads were coupled to both DH5α/pMALp2intB2 lysate and to BSA as follows. Next, 720 μg of DH5α/pMALp2intB2 lysate and 600 μg of BSA were separately added to 1 ml

of borate buffer (Polysciences, Inc) and mixed (end to end) for 22 hours at room temperature. Tubes were then centrifuged (16,060 x *g*, 10 minutes, 4°C) to pellet beads, the supernatant was removed carefully and then centrifuged as above for an additional 10 minutes, and the protein concentration was determined. Following completion of the protocol, beads were stored at 4°C until use.

Immediately prior to use, beads were washed in 1 ml of sterile PBS to remove any traces of storage buffer (16,060 x *g*, 10 minutes, 4°C). To remove non-specific antibodies from the MagA immunized rabbit antiserum, 1 ml of antiserum was incubated with BSA coated beads (mixed end to end) for 45 minutes at room temperature and the beads were pelleted by centrifugation (16,060 x *g*, 6 minutes, 4°C). This supernatant was then transferred to the tube containing DH5α/pMALp2intB2 crude lysate coupled beads, incubated for 45 minutes at room temperature and beads were pelleted as described for BSA. The purified antiserum was then diluted 1:750 in TTBS (see below) for use as a primary antibody.

2.17 Western Blotting

Approximately 10^8 bacterial cells from BCYEα were washed in ddH_2O, and resuspended in LDS buffer with 10% β-mercaptoethanol, and the samples were placed in boiling ddH_2O for 10 minutes. Samples were then cooled on ice, and centrifuged at 16,060 x *g*, 5 minutes at room temperature. For developmental expression Western blot of IHF, Svir MIFs (1.0 O.D.) (Gift, Elizabeth Garduno, Dalhousie University, Halifax)

were added to 5 ml of prewarmed BYEα broth, and grown at 37°C with aeration (100 rpm). Samples were taken at various time points, standardized to an $O.D._{620}$ of 0.2 in ddH_20, resuspended in sample buffer, and treated as described above. For all experiments, 10 µl of sample was then run on NuPage (Invitrogen), 4-12% Bis-Tris polyacrylamide gels for 1 hour at 100 V, along with a prestained molecular weight marker. For MagA western blot experiments using intracellularly grown bacteria, a sample of each strain was serially diluted and plated on BCYEα with appropriate selection to enumerate the bacteria. Then, 5×10^4 bacterial cells were prepared as described above. The gel was then removed from the plastic case, the lanes were trimmed, and placed on a nitrocellulose membrane soaked in transfer buffer (0.192 M glycine, 0.025 M Tris, 20% methanol, pH 8.2-8.3). The gel/membrane was then placed between filter paper soaked in transfer buffer and placed in the Western transfer apparatus, filled with transfer buffer. Transfer was done for 200 V hours. The nitrocellulose was then removed, washed with ddH_2O and stained for 10 minutes with Ponceau S stain (10X in 100 ml ddH_2O: 2 g Ponceau S (Allied Chemicals), 30 g Trichloroacetic and 30 g sulfosalicylic acid, diluted to 1X in ddH_2O). Excess stain was removed with ddH_2O, and a digital image of the membrane acquired. Stain was removed with 3 ten minute PBS washes. The membrane was then blocked overnight at 4°C in blocking solution (10 ml TTBS (0.5% Tween 20, 0.15 M NaCl, 0.005 M EDTA and 0.05 Tris HCl pH 7.4), 100 mg Skim milk powder, 100 mg BSA). Blocking solution was removed with 2, 5 minute TTBS washes, followed 1 hour incubation with the primary antibody diluted 1/5000 in TTBS supplemented with 100 mg BSA. 2 different primary antibodies were used, rabbit anti-IHF antibody (gift from Dr. S Goodman, University of

Southern California, USA), and rabbit anti-MagA antibody. Primary antibody solutions were reused 5 times, with 0.1% sodium azide being added after the first use. Membranes were then washed with Tris-NaCl solution (2 ml of 1.0 M Tris HCl pH 7.5, 1.25 ml 4 M NaCl and 96.75 ml ddH_20) 3 times for ten minutes, followed by incubation for one hour with goat anti-rabbit antibody dissolved in 10 ml of Tris-NaCl solution. Secondary antibody was removed with 2, 10-minute Tris-NaCl washes, followed by incubation in AP buffer (0.1 M Tris HCl pH 9.5, 0.1 M NaCl, 0.05 M $MgCl_2 \cdot 6H_20$) for 10 minutes. For western blotting, developing was done using 44 µl of Nitro blue tetrazolium (NBT, made as a 75 mg/ml solution in 70% dimethylformamide (DMF)), and 33 µl of 5-bromo-4-chloro-3-indolyl phosphate (BCIP, made as a 50 mg/ml in DMF solution) in 10 ml of AP buffer. The reaction was stopped with three 5 minute PBS washes. Digital images of the blots were then captured using an ES-1200C flatbed scanner (Epson). Densitometric analysis was done using Gel-Pro analysis software (Media Cybernetics, USA). To standardize for protein loading differences, the densitometry of two bands present in the Ponceau stained gel (not MagA) was calculated, and the intensity standardized to the densitometric values of the corresponding bands in the Lp02 lane. The resulting standardizing factor was then used to correct the intensity of the MagA protein. Error bars for Lp02 values represent a corrected intensity for the MagA protein based on the average of the two standardizing bands.

2.18 Preparation of Crude Cell Lysates

L. pneumophila overnight plate growth was used to inoculate 500 ml of BYEα broth with appropriate selection, and the culture was grown at 37°C with aeration (100 rpm) for various times. For sodium challenge, 0.075 moles of NaCl of KCl were added and the cultures allowed to grow for an additional hour. Cultures were then harvested at 4,800 x *g*, 6 minutes, 4°C, resuspended in cold 50 mM Tris HCl pH 7.5, and harvested as before. Pellets were then resuspended in sonication buffer (50 mM Tris-HCl pH 7.5, 1 mM EDTA pH 8.0, 50 mM NaCl, 10% glycerol with 0.1 mM PMSF and 1 mM DTT added fresh), and placed on ice. Cells were then lysed using a French pressure cell and subjected to ultracentrifugation at 100,000 x *g*. Lysate was then aliquoted and stored at -75°C. A sample of this extract was set aside for protein concentration determination using Bradford reagent (BioRad). Lysates were serially 10-fold diluted in ddH_2O, and 800 µl of diluted lysate received 200 µl of Bradford reagent. The samples were then mixed and read spectrophotometrically at 595 nm using a Unico UV-2100 spectrophotometer (Unico). As a blank, 200 µl of Bradford reagent (BioRad) was added to 800 µl of ddH_2O. Protein concentration was estimated from a BSA standard curve.

2.19 Two-dimensional SDS Poly acrylamide gel electrophoresis (SDS PAGE)

Isoelectric focusing was done using Immobiline DryStrips NL pH 3-10 (Amersham Biosciences) as per the manufacturer's instructions. Crude protein lysate (50 µg) was added to rehydration buffer (8 M Urea, 2% CHAPS, 0.5% IPG Buffer, Bromophenol Blue (trace) and 7 mg DTT per 2.5 ml of rehydration buffer added immediately prior to use) to a final volume of 0.25 ml, and placed in a ceramic strip holder. The Immobiline

DryStrip was then placed in the strip holder and overlayed with mineral oil. The strips were then focused overnight (12 hours rehydration, 500 V for 1 hour, 1000 V for 1 hour, 8000 V for 2 hours). Strips were then equilibrated in SDS equilibration buffer (in ddH_2O: 50 mM Tris-HCl pH 8.8, 6 M Urea, 30% glycerol and 2% Bromophenol blue) for 10 minutes. Gradient acrylamide gels (5 or 10 ml of 30% acrylamide, 3.75 ml of 1.5 M Tris HCl pH 8.8, 1.05 or 6.05 ml of ddH_2O, 0.15 ml of SDS, 50 µl of 15% Ammonium Persulfate and 3 µl of TEMED) were cast using a gradient mixer, and overlayed with water saturated butanol. Butanol was removed, and strips were applied to the top of the gels, held in place using moistened Kimwipes. Gels were run at constant voltage (30 V) until the dye front reached the end of the gel. Gels were then placed in fixative buffer (50% methanol, 10% Acetic acid) overnight at room temperature.

2.20 Silver Staining

All washes were performed with 250 ml of each respective solution. After fixing, gels were washed in 50% methanol for 15 minutes, followed by 5 x 5 minute washes in ddH_2O. Gels were then sensitized with 0.02% sodium thiosulfate for 1 minute, followed by 2 x 1 minute washes with ddH_2O. The gels were then treated with 0.2% cold silver nitrate solution for 25 minutes. Developer (3% sodium carbonate, 0.025% formalin) was added, removed upon colour change, and then replaced with an equal volume of developer. Once the desired staining intensity had been achieved, 1.4% EDTA (in ddH_2O), was added to stop the reaction. Gels were then washed twice with ddH_2O for one minute each, digital images were acquired taking extreme precautions to avoid

contamination, and then stored in ddH_2O at 4°C until proteins were removed for sequencing. Protein removal was done in a laminar flow hood using a new, clean scalpel blade for each protein. Proteins were sequenced by trypsin digestion followed by LC-MS at the DalGEN Microbial Genomics Center, Proteomics facility by Elden Rowland at Dalhousie University, Halifax. Mass spectral data were analyzed using the MASCOT (Matrix Science, Boston, USA) database search program. Statistical significance was performed using a two-tailed student's t-test ($p < 0.01$).

2.21 Electrophoretic Mobility Shift Assay (EMSA)

Radiolabeled amplicons were prepared as follows. Approximately 1 µg of template DNA was used in PCR (MBI Fermentas), using radiolabeled α-^{32}P dCTP (3000 Ci/mmol) (Amersham Biosciences). All other PCR conditions were done as described above. Three amplicons were generated for mobility shift reactions; R6/F6 (standard PCR conditions), R6/F9 (47°C anneal), and prom Rev/F6 (hot start, 46°C anneal). Amplicons were then purified using a nucleotide removal kit (Qiagen) as per the manufacturer's directions. Amplicons were eluted in 101 µl of elution buffer. The eluted amplicon (1 µl) was used for scintillation counting to determine the cpm/µl. Working stocks were prepared in ddH_2O to a concentration of 30,000 cpm/µl.

Each reaction contained 10 µl of crude protein lysate, or purified *L. pneumophila* IHF subunits (Gift from Dr. Ann Karen C. Brassinga, University of Virginia, USA). For purified IHF subunit mobility shifts, 0.75, 3.75 and 7.5 µg of protein were used, and for

reactions using both subunits, a 1:1 ratio of each subunit was used. All protein samples were diluted in ddH_2O to the desired concentrations and added to 10 µl of binding reaction mix (4 µl of 50% glycerol, 3 µl of salmon sperm DNA, radiolabeled amplicon totalling 5000 cpm, 2 µl of 10x binding mix (in 250 µl - 25 µl 100x BSA, 25 µl 1 M HEPES pH 7.9, 62.5 µl 4 M KCl, 1 µl EDTA pH 8.0, 10 µl 0.5 M DTT, 10 µl 0.5 M PMSF and 116.5 µl ddH_2O) and ddH_2O up to 10 µl). The protein/binding reaction mix was then incubated for 30 minutes at room temperature before receiving 2 µl of 10X loading dye (0.3% glycerol and 0.01% bromophenol blue diluted in 10x Tris borate buffer. Reactions were then run on 6% polyacrylamide gels (in 40 ml – 8 ml 30% acrylamide (29% acrylamide, 1% bisacyrlamide), 29.7 ml of ddH_2O, 4 ml 5x TBE buffer, 0.25 ml 10% APS) were cast and prerun for 1 hour at 20 mA. Samples were then loaded on the gel and run for 3 hours at 20 mA. Gels were then dried onto filter paper, placed into a cassette with intensifying screens, and exposed to autoradiograph film overnight at -75˚C. Films were then developed.

2.22 Anion exchange chromatography

Late exponential phase Svir lysate was partially purified using a DE-52 (diethylaminoethyl cellulose) pre-swollen microgranular anion exchange column (Whatman Bio Systems Ltd, Kent, England). Approximately 50 g of DE-52 was added to 500 ml of suspension buffer (100 mM Tris-HCl pH 7.6) and mixed at room temperature to form an even suspension. The column (a sterile 60 ml syringe) was packed with glass wool, and then 10 ml of suspension buffer was passed through the glass wool to remove

any trapped air. The DE-52 slurry was then slowly added to the syringe, each time allowing the granules to settle, and finally topped off with 10 ml of suspension buffer before allowing the column to settle. The column was then cleaned and charged by passing 50 ml of 500 mM Tris-HCl, pH 9.0, and then equilibrated by passing 50 mM Tris-HCl, pH 7.5 through it until the eluate reached pH 7.5. Following equilibration, 100 ml of running buffer (buffer X (50 mM Tris-HCl, pH 7.6, 35 mM potassium chloride, 25 mM ammonium chloride, 1 mM DTT, 1 mM PMSF in 95% ethanol and 5 mM EDTA) was passed through the column. Svir lysate was diluted 1:3 in buffer X, and 15 ml of this mixture was slowly and carefully added to the top of the column. The column was then allowed to run until all the lysate solution had entered the column. To determine when the void fraction had been completely eluted, the eluate was monitored spectrophotometrically using an ultraviolet detector, and absorbance profiles were recorded using a stripchart recorder. Once the void fraction was completely removed, a salt gradient (100 ml each of buffer X containing either 35 mM or 500 mM potassium chloride) was run through the column to elute bound proteins. 3 ml fractions were collected until all 200 ml of the salt gradient had been run. To remove any residual proteins, 100 ml of buffer X containing 500 mM potassium chloride, followed by 100 ml of buffer X containing 1 M potassium chloride were additionally run through the column, with 3 ml fractions collected as before. All fractions were kept at 4°C until used in EMSA.

Fractions identified as containing *ompS* promoter binding proteins as determined by EMSA were pooled, and dialysed in an attempt to further purify the binding proteins.

Combined fractions were placed in 6,000 - 8,000 molecular weight cut off dialysis tubing (Spectrapor, Spectrum Medical Industries, Los Angeles, CA), and dialysed against buffer X overnight at 4°C. Dialysed samples were then re-run on the column as described above and eluted with the same salt gradient. These fractions were then re-tested for their ability to bind the radiolabeled R6/F6 *ompS* promoter amplicon (described above).

2.23 Sodium Sensitivity Assays

Overnight plate growth of *L. pneumophila* was subcultured onto BCYEα plates containing appropriate selection and incubated at 37°C for 72 hours. After 72 hours, bacteria were harvested using a disposable loop into 1 ml of ddH_2O and vortexed until evenly suspended. 10 µl of this suspension was then added to 990 µl of ddH_2O (i.e. a 1/100 dilution), and the optical density determined at 600 nm. Suspensions were then standardized to an optical density of 4.0, and serially diluted 10-fold. Three 20 µl drops of each dilution were then plated on BCYEα (Str100, Thy100 and Cm4 when appropriate). Chloramphenicol was used to ensure maintenance of plasmids. In addition, 3 x 20 µl of each dilution was plated on BCYEα (Str100, Thy100 and Cm4 when appropriate) containing either 100 mM or 145 mM sodium chloride. Plates were then incubated at 37°C for 5-7 days. Bacteria were then enumerated and the percent viability calculated as follows: (c.f.u./ml on media with sodium chloride / c.f.u on media without sodium chloride) x 100. Statistical analysis was performed using a two-tailed student's t-test ($p < 0.01$).

2.24 Green Fluorescent Protein Reporter Assays

Green fluorescent protein (GFP) reporters were constructed by PCR amplifying the promoters of three virulence genes using standard conditions (*dotA* (546 bp, including *dotV* and 148 bp upstream) (58°C anneal), *dotD* (547 bp) and *icmT* (502 bp) (all 40 second extensions)) from Lp02 chromosomal DNA, and cloned into the GFP reporter plasmid pBH6119. All promoter sequences contained 10-30 bases of coding sequence. These plasmids were electroporated into Lp02 and into Lp02Δ*ihfAB* and selected for on media not supplemented with thymide (due to the thymidylate synthetase gene located on the reporter plasmid). Overnight plate growth of these strains was then used to inoculate BYEα (Str100) media at an OD_{620} of 0.2 and these were incubated at 37°C with aeration (100 rpm) for 30 hours. After 24 hours, 1 ml samples were removed, transferred to 1.5 ml eppendorf tubes, and centrifuged at 4,800 x *g*, 6 minutes, room temperature. Pellets were then resuspended in 1 ml sterile PBS, diluted 1/10 in PBS and the optical density determined at 620 nm. The PBS bacterial suspensions were then diluted to an $O.D._{620}$ of 0.5, placed in a 0.25 ml cylindrical cuvettes and the fluorescence read at excitation wavelengths of 488 and 510 nm using a TD-700 (Turner Designs Inc.) fluorescence spectrophotometer.

2.25 Tissue culture

HeLa cells were grown and passaged in 30-ml cell culture flasks in Minimal Essential Medium (MEM) supplemented with antibiotic-antimitotic mix (Gibco BRL),

10% Newborn Calf Serum (NCS) and 0.3 mg/ml glutamine (made as a 30 mg/ml stock solution, heated to 37°C and vortexed until clear). Cells were grown to confluence in flasks, and removed from the flask using trypsin-EDTA and shaking. Cells were counted using a Neubauer hemocytometer. L929 cells were grown in 30 ml flasks in MEM supplemented with 10% Fetal Bovine Serum (FBS) and antibiotic-antimitotic mix.

2.26 L929 plaque assay

L929 cells were seeded in 24 well plates and allowed to form monolayers. Cells were washed once with PBS for HeLa cells (in 500 mls, 4 g NaCl, 0.07 g NaH_2PO_4) and received 0.2 ml of MEM (1% fetal bovine serum (FBS)). Two types of bacterial innoculum were used. Overnight plate growth was harvested in 1 ml of ddH_2O and standardized to an O.D. of 0.5 at 620 nm in 1 ml of MEM (1% FBS). Cells were then serially diluted 10-fold in 0.9 ml of MEM (1% FBS). For MIF experiments, HeLa cells were grown in 30-ml flasks overnight in MEM (10% NCS and Glutamine), and the flasks were examined prior to infection to ensure monolayers had formed. Overnight plate growth of *L. pneumophila* was harvested into 1 ml ddH_2O, the optical density determined at 620 nm and cells were pelleted (4,800 x *g*, 6 minutes, room temperature) and resuspended in MEM (10% NCS and glutamine). Monolayers were then infected with 1 ml of a bacterial suspension with an O.D. of 1.0 (~10^9 legionellae), and the flasks were incubated overnight at 37°C, 5% CO_2. Monolayers were then washed six times with PBS for HeLa cells, and then received 3 ml of MEM (10% NCS and glutamine, supplemented with Str100, Thy100 and Cm4 where appropriate), and were incubated for an additional 3

days at 37°C, 5% CO_2. After 4 days of infection, monolayers were removed using a tissue culture scraper and transferred along with the supernatant into 13 ml tubes. After centrifugation (3,000 x *g*, 10 minutes, room temperature), pellets were resuspended in 1 ml ddH_2O, and HeLa cells were lysed by passage through 26g1 needles. The lysed HeLa cells (100 µl) were then serially diluted 10-fold in 0.9 ml of MEM (1% FBS).

A fraction of each innoculum dilution (400 µl) was then added to two wells of L929 cells immediately after removing the 0.2 ml of MEM (1% FBS), for a total of 6 dilutions per strain. Plates were then centrifuged (3,000 x *g*, 10 minutes, room temperature) and incubated for 3 hours at 37°C, 5% CO_2. Monolayers were then washed six times with PBS for HeLa cells, before adding 1 ml of 0.6% agarose in MEM (1% FBS) supplemented with glutamine, Str100, Thy100, sodium bicarbonate, and Cm4 where appropriate. Cells were then incubated for four days at 37°C, 5% CO_2 in a Tupperware container with damp paper towels. Two drops of each innoculum dilution (50 µl) were then plated on BCYEα with appropriate selection to determine viable counts. After four days each monolayer then received 1 ml of PBS containing 10% formalin, and was further incubated for an additional 24 hours at room temperature. The PBS/formalin solution was then washed off with tap water, the agarose plugs were removed, and the monolayers were stained for 1 hour with crystal violet. Excess stain was then removed with tap water, and the plates air-dried. Plaques in the monolayer were then enumerated along with the innoculum viable counts. Plaquing efficiency was then calculated as (# of plaques formed per ml / c.f.u per ml) x 100. Statistical analysis was performed using a two-tailed student's t-test ($p < 0.01$).

2.27 Attachment and Invasion assays

HeLa cells used in attachment and invasion assays were grown in spinner bottles in MEM with antibiotics (10% NCS and glutamine). Prior to assays HeLa cells were seeded in MEM (10% NCS and glutamine) at 1 million cells per well in 12 well plates, as estimated by counting with a Neubauer Hemocytometer. Cells were allowed to attach to the wells for at least three hours, and monolayer formation was monitored using an inverted microscope. Monolayers were then washed once with PBS for HeLa cells, and received 0.9 ml of MEM (10% NCS and glutamine). Overnight plate growth of *L. pneumophila* was then standardized to an optical density of 1.0 in MEM (10% NCS and glutamine), and 0.1 ml of this innoculum was added to each well of duplicate plates. Plates were then centrifuged (3,000 x *g*, 10 minutes, room temperature) and incubated for three hours at 37°C, 5% CO_2. One plate was then washed 6 times with PBS for HeLa cells, and then monolayers were lysed with 1 ml of ddH_2O. Bacteria were then serially diluted 10-fold and plated on appropriate media, incubated at 37°C, 5% CO_2 for 4-6 days, and enumerated. The second plate was washed three times with PBS for HeLa cells, and then received 1 ml of MEM (10% NCS and glutamine) supplemented with 100 μg/μl gentamicin, followed by a further 90 minute incubation at 37°C, 5% CO_2. These monolayers were then washed three times with PBS for HeLa cells, and lysed with repetitive pipetting in 1 ml ddH_2O. Cells were then serially diluted 10-fold and plated on appropriate media, incubated at 37°C, 5% CO_2 for 4-6 days, and the colonies were enumerated. Statistical analysis was performed using a one-way ANOVA test ($p < 0.01$).

2.28 Intracellular growth curves

HeLa cells were seeded in 12-well plates at 1 million cells per well, and allowed to form monolayers overnight in MEM with antibiotics (10% NCS and glutamine) at 37°C, 5% CO_2. The following morning, cells were examined by inverted microscope to ensure monolayers had formed. Monolayers were then washed with 1 ml PBS for HeLa cells, and then covered with 0.9 ml of MEM (10% NCS and Glutamine). Overnight plate growth of *L. pneumophila* was harvested into ddH_2O and the $O.D._{620}$ determined. Bacteria were then pelleted (4,800 x *g*, 6 minutes, room temperature), and resuspended in MEM (10% NCS and glutamine). 0.1 O.D. of bacteria were added to each well, and the plates were centrifuged at 500 x *g* for 10 minutes at room temperature to increase bacterial contact with the monolayer. Plates were then incubated for 3 hours at 37°C, 5% CO_2. Following incubation, monolayers were washed 6 times with 0.5 ml PBS for HeLa cells to remove extracellular bacteria, and three of the plates received 1 ml of MEM (10% NCS and glutamine supplemented with Str100, Thy100 and Cm4 where appropriate). The fourth plate was used as the time 0. Lysis of all monolayers for bacterial viable counts was then done using the same procedure. One mililiter of ddH_2O was added to each well, the HeLa cells were lysed with repetitive pipetting and then the cells were transferred to a 1.5 ml eppendorf tube. Cells were then vortexed to promote further HeLa cell lysis, and serially diluted in ddH_2O. Bacteria were then plated on BCYEα containing appropriate selection, incubated at 37°C, 5% CO_2 for 4-6 days, and enumerated. Statistical analysis was performed using a two-tailed student's t-test ($p < 0.01$).

2.29 Isolation of MIFs

Ten 250 ml flasks were seeded with HeLa cells and allowed to grow to ~ 80% confluence in 10 ml of MEM supplemented with 10% NCS and glutamine, and containing antibiotics. Prior to infection, monolayers were washed with 5 ml of PBS for HeLa cells, and then covered with 10 ml of MEM (10% NCS and glutamine). Twenty four hour plate growth of *L. pneumophila* was resuspended in ddH_2O, the optical density was determined at 620 nm as described above, and resuspended in MEM supplemented with 10% NCS. A bacterial suspension of 1.0 O.D. was added to each monolayer, and the flasks were then incubated at 37°C at 5% CO_2 overnight to allow the bacteria to become intracellular. The next morning, the monolayers were washed 3 times with PBS for HeLa cells, and then they received MEM (10% NCS, glutamine, Str100, Thy100 and Cm4 where appropriate). Infections were then allowed to proceed for an additional 48 hours. After 72 hours, flasks were shaken to remove loose cells and the media removed and pooled into 50 ml centrifuge tubes. Cells were then pelleted at 3500 x *g* for 15 minutes at room temperature. Supernatants were then removed, and the pellets were resuspended in 1 ml of ddH_2O and transferred to 1.5 ml eppendorf tubes. These tubes were then centrifuged at 13,000 rpm for 1 minute at room temperature. The supernatants were removed, and the pellets again were resuspended in ddH_2O. To promote complete lysis of the remaining HeLa cells the cell suspension was passaged 15-20 times through a 26G1 needle fitted into a 24G1 needle. The needle junction was wrapped in parafilm and then flamed briefly to ensure no leaks. After passage, cells were removed and added to 5 ml of MEM and 3 ml of Percoll (in 50 ml: 5 ml of 1.5 M NaCl and 45 ml Percoll) in 10 ml

pyrocarbonate tubes. The resulting 9 ml suspension was then centrifuged at 20,400 x *g* for 15 minutes at 4°C, with no deceleration set. After centrifugation, two layers formed towards the bottom of the tube, the upper layer containing HeLa cell debris, and the lower layer containing MIFs. Very carefully, ~ 5 ml of the supernatant was removed to make access to the two layers easier. A Pasteur pipette attached to a manual pipette aid was then inserted to the very bottom of the tube, and the lower layer was carefully taken up into the pipette, and removed before coming in contact with the top layer. The MIFs were then transferred to a 1.5 ml eppendorf tube, diluted to capacity with PBS, and centrifuged at 13,000 rpm for 1 minute at room temperature. The MIFs were then washed twice more in PBS, and finally resuspended in 1 ml PBS. The optical density was then determined at 620 nm. Enough of the MIFs were put aside make 1 ml of a 1.0 O.D./ml suspension; the remaining cells were pelleted at 16,060 x *g* for 1 minute at room temperature, and then stored at -75°C until required.

2.30 Resilience Testing

Resilience of MIFs was assessed by determining the cells resistance to a variety of stresses including pH 11, ability to resist lysis by detergents, and ability to survive in ddH_2O for 8 days. All statistical analysis was performed using a two-tailed student's t-test ($p < 0.01$). Detergent resistance was done using purified MIF cells that had been stored at -75°C. One ml of a bacterial cell suspension with an OD of 1.0 units was pelleted at ~4500 x *g* for 6 minutes at room temperature and resuspended in 0.1% Triton X-100 in 1 M Tris-HCl pH 7.5. The optical density of the solution was monitored at 620

nm for up to 96 hours. Sensitivity was measured as the time required to reduce the optical density to 0.5. For resistance to pH 11, approximately 10^5 MIFs were suspended in 100 mM Tris base, pH 11, and incubated for 16 hours at 37°C. Samples were then centrifuged at 16,060 x *g* for 1 minute and the ability to form pellets evaluated.

For ddH_2O testing, 0.1 O.D. of bacteria grown for 48 hours on BCYEα (Str100, Thy100 and Cm4 where appropriate) was suspended in ddH_2O, and then incubated at 37°C for 8 days. Serial 10-fold dilutions of the suspension were plated on BCYEα (Str100, Thy100 and Cm4 where appropriate) and incubated for 5-7 days, after which colonies were enumerated.

Resistance to heat was determined using 0.1 OD of 48-hour bacteria grown on BCYEα (Str100, Thy100 and Cm4 where appropriate). Cells were then suspended in ddH_2O, standardized to an optical density of 620 nm, and tubes were put in a 50°C water bath for 30 minutes. Cells were then serially diluted 10-fold and plated on BCYEα with appropriate selection. As a control, similar tubes were set up and left at room temperature before being serially ten-fold diluted and plated.

2.31 Preparation of MIF lysates for 2D gels

MIFs isolated by Percoll density centrifugation were frozen at -75°C, and then 3 ODs were removed, and sonicated in lysis buffer (in ddH_2O: 8 M urea, 4% CHAPS and 40 mM Tris base). The lysate was then centrifuged at 50,000 x *g* for 30 minutes at 4°C. Fifty

to seventy micrograms of protein were then added to rehydration buffer and subjected to isoelectric focusing on pH 3-10 NL Immobiline dry strips. 2D gels were then run as described above.

2.32 Growth of Intracellular bacteria for Light and Electron Microscopy

Hela cells were grown in 30 ml flasks as described until monolayers were ~80% confluent. Monolayers were washed once with 5 ml of PBS for HeLa cells, and then received 2.7 ml of MEM (10% NCS and glutamine). Overnight plate growth of *L. pneumophila* was harvested into ddH_2O using a sterile loop and vortexed. The optical density was then determined and suspensions standardized to 3 O.D. Cells were then harvested at 4,800 x *g*, 6 minutes, room temperature and resuspended in 1 ml of MEM (10% NCS and Glutamine). 0.3 ml of the suspension was then added to the flask, and incubated at 37˚C at 5% CO_2 for 3 hours. Cells were then washed 6 times with PBS for HeLa cells, and received 5 ml of MEM (10% NCS and Glutamine) with appropriate selection. After 48 or 72 hours, flasks were examined using at 400x using an Olympus IX-71 (Olympus) inverted microscope equipped with a 12-bit Evolution QET Monochrome camera (Media Cybernetics), and digital images captured using Image Pro software (Media Cybernetics). Following light microscopy, monolayers were agitated by shaking to loosen infected cells, and the supernatant was removed and transferred to 13 ml polystyrene tubes. Tubes were centrifuged at 3,000 x *g*, 10 minutes, room temperature and the supernatants were discarded. Cacodylate buffer (1.5 ml of a 0.1 M solution), pH 7.2, containing 10% glutaraldehyde was added gently to the pellet, and the tube was left

at 4°C overnight. The fixative solution was removed and replaced with 0.1 M cacodylate buffer, pH 7.2, and stored for 2-3 days until being submitted for electron microscopy.

2.33 Electron Microscopy

Electron microscopy was performed at the Electron Microscopy Laboratory in the Tupper building. All samples were prepared and thin-sectioned by the Electron Microscopy Laboratory. Samples were post-fixed with 1% wt/vol osmium tetroxide (OsO_4) in cacodylate buffer for 2 hours, followed by overnight incubation at 4°C in 0.25% uranyl acetate. Following dehydration, samples were embedded in Epon Araldite Resin, and then thin-sectioned using an LKB Huxley Ultramicrotome to a thickness of approximately 100 nm. Once the sample preparations were completed, they were viewed using a JEOL JEM 1230 transmission electron microscope at 80 kV. Images were captured using a Hamamatsu ORCA-HR digital camera, and saved using AMT v.450 software.

CHAPTER 3: RESULTS

3.1 Confirmation of the presence of the sodium sensitive regulator in Lp02

To confirm that the sodium sensitive regulator was also present in the Lp02 strain, duplicate cultures were grown to late exponential phase; one was challenged with 145 mM NaCl for one hour, while the other served as a control and received no sodium chloride. A schematic representation of the 285 bp *ompS* promoter, including primer binding sites, is shown in figure 2. Cellular extracts were prepared by French Pressure cells, and used in Electrophoretic Mobility Shift Assays (EMSA) with a 285 bp region of the *ompS* promoter region. Figure 3A depicts a schematic representation of the *ompS* promoter region. As seen in figure 3B, cell-free extract prepared from unchallenged Lp02 cultures produced a mobility shift, similar to that seen in the *Legionella pneumophila* Philadelphia-1 strain Svir, as demonstrated by Weeratna (Ph.D. thesis, Dalhousie University, 1995). Cell-free extracts prepared from bacteria challenged for one hour with sodium chloride did not produce a gel shift at the same protein concentrations. Much higher levels of protein were required to achieve a gel shift. Even at the highest concentrations, the mobility shift band was more diffuse than that seen in the respective unchallenged lane. This suggests that the OmpT protein(s) is no longer able to bind as stringently to the *ompS* promoter upon exposure to sodium chloride.

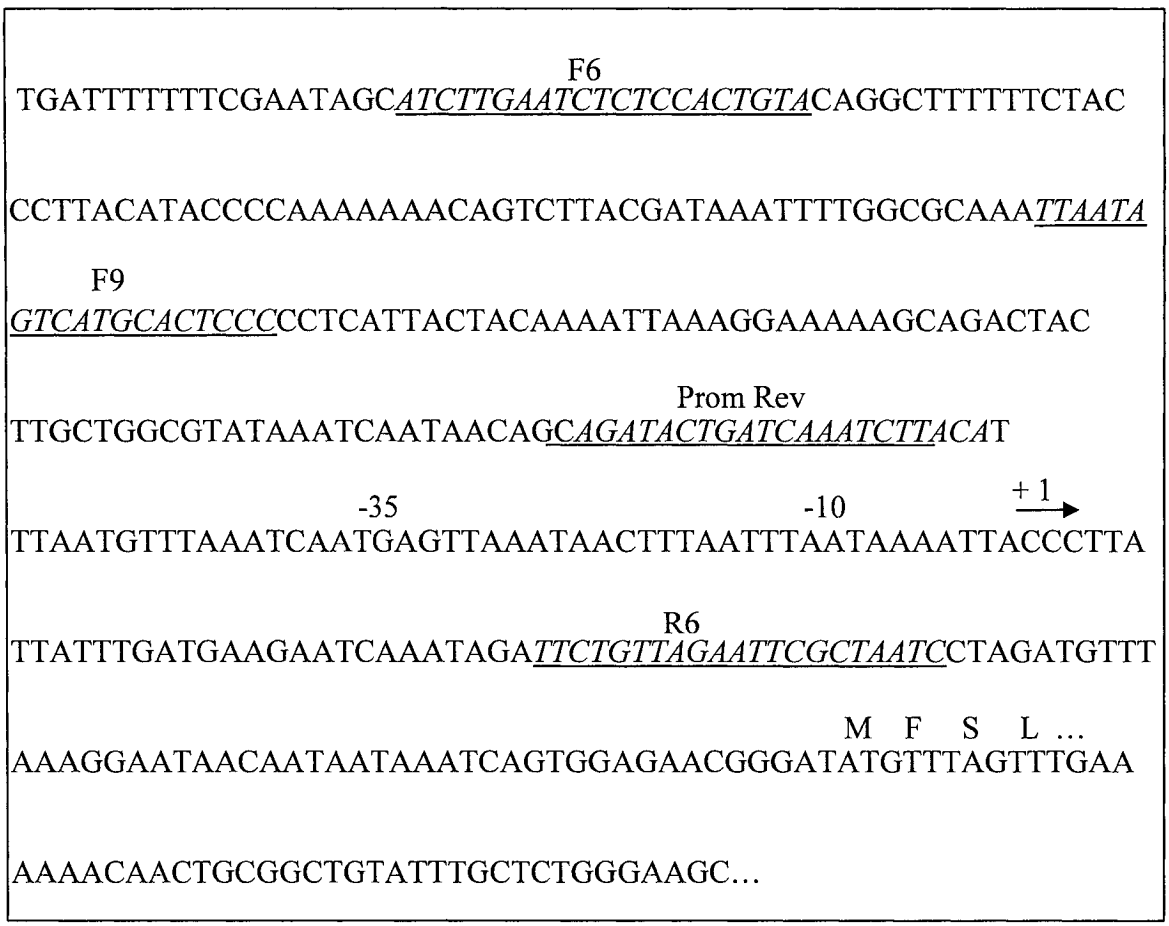


Figure 2. Schematic representation of the *ompS* 285 bp promoter region illustrating the position of primer binding sites. The nucleotide sequence of the *ompS* promoter region is shown above, taken from the published genome sequence. –10 and -35 sites are labelled appropriately, including the transcription start site, which has been previously defined (Hoffman *et al.*, 1992). Primer binding sites are underlined, in italics, and labeled. Also shown are the first four amino acids in the coding sequence.

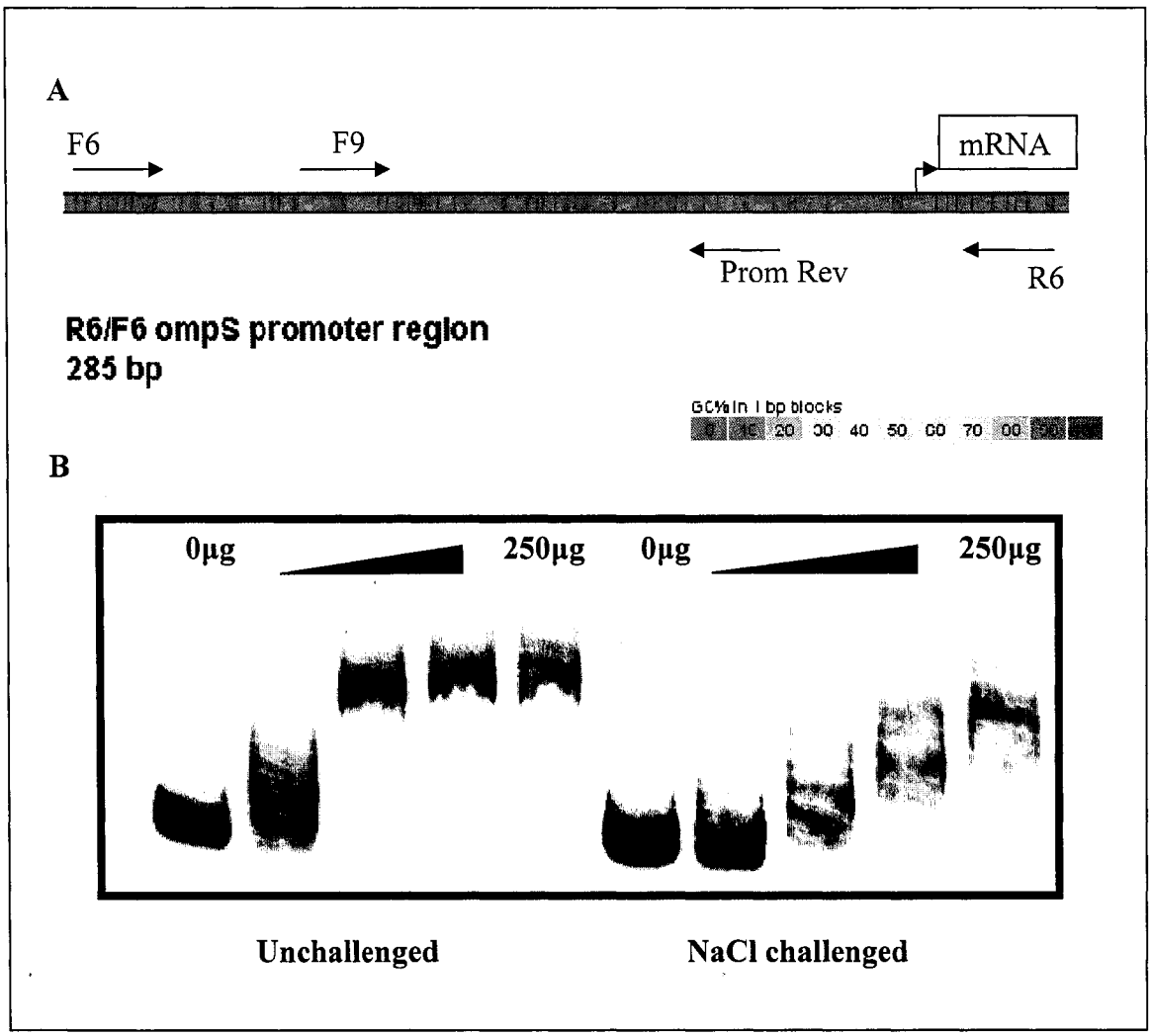


Figure 3: Presence of the sodium sensitive regulator OmpT in Lp02. Panel A. Schematic representation of the 285 bp R6/F6 *ompS* promoter region, including mRNA start site and the approximate location of F9, F6, R6 and prom Rev primer binding sites. Panel B. Electrophoretic Mobility Shift Assay, run on an 8% polyacrylamide gel of the 285 bp *ompS* promoter region using crude lysates from both unchallenged and sodium challenged cultures. Increasing equal amounts of crude lysate were used. At equivalent amounts of protein, the NaCl challenged lysate was unable to produce the same retardation as that of the unchallenged culture. µg amounts indicate µgs of total protein per lane.

3.2 Attempted isolation of the sodium sensitive regulator by anion exchange chromatography

In an attempt to identify OmpT, and to isolate any other DNA binding proteins that may affect the expression of OmpS, cell-free extracts from the Philadelphia-1 strain Svir were subjected to anion exchange column chromatography. The Svir strain was used in these experiments in order to eliminate the presence of exogenous thymidine from the column. Increasing concentrations of KCl (35 to 500 mM) were used to elute proteins from the column. The protein content of the eluent was measured using a UV spectrophotometer. Fractions from the column were then assessed for their ability to retard the *ompS* amplicon. Two fractions contained *ompS* DNA binding components. These fractions are indicated by red arrows in figure 4. Fractions containing DNA binding activity were combined, concentrated by dialysis, and again subjected to purification by anion exchange column chromatography under identical conditions. No DNA binding activity was obtained from the second column run. The lack of mobility shift after rechromatography suggests either instability in the proteins responsible for the shift, or that multiple components are required for the shift, and the second purification step effectively separates the two.

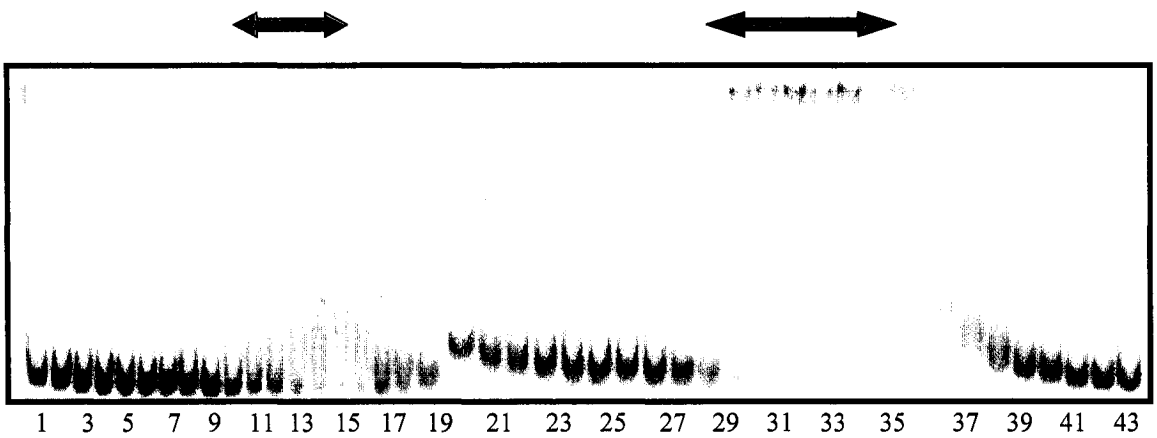


Figure 4: Multiple *ompS* binding proteins are present in anion exchange separated fractions of Svir crude lysate. 10 µl of each fraction derived from a DE-52 (diethylaminoethyl cellulose) anion exchange column were tested for their ability to retard the *omp*S amplicon in 8% polyacrylamide gels. The red arrows indicate fractions that were able to cause a mobility shift of the *ompS* promoter region (fractions 10-18 and 30-38). Lane 1 is unpurified Svir lysate as a positive control.

3.3 Determining the global effects of sodium on gene expression using two-dimensional SDS PAGE and identification of regulated proteins.

Due to the difficulties in the attempted identification of OmpT and the possibility that the protein turns over rapidly, a proteomic strategy was employed to identify OmpT, and any other proteins whose expression is affected by sodium. This strategy may also identify proteins whose mobility changes might reflect phosphorylation state. This technique was also employed to assess the role of sodium in global modulation of gene expression. Lysates from late exponential Lp02 cultures that had been challenged with sodium chloride or potassium chloride were subjected to isoelectric focusing between pH 3-10, followed by electrophoresis on 10-20% gradient polyacrylamide gels. Potassium chloride challenged cultures were included to identify proteins whose expression was altered due to changes in osmolarity, and were thus not sodium specific. As a control, late exponential phase extracts from unchallenged Lp02 cultures were also prepared. Duplicate gels for each treatment were then silver stained and the gels compared for differences in protein profiles. As is seen in figure 5, there were no significant differences between lysates from sodium (panel B) and potassium (panel C) challenged cultures. When compared to the unchallenged Lp02 gel, several proteins showed differences in expression, and are pictured in panel A. Proteins identified as being alternatively expressed after salt challenge were subjected to trypsin digestion and LC-MS analysis (see Table 3). While the expression level of the protein #1 remained unchanged, the isolelectric point shifted to a more acidic pH, indicative of a post translational modification. Attempts to identify this modification based on the generated mass spectra

were unsuccessful. Two proteins, the 27-kDa outer membrane protein (spots 5 and 9), and the succinyl CoA synthetase Beta chain (spots 7 and 8), were identified more than once from distinct spots. Thus, there were no sodium-specific differences identified by two-dimensional gel electrophoresis, and this experiment was unable to identify OmpT.

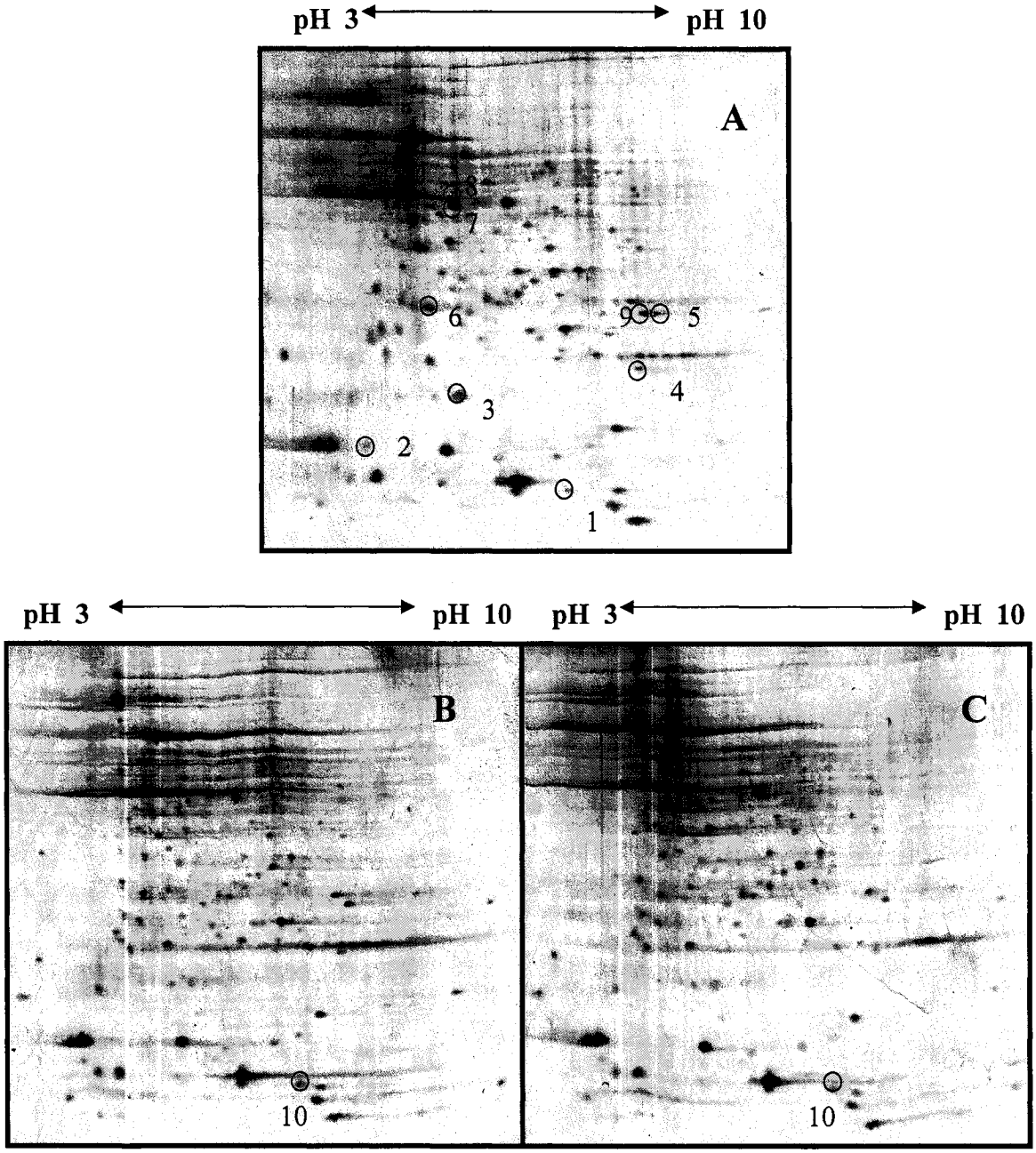


Figure 5. Sodium and potassium induced changes in late exponential phase, Lp02 lysates. Two-dimensional SDS PAGE gels of lysates were focused on pH 3-10 IEF strips, and run on 10-20% gradient polyacrylamide gel. Lysates were derived from unchallenged (A), NaCl challenged (B), and KCl challenged (C) Lp02 cultures. Sodium or potassium chloride challenges were conducted for one hour prior to harvesting. Proteins circled in panel A indicate proteins whose expression has been altered after salt challenge, while circled spot 10 in gels B and C represent a protein whose isoelectric point has changed.

Spot number	ID	% Coverage	MW (kDa)	Calc pI	% exp KCl	% exp NaCl
1	lpg0406	16	12.3	6.1	117.0*	191.5*
2	lpg1585	22	18.4	5.14	87.6	72.4
3	GspA GspA	44 40	19.0 20.0	6.12 6.12	50.8	19.0*
4	Ribosome Recycling Factor	31	20.9	6.35	51.4	61.8
5	27 kDa Outer Membrane Protein	36	28.5	6.92	30.2	50.5
6	ATP transporter, ABC binding component, ATP-binding protein	24	27.7	5.26	53.5*	53.0
7	Succinyl CoA Synthetase Beta Chain	16	44.0	6.08	47.3	34.0
8	Succinyl CoA Synthetase Beta Chain	11	44.0	6.08	48.5	74.6
9	27 kDa outer membrane protein	54	28.5	6.92	50.5	66.4

Table 3. LC-MS identification of proteins whose expression is altered upon exposure to NaCl or KCl. Proteins identified as being differentially expressed after salt challenge were excised, digested with trypsin, and the resulting polypeptide fragments were sequenced by LC-MS. Asterisks indicate P-values of < 0.1. Spot 10, not listed here, was identified as spot 1. % coverage = percentage of total amino acid sequence identified in trypsonized fragments. MW = Molecular weight. Calc pI – Predicted isolectric point from sequence data. % Exp NaCl or KCl = % expression of the protein after sodium or potassium challenge respectively.

3.4 Mapping of the DNA binding sites in the *ompS* promoter.

In order to define the sequence elements necessary for binding of OmpT to the *ompS* promoter region, 5' and 3' truncations were created in the 285 bp promoter amplicon. Oligonucleotides were designed (prom Rev and F9, see figure 2) to create two overlapping fragments of the 285 bp amplicon. These fragments were then subjected to EMSA using the unchallenged Lp02 lysate. Figure 6 shows the results of these experiments. EMSA using the 5' and 3' truncated probes with Lp02 unchallenged lysate failed to produce any significant mobility shifts, and only slight shifts at the highest protein concentrations. The lysates used in this study were still able to shift the full 285 bp promoter region. Arrows designate the location of the unshifted amplicon (lower arrow), and the slight shift arising at the highest protein concentrations (upper arrow). These results suggest that either the motif recognized by the binding protein spans both fragments (and is thus destroyed by the separation), or that there are multiple sequences required for OmpT binding. To address this issue, the 285 bp motif was analyzed bioinformatically for DNA binding protein domains based on consensus sequence data. Analysis revealed the presence of Integration Host Factor (IHF) binding sites in both of the fragments. The locations of these IHF consensus sequences are schematically represented in figure 7.

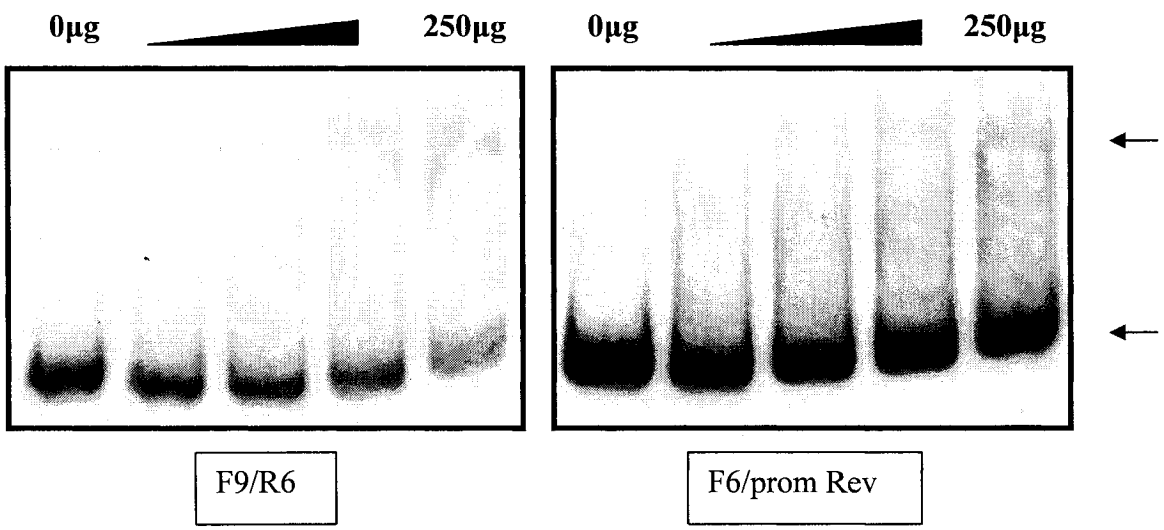


Figure 6: EMSA using radiolabeled fragments of the 285 bp promoter and Lp02 unchallenged lysate. Truncated versions of the 285 base pair *ompS* promoter region were incubated with increasing concentrations of unchallenged Lp02 lysate, and run on 8% polyacrylamide gels. The Lp02 lysate was essentially unable to shift the truncated products (lower arrow), except at the highest protein concentrations, where a small amount of the radiolabeled amplicon was retarded (indicated by the upper arrow).

A

F6 1

TGATTTTTTTCGAATAGC*ATCTTGAATCTCTCCACTGTA*CAGGCTTT**TTTCTAC**

CCTTACATACCCCAAAAAAACAGTCTTACGATAAATTTTGGCGCAAA*TTAAT*

F9

*AAGTCATGCACTCCC*CCTCATTACTACAAAATTAAAGGAAAAAGCAGACTAC

Prom 2

TTGCTGGCGTATAAATCAATAACAGC*AGATACTG***ATCAAATCTTAC***A*T

-35 -10 +1

TTAATGTTTA**AATCAATGAGTTA**AATAACTTTAATTTAATAAAATTACCCTT

3 R6

ATTATTTGATGAAGAATCAAATAGA*TTCTGTTAGAATTCGCTAATC*CTAGATGT

M F S L ...

TAAAGGAATAACAATAATAAATCAGTGGAGAACGGGATATGTTTAGTTTGAA

AAAACAACTGCGGCTGTATTTGCTCTGGGAAGC...

B

Consensus:
(A/T)ATCAANNNNTT(A/G)
Site 1 (+19 to +31):
AATCAAATAGATT
Site 2 (–42 to –30):
AATCAATGAGTTA
Site 3 (–60 to –48):
GATCAATCTTTAC

Figure 7. Schematic representation of the 285bp promoter region illustrating the position of IHF binding sites. Panel A. Nucleotide sequence of the *ompS* promoter region. IHF binding sites are indicated in bold, –10, -35 sites and labelled appropriately, and primer sequences shown in italics. Panel B. Comparison of the three IHF binding sites to the *E. coli* consensus sequence. Areas in blue indicate matches, areas in red indicate mismatches.

3.5 Integration Host Factor binds the *ompS* promoter region

To determine whether the predicted IHF binding sites are indeed bound by integration host factor, His tagged *L. pneumophila* IhfA and IhfB subunits (gift from Dr. Ann Karen C. Brassinga, University of Virginia, USA) were purified by nickel chromatography, and used in EMSA with the *ompS* promoter. The results of this experiment are shown in Figure 8. Purified IhfA protein was capable of producing only a slight shift (lanes 2-4), even at the highest concentrations used, when compared to the purified IhfB protein (lanes 5-7). The greatest shift was obtained when equal amounts of both subunits were used in the binding reaction (lanes 8-10), specifically in lane 10. Thus, IHF is capable of binding the *ompS* promoter, and both subunits are required for maximal retardation of the *ompS* promoter.

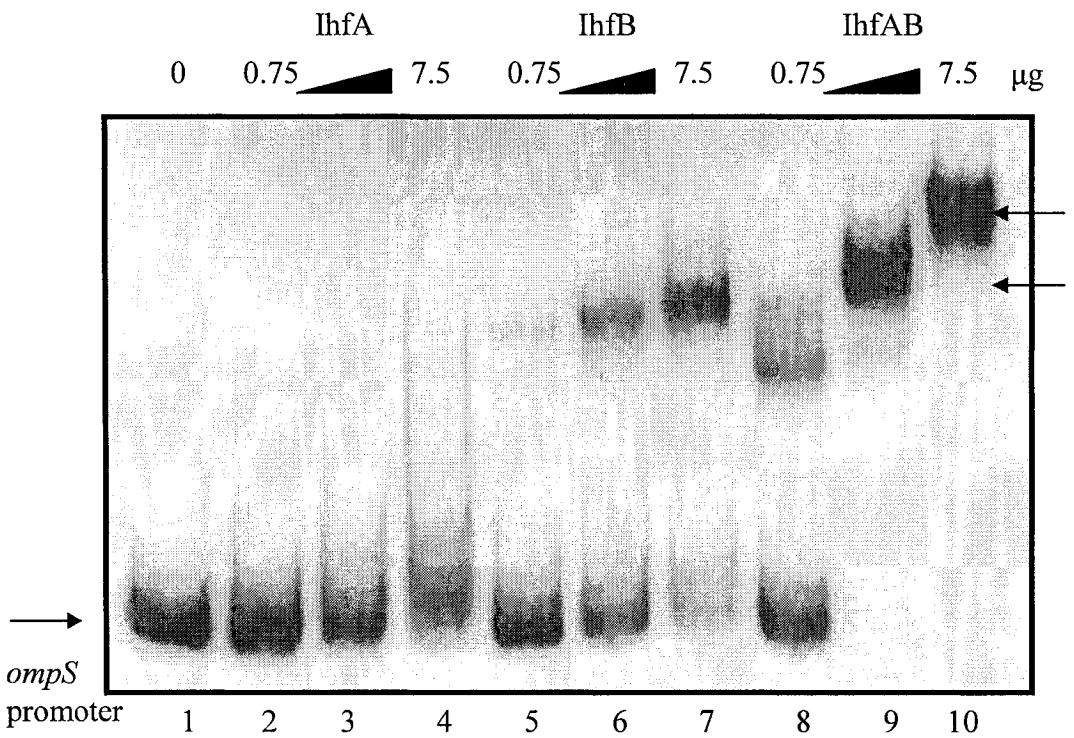


Figure 8. EMSA using purified IHF subunits and the *ompS* promoter region. The *ompS* promoter was incubated with increasing amounts (0.75, 3.75 or 7.5 µg) of purified IhfA (lanes 2-4), IhfB (lanes 5-7) and equal amounts of both IhfA and IhfB subunits (1:1 ratio), and then run on an 8% polyacrylamide gel. Purified IhfA was not able to cause a significant shift at the concentrations used, while IhfB did produce a significant shift. The greatest mobility shift occurred in the presence of both α and β subunits (lanes 8-10, indicated with upper arrow). Performed in collaboration with Dr. Ann Karen C. Brassinga, figure adapted from from Brassinga *et al.*, 2005, manuscript in preparation.

3.6 The *L. pneumophila* Integration Host Factor

Integration Host Factor (IHF) is a small, basic, heterodimeric DNA-binding protein implicated in the regulation of several genes and global chromosome structuring. The *Legionella pneumophila* IhfA and IhfB genes bear extensive sequence identity to the *E. coli* homologues, with the IhfB proteins having 61% identity, 72% similarity over the first 90 amino acids (both 103 amino acids total length). Similarly, the IhfA proteins share 71% identity and 82% similarities over the first 91 amino acids (both 99 amino acids total length). The *L. pneumophila* homologue also contains the four IhfA residues (S47, R60, P65 and R63) and two IhfB residues (R46 and P64) previously shown in *E. coli* to be required for proper contact with the consensus site (Ellenberger and Landy, 1997). The genes encoding both subunits also show 100% conservation to the homologues from the *L. pneumophila* Paris and Lens strains, except for 1 amino acid change in the IhfA protein, where there is a V to M mutation in position 3.

While sequence identity remains conserved, location on the chromosome is different between *E. coli* and *L. pneumophila*. In both bacteria, the *ihfA* gene overlaps the last 10-20 nucleotides of the upstream phenyl tRNA synthetase. Downstream of the *E. coli ihfA* gene lies the vitamin B-12 uptake operon; however in *L. pneumophila*, 100 base pairs downstream lies a ferredoxin 2Fe-2S protein. The *ihfB* genes show no similar surrounding regions between both bacteria. The *E. coli ihfB* gene is flanked upstream by a cytidylate kinase and 50S ribosomal protein S1 (both in the same orientation as *ihfB*), and a *rec2* homologue of *Neisseria* downstream. The *L. pneumophila ihfB* gene is flanked

upstream by two hypothetical proteins (lpg2953 and lpg2954, again in the same orientation as *ihfB*) and downstream by a deoxycytidine triphosphate deaminase.

3.7 Allelic replacement mutagenesis of the *ihfA* and *ihfB* genes

To assess the role of IHF in binding of the *ompS* promoter, and its role in the sodium response, chromosomal deletion mutants were created in both the *ihfA* and *ihfB* genes, which encode the IhfA and IhfB protein products respectively, using the pBOCRdxA vector. During allelic replacement, it becomes necessary to select between clones that are resistant due to chromosomal insertion of the replacement construct (double crossover), and clones that contain the unrecombined vector (merodiploid). To increase the probability of obtaining clones that have undergone recombination, a gene that confers lethal sensitivity to some compound is introduced into the delivery plasmid. The suicide plasmid created in collaboration with Dr. Ann Karen C. Brassinga used the *Helicobacter pylori rdxA* nitro reductase gene, discovered in this lab (Goodwin *et al.*, 1998). RdxA converts the prodrug metronidazole (2-Methyl-5-nitroimidazole-1-ethanol) to the hydroxyl radical, a reactive radical species that causes damage to DNA (Sisson et al. 2000). Therefore bacteria harbouring the *rdx*A gene are rendered sensitive to metronidazole. The suicide vectors used in this work, and the plasmids in which the allelic replacement constructs were made are shown in figure 9. The vector was electroporated into Lp02 and transformants plated on the appropriate antibiotic selection. The resulting colonies were replica plated on media containing appropriate selection and

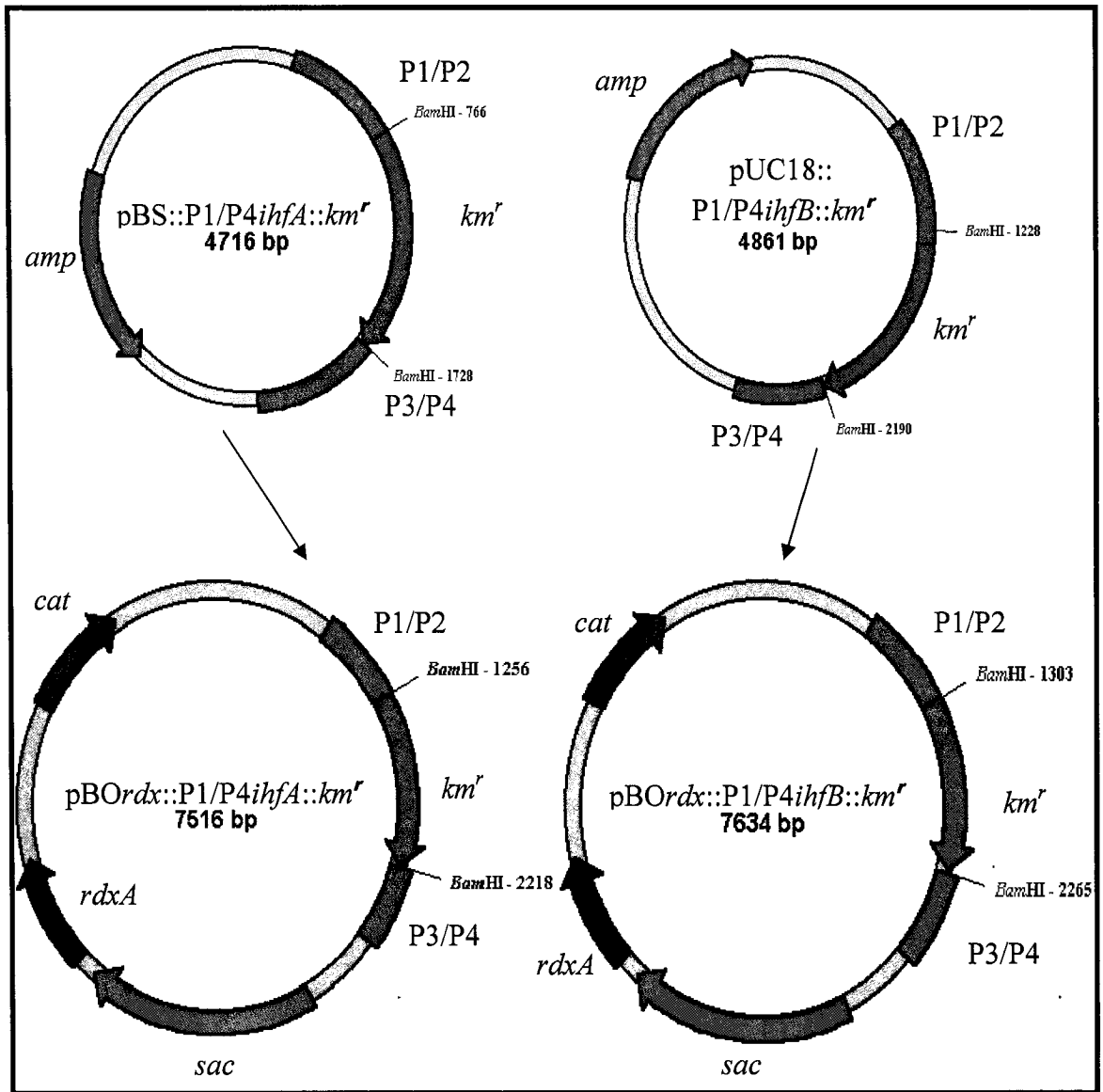


Figure 9. Schematic representation of the *ihfA* and *ihfB* chromosomal deletion constructs and the vectors in which they were created. Sections in blue indicate the exact location of the deletion construct, which includes the 5' (P1/P2) and 3' (P3/P4) flanking regions, and the antibiotic resistance marker (*km*^{r}). Ampicilin resistance (*amp*, top) and sucrose sensitivity (*sac*, bottom) markers shown in orange. Chloramphenicol resistance (*cat*) shown in dark blue. pBS and pUC18 plasmids are shown in grey, pBOCrdxA in green. The gentamicin resistance constructs were created by removal of the kanamycin cassette by BamHI restriction endonuclease digestion (sites indicated in diagram above) followed by insertion of the new resistance gene.

metronidazole, to eliminate clones that had not undergone recombination. This plating step generally reduced the number of possible recombinants by ~20%. Screening for loss of the gene was done by PCR using oligonucleotides specific for the regions that were deleted (see figure 12, panel B). For each of the mutants, 20 colonies were screened, with 6 and 8 mutants being identified for the *ihfA* and *ihfB* genes respectively.

3.8 Determining the role of individual IHF subunits in the gel retardation of the *ompS* promoter.

EMSA was used to evaluate IHF mutants for their ability to cause a retardation of the *ompS* promoter. EMSA were performed using crude cell lysates from *Legionella pneumophila ihfA* and *ihfB* single deletion mutants grown to late exponential phase. The lysates were analysed for their ability to cause a mobility shift. Figure 10, panel A, represents increasing concentration of Lp02 unchallenged lysate as a positive control. Neither of the deletions in *ihfA* or *ihfB* caused any alteration in the retardation profile of the *omp*S amplicon (Figure 10, Panels B and C). Thus, despite the fact that the purified IHF subunits are capable of binding the *ompS* promoter, neither subunit is solely responsible for the OmpT mediated shift seen in the control lanes.

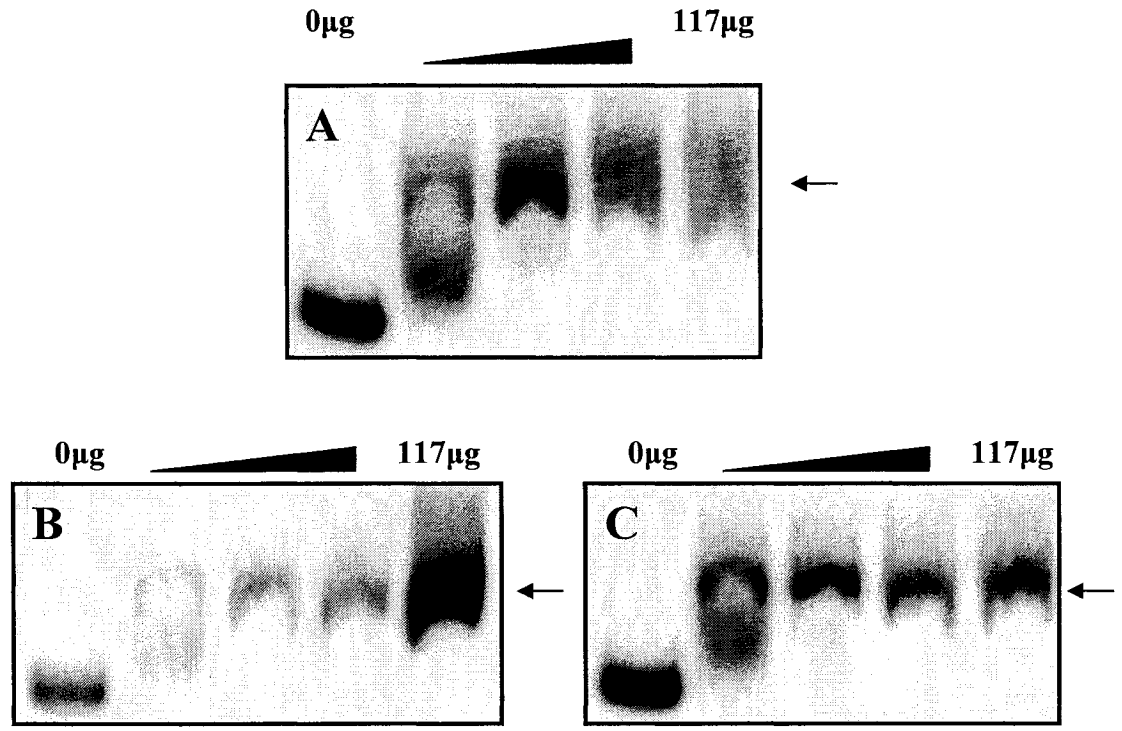


Figure 10. EMSA using cell-free crude lysates derived from *ihf* single deletion mutants. Increasing amounts of crude cell lysates from Lp02 (panel A), Lp02Δ*ihfA* (panel B), and Lp02Δ*ihfB* (panel C) were incubated with radiolabeled, 285 bp *ompS* promoter, and run on 8% polyacrylamide gels. Similar shifts were produced in the single *ihf* deletion mutants as was seen in the Lp02 gels (mobility shifts are indicated by arrows).

3.9 Sodium sensitivity of the *ihfA* and *ihfB* single deletion mutants.

To further assess whether there were any defects caused by the deletion of individual subunits, the sodium sensitivity of the strains was determined. It had been shown previously that mutations in several virulence genes (*dot/icm*) (Berger and Isberg, 1993), and several regulatory genes (*rpoS*, *letA/S*) (Bachman and Swanson, 2001 and Hammer *et al.*, 2002) led to decreased sodium sensitivity when plated on media containing 100 mM sodium chloride. Percent viability is calculated as (c.f.u. per ml on media + NaCl / c.f.u per ml on media without NaCl) x 100. Thus, this assay was employed to determine whether there were any differences in the sodium sensitivity of the single mutants when compared to wild type, which could suggest possible defects in *dot/icm* expression. As seen in Figure 11, the wild type strain Lp02 displayed a typical sodium sensitivity phenotype, with a percent survival of 0.021. Similarly, both of the single IHF mutants were statistically indistinguishable from the wild type ($p > 0.1$, student's t-test). Therefore, the deletion of either IHF subunit does not affect sodium sensitivity.

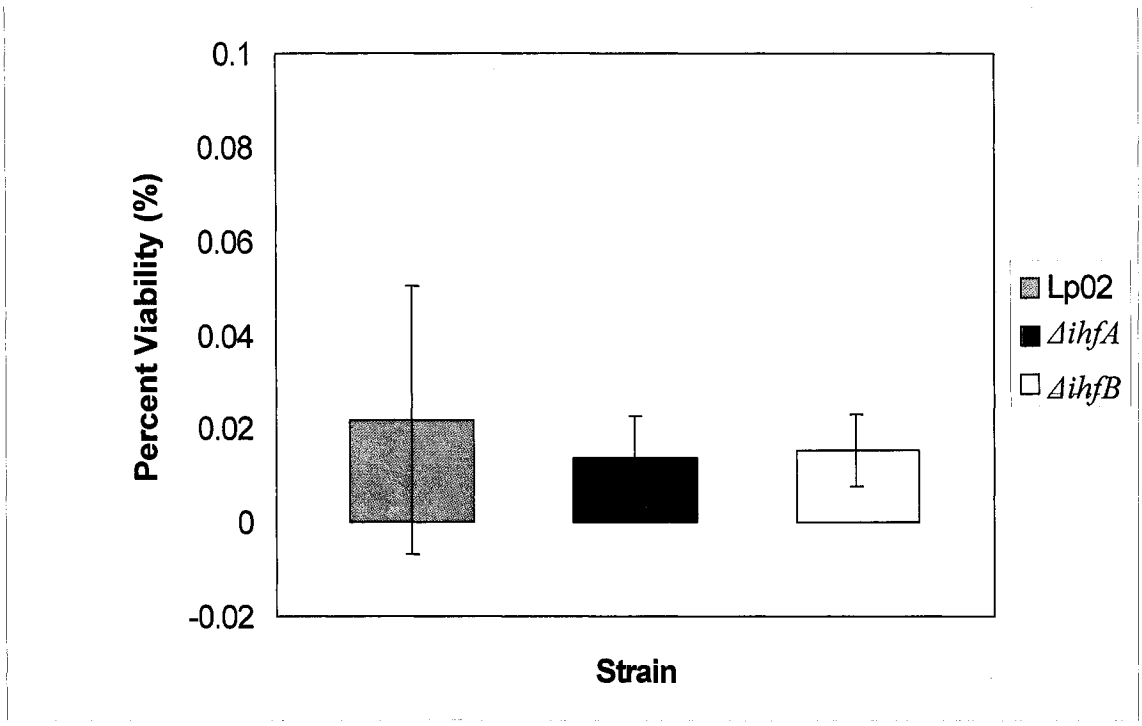


Figure 11: Sodium sensitivity of the single deletion mutants. 72-hour plate growth of Lp02, *ihfA* and *ihfB* was plated on media with and without 100mM NaCl. Percent viability is calculated as (c.f.u per ml on media containing 100 mM NaCl / c.f.u per ml on media without NaCl) x 100. Similar viabilities were obtained for all three strains. Data are based on duplicate experiments.

3.10 Construction of the Lp02Δ*ihfAB* deletion strain, Lp02Δ*ihfAB*::pMMB206::*ihfAB*, Lp02Δ*ihfAB*::pMMB206, Lp02/pMMB206::*ihfAB* over expressing strain and the Lp02/pMMB206 strain.

Because there were no apparent differences between the single deletion mutants and the wild type strain, a mutant containing deletions in both *ihf* genes was created. The Lp02Δ*ihfAB* mutant was confirmed by RT-PCR and Western blot (Figure 12). The Lp02Δ*ihfAB* mutant strain was complemented by introduction of a plasmid containing both genes. Initial attempts involved cloning the coding sequences, along with ~700 base pairs of promoter region, in tandem, into the vector pMMB206. Close examination of this construct revealed no expression of the IhfB protein. To ensure expression of both subunits, an operon was created by fusing the coding sequence of the *ihfB* gene, including 12 base pairs containing the Shine-Delgarno sequence immediately 3' to the end of the *ihfA* coding sequence (illustrated in figure 12, panel A). Expression of the fusion construct was driven off of the IPTG inducible *lacZ* promoter present in the pMMB206 vector. Constructs were confirmed by PCR (figure 12, panel B). Western blot analysis of this construct revealed expression levels similar to that of wild type (Figure 12, panel C). Expression of both subunits was confirmed by RT-PCR using RNA from an exponential phase, 2 mM IPTG induced broth culture of Lp02Δ*ihfAB*/pMMB206::*ihfAB* (Figure 12, panel D). In the absence of IPTG, there was still some expression of IHF, as judged by western blotting (Figure 12, panel C).

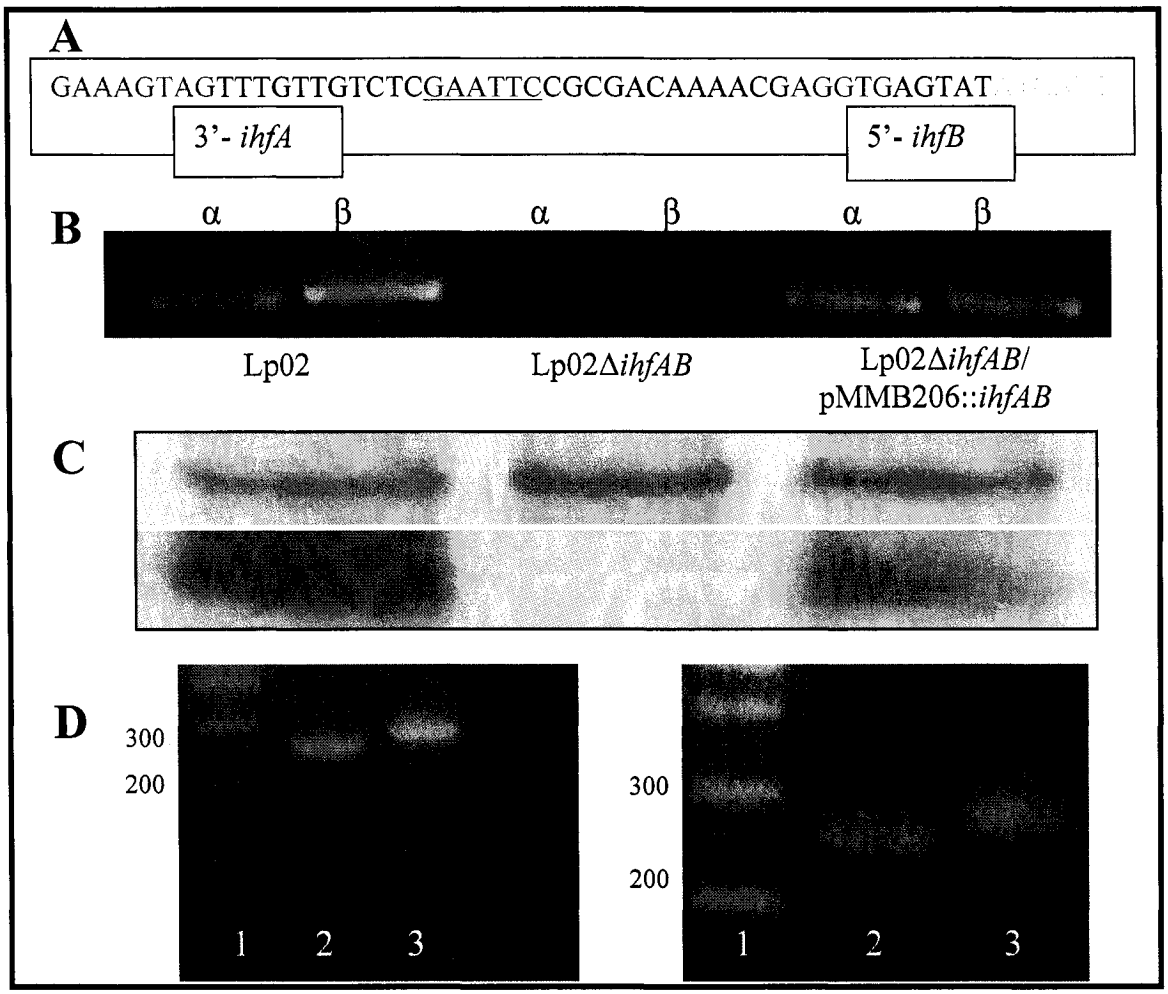


Figure 12: Confirmation of allelic replacement of both the *ihfA* and *ihfB* genes, and creation of the complementing construct. Panel A; Schematic representation of the *ihfAB* fusion used to make the complementing construct. The end of *ihfA* coding sequence is shown in blue, the start of *ihfB* coding sequence in green. The –10 (purple) and –35 (red) of the *ihfB* gene is also shown. Panel B; PCR of the α and β subunits using genomic DNA from Lp02, Lp02Δ*ihfAB* and Lp02Δ*ihfAB*/pMMB206:: *ihfAB*, using the RT IHFα For/Rev and RT IHFβ For/Rev primer sets where appropriate. Panel C; Western blot of the same three strains listed above using the anti-IHF antibody. The top band is a non-specific band that occurred during developing, used to demonstrate equal loading, the lower band is IHF. Panel D; RT-PCR of in vitro grown Lp02 and IPTG induced Lp02Δ*ihfAB*/pMMB206::*ihfAB*. The expression of both subunits was confirmed using the RT IHFα For/Rev (lanes 2) and RT IHFβ For/Rev (lanes 3) primer sets where appropriate. Lane 1 = MW markers.

3.11 Determining whether IHF binds the *ompS* promoter.

If the bioinformatic predictions made earlier were accurate, then extracts from late exponential and stationary phase cultures of the Lp02Δ*ihfAB* strain should no longer be able to retard the *ompS* promoter in gel shift assays. This hypothesis was addressed, and figure 13 illustrates the results of these experiments. As was the case for the single deletion mutants, there was no difference in mobility shift profile using the Lp02Δ*ihfAB* late exponential phase lysate (Figure 13, panel A). The lysates examined from stationary phase Lp02Δ*ihfAB* cultures, however, did show substantial differences when compared to those from both the late exponential phase extracts (figure 13, panel A), and those from the wild type stationary phase lysate (figure 13, panel B, lanes 1-5). Lp02 stationary phase lysates are capable of producing a greater retardation of the *ompS* amplicon than lysates from the late exponential phase Lp02 cultures. The shift produced by the Lp02Δ*ihfAB* stationary phase culture lysates was not as great as that produced by Lp02 in stationary phase, yet greater than that in late exponential, indicating that IHF is partially required for the shift observed during this point in the growth cycle. Thus, this experiment confirms the bioinformatic predictions made earlier, that IHF does bind the *ompS* promoter region, and demonstrated that this binding occurs during stationary phase, and not during exponential phase. OmpT binding was not observed in extracts harvested during stationary phase from either the Lp02 or the IHF mutant strains. In contrast, IHF binding was more evident with Lp02 in stationary phase than in the IHF mutant.

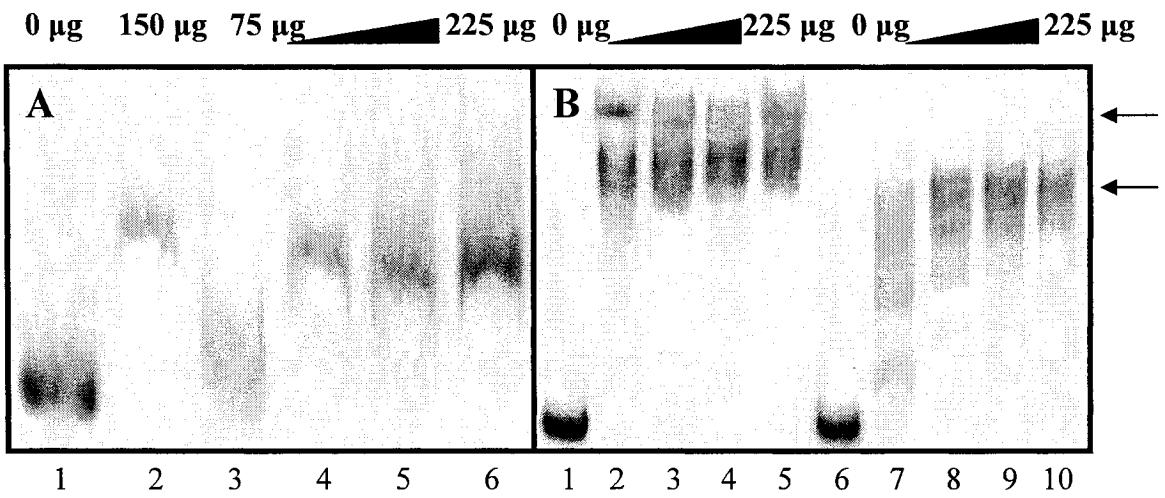


Figure 13. IHF does bind the *omp*S promoter in vitro as determined by EMSA. Panel A. Late exponential phase crude lysate of Lp02 (lane 2), and increasing amounts of Lp02Δ*ihfAB* crude lysate (lanes 3-6) were incubated with radiolabeled 285 bp *ompS* amplicon and run on 8% polyacrylamide gels. The mutant extracts were able to retard the *omp*S amplicon similar to wild type. Panel B. Increasing amounts of crude lysate from stationary phase cultures of Lp02 (lanes 2-5) and Lp02Δ*ihfAB* (lanes 7-10) were incubated with radiolabeled 285 bp *ompS* amplicon and run on 8% polyacrylamide gels. The upper arrow indicates the wild type shift, while the lower arrow indicates the altered shift profile seen in the mutant background. µg amounts indicate the µgs of total protein.

3.12 IHF is developmentally regulated

In other bacteria, IHF levels cycle during bacterial growth, with levels being most abundant post-exponentially. Because IHF activity was present in stationary phase extracts of *L. pneumophila* and not in exponential phase extracts, suggesting it may be growth phase regulated, the levels of IHF were followed during the growth cycle by Western blot. This experiment was conducted in collaboration with Dr. Ann Karen C. Brassinga, University of Virginia, USA. Using antibody raised against the *E. coli* IHF proteins, cross reactivity was established with the *L. pneumophila* proteins, including HU-1, a closely related paralog. Preliminary studies revealed that MIFs contain elevated levels of IHF, and thus the abundance of IHF and HU-1 were monitored during MIF entry into exponential phase and following in vitro growth. As shown in figure 14, the expression of IHF (upper band) is concentrated in the MIF, and decreases sharply upon entry into exponential phase. Upon entry into stationary phase, IHF levels increases substantially to levels slightly lower than those seen in MIFs. Thus, IHF is growth phase regulated, as well as differentially regulated. HU-1, is regulated oppositely to IHF, with undetectable levels in the MIF, and increased levels during exponential phase.

3.13 Sodium sensitivity of IHF mutants, and over expressing strains.

The expression of virulence traits in *L. pneumophila* during stationary phase, including sodium sensitivity, has been firmly established. To this end, the Lp02Δ*ihfAB*

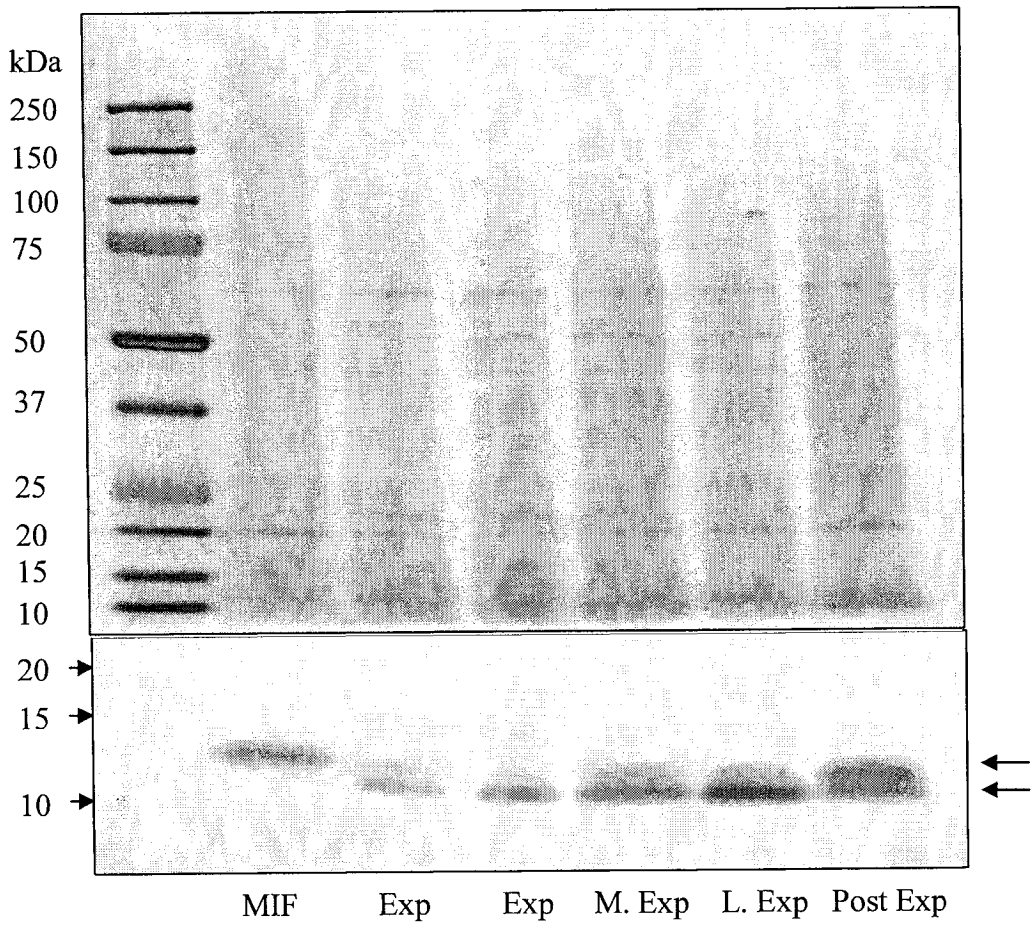


Figure 14. Growth phase regulation of IHF. Western blot, run on a 12% polyacrylamide gel, of *L. pneumophila* cells harvested at various time points during the growth cycle, Mature Intracellular Form (MIF), Exponential (Exp.), Mid Exponential (Mid Exp.), Late Exponential (Late Exp.) and Post Exponential (Post Exp.). The upper arrow indicates IHF, whereas the lower arrow indicates the presumed HU-1. Shown above is the Ponceau S stain to show equal loading. Adapted from Brassinga *et al.*, 2005, manuscript in preparation.

strain was examined for its ability to replicate in the presence of sodium in the growth media, the results of which are shown in Table 4. The Lp02Δ*ihfAB* strain did show a slight, but statistically significant ($p < 0.01$, student's t-test) increase in percent viability when plated on 100 mM NaCl. In trans complementation of the Lp02*ihfAB* mutant did restore wild type sodium sensitivity to the Lp02Δ*ihfAB* strain. The mock complemented strain showed a further ~ 10 fold decrease in viability on sodium containing media when compared to wild type, indicating that the presence of the pMMB206 vector increases the sodium sensitivity of the Lp02Δ*ihfAB* strain. Lp02/pMMB206::*ihfAB* showed a significant increase in percent viability compared to both the wild type and mock over-expressing strains. The *L. pneumophila* Philadelphia-1 strain Svir was included as a positive control, as it had been previously shown to be virulent. As a negative control, the avirulent Lp02 *dotB* mutant was also included, since avirulent strains are capable of growth in the presence of sodium. This strain showed the three orders of magnitude increase in percent viability typical of avirulent mutants. Because *L. pneumophila* is the only bacteria whose growth is inhibited by physiological concentrations of sodium chloride, the strains were also plated on media containing 145 mM NaCl, the same NaCl concentration used in the mobility shift experiments performed above, to determine whether this concentration could increase the difference in viability between virulent and avirulent mutants. All strains, excluding the IHF complementing construct, showed reduced growth in the presence of 145 mM sodium chloride. In contrast to the results of the 100 mM experiments, where the Lp02Δ*ihfAB* and Lp02Δ*ihfAB*/pMMB206 showed an increase in percent viability, these strains were not capable of growth under the increased concentration of sodium. Thus, sodium sensitivity is in part regulated by IHF.

Strain	100 mM NaCl	145 mM NaCl
Lp02	0.07±0.008	0.009±0.0002
Lp02Δ*ihfAB*	0.23±0.049	0
Lp02Δ*ihfAB*/pMMB206::*ihfAB*	0.05±0.038	0.06±0.032
Lp02Δ*ihfAB*/pMMB206	0.007±0.003	0
Lp02/pMMB206::*ihfAB*	12.31±3.80	0.100±0.135
Lp02/pMMB206	0.010±0.011	0.012±0.012
Svir	0.015±0.005	0.005±0.003
Lp02 *dot*B⁻ mutant	99.57±139.1	0.05±0.077

Table 4. Determining the role of IHF in sodium sensitivity. The viability of 72 hour plate growth of all strains was determined on 100 mM NaCl, and 145 mM NaCl. Percent viability is calculated as (c.f.u per ml on media containing 100 or 145 mM NaCl / c.f.u per ml on media without NaCl) x 100. While the IHF deletion mutant strain did show a slight increase in viability, the Lp02/pMMB206::*ihfAB* strain displayed the greatest increase in viability, at 12.3 percent. Lp02Δ*ihfAB* and Lp02Δ*ihfAB*/pMMB206 were not viable on 0145 mM NaCl, whereas the other strains were capable of some growth.

3.14 Two-dimensional SDS polyacrylamide gel electrophoresis of stationary phase, in vitro grown Lp02Δ*ihfAB*.

In an attempt to determine how IHF might function in sodium sensitivity, and to determine whether there are genes specifically regulated by IHF, stationary phase cultures of Lp02Δ*ihfAB* mutants were examined for changes in protein profiles when compared to wild type. Figure 15 (panel A) displays the protein profile of Lp02 bacteria, while panel B represents the Lp02Δ*ihfAB* mutant strain. For these experiments, gels of mutant lysates were over stained slightly to make identification of differentially expressed proteins easier. Expression levels were measured using densitometric analysis of protein spots, and P-values (student's t-test) ascertained from quadruplicate gels. To standardize for the over staining, random spots were chosen, and a ratio was determined to normalize the gels. Proteins 11, 13, 14, 16 and 18 are upregulated in the mutant background, while the remaining proteins are decreased. The quartet of proteins indicated by arrows in the gels represent MagA, and the multiple spots suggest some form of modification, resulting in a variety of isoelectric points. Densitometry analysis of these proteins was not performed due to difficulty in selecting all spots and the substantial background. The IHF protein was not visible using the silver staining technique. These results demonstrate that IHF is specifically involved in the expression of a number of proteins in stationary phase.

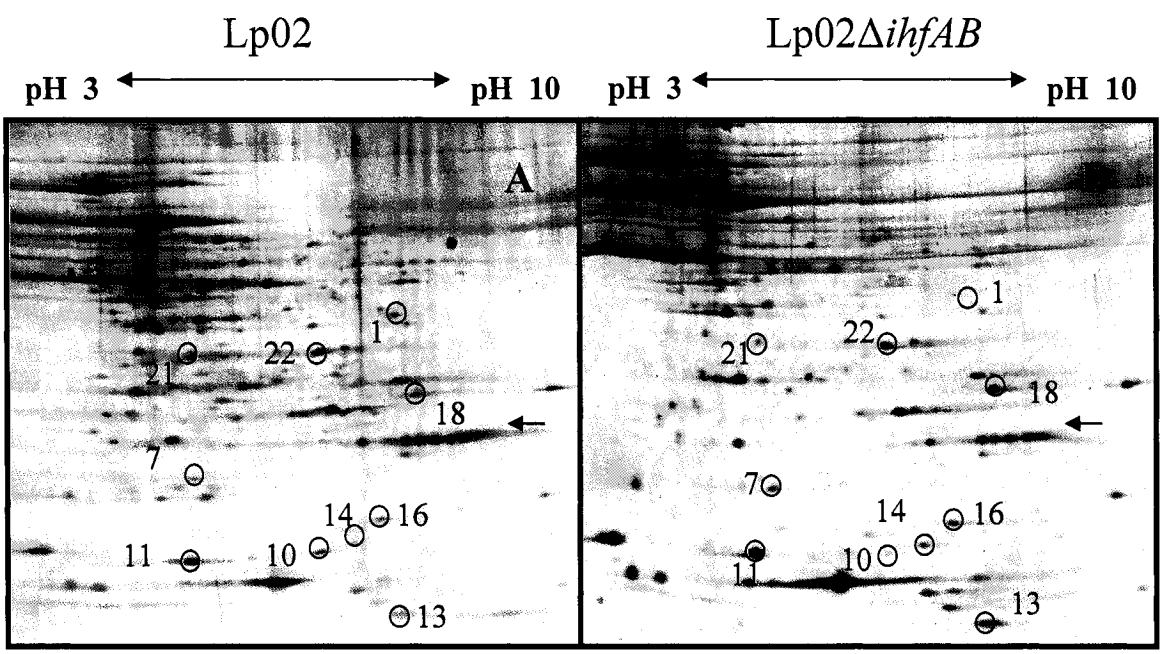


Figure 15. Identification of proteins whose expression is altered in the Lp02Δ*ihfAB* strain when grown in vitro to stationary phase. Two-dimensional SDS PAGE of lysates of stationary phase broth cultures of Lp02 (panel A), and Lp02Δ*ihfAB* (panel B) were focused on pH 3-10 IEF strips, and run on 10-20% gradient polyacrylamide gel. Protein differences between both gels are circled. Arrows are used to identify the MagA protein.

Spot number	ID	MW (kDa)	Calc pI	% Difference	P-Value (student's t-test)
1	Lipase	35.5	6.8	58.1	0.017
7	Transcription elongation factor GreA	17.7	5.4	89.3 (IHF)	0.0056
10	Universal Stress Protein A UspA	15.8	5.79	80.2	0.0021
11	Nucleoside Diphosphate Kinase	15.6	5.3	11.8 (IHF)	0.2188
13	Cold Shock Protein CspE	7.39	6.5	58.6 (IHF)	0.0026
14	DNA Binding Stress Protein	16.6	6.1	69.4 (IHF)	0.0005
16	50S Ribosomal Protein L9	16.5	6.10	75.2 (IHF)	0.0101
18	27-kDa outer membrane protein	28.5	6.92	57.5 (IHF)	0.0069
21	Thioredoxin Reductase	37.8	6.14	55.4	0.0274
22	Enoyl Reductase	28.5	5.6	10.1	0.1074

Table 5. LC-MS identification of proteins whose expression is altered in the IHF mutant background. Proteins identified as being differentially expressed were excised, digested with trypsin, and the resulting polypeptide fragments were sequenced by LC-MS. % coverage = percentage of total amino acid sequence identified in trypsonized fragments. MW = Molecular weight. Calc pI – Predicted isolectric point from sequence data. % Difference = % difference in expression of the protein. Proteins whose expression is increased in the mutant background are marked in brackets (IHF).

3.15 Expression of the *dot/icm* genes in the Lp02Δ*ihfAB* mutant strain as judged by green fluorescent protein.

As most of the *dot/icm* genes have been implicated in sodium sensitivity, and the above work demonstrated IHF mutants tolerate the presence of sodium chloride in the growth media, the role of IHF in the expression of the *dot/icm* virulence genes was investigated. GFP reporter plasmids containing promoters of 3 representatives of the 9 *dot/icm* operons (*dotA-IcmV*, *dotDCB* and *icmTS*) were cloned into the wild type and IHF deletion mutant strains, and fluorescence measured at various times. The results of this experiment are shown in figure 16. For the *icmT* construct, no differences in fluorescence were observed in the mutant background at any time taken. There was a slight decrease in expression of the *dotA* reporter in the mutant background at 48 hours, suggesting a possible defect in the maintenance of *icmV-dotA* expression late in stationary phase although the difference was not statistically significant ($p = 0.06$, two-tailed student's t-test). Also, the data reveal that the three operons examined are expressed at different levels when compared to each other, with the *dotD* promoter being most highly active, followed by *dotA* and finally *icmT*. This order was unchanged in the mutant strain. Of particular interest is the decrease in *dotDCB* expression beginning at 32 hours, following the slight increase observed over the preceding four hours. There was a statistically significant difference between the mutant and wild type strains in *dotDCB* expression after 48 hours ($p < 0.001$, student's t-test). Thus IHF is not required for full expression of the three *dot/icm* operons studied herein, but does play a role in expression of the *dotDCB* operon late in the growth cycle.

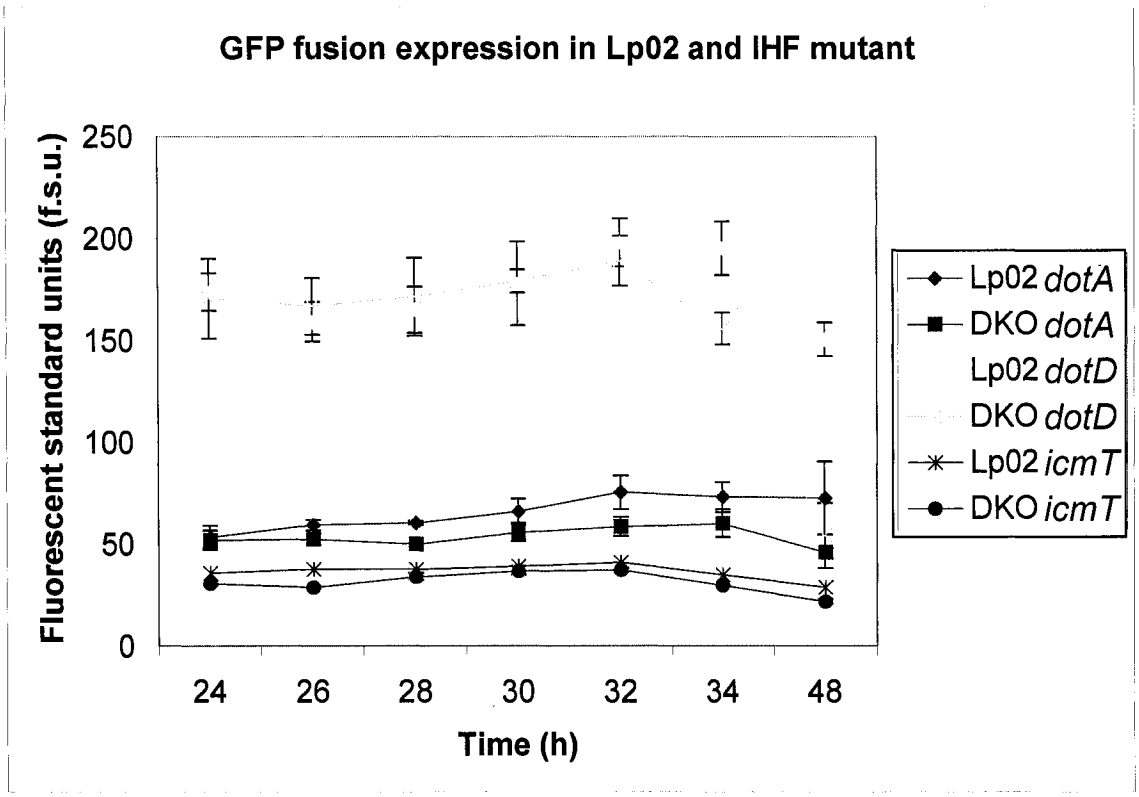


Figure 16. Expression of selected *dot/icm* genes in the wild type and Lp02Δ*ihfAB* (DKO) strain as determined by GFP reporter assays. The fluorescence of 0.5 O.D. samples, taken from triplicate broth cultures were grown for 24 hours, and 0.5 O.D. samples were removed for up to 48 hours. The fluorescence of these cultures was then determined at 488 and 510 nm. No differences in expression were seen between the wild type and mutant strains, except in the late stages of *dotD* expression, and the 48-hour time point for *dotA* reporters.

3.16 Infectivity and Intracellular Growth Assays

3.16.1 Attachment and invasion assays

To determine whether the difference in sodium sensitivity in the Lp02Δ*ihfAB* strain correlated with changes in virulence, the ability of the strains to attach to and invade the cervical carcinoma HeLa cell line was examined. Attachment (cell-association) and invasion assays were conducted using 24-hour agar grown bacteria that were centrifuged at 500 x *g* to increase contact with the monolayer. Cell-association is judged as the number of bacteria recovered from the monolayer incubated with bacteria for three hours after six PBS washes. To determine the number of bacteria that were intracellular, monolayers were treated with 100 μg/ml gentamicin to kill extracellular bacteria. As is shown in figure 17, there were no statistically significant differences seen between the wild type Lp02 strain, and the Lp02Δ*ihfAB* strains in attachment (panel A) or invasiveness (panel B), (one-way ANOVA, $p > 0.1$). Due to difficulties in obtaining consistent monolayers using HeLa cells grown in spinner bottles, there was considerable variability between experiments. Despite this, these results demonstrate there is no statistically significant difference in cell-association or invasion between the Lp02 and IHF mutant strains.

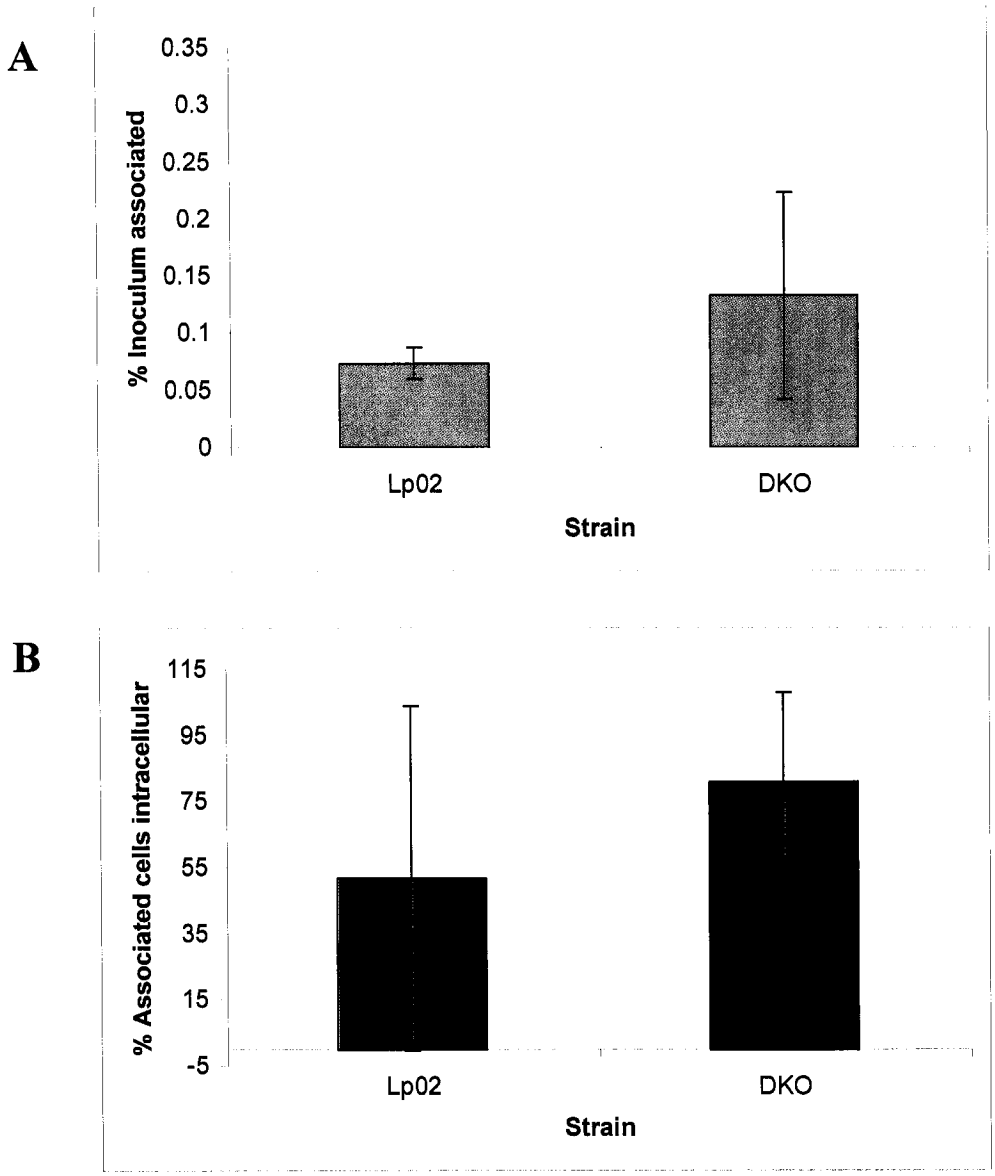


Figure 17. Attachment and invasion assay comparing Lp02 and Lp02Δ*ihfAB* (DKO). 1.0 O.D. suspensions of bacteria were used to inoculate triplicate wells of HeLa cells. The percent of the inoculum that resisted six PBS washes (cell-association) (panel A) after three hours, or 90 additional minutes of gentamicin treatment (invasion) (panel B) are shown above. These experiments were performed twice, each time using triplicate wells. No statistical differences were observed between the strains in either attachment or invasion assays.

3.16.2 Intracellular growth curves in HeLa cells

Since there were no apparent defects in infectivity between the wild type Lp02 and the Lp02Δ*ihfAB* mutant, the ability of the mutant strain to replicate intracellularly was examined. This was done by infecting HeLa cells with agar grown bacteria, and taking time points every 24 hours. The results of one representative assay are shown below in figure 18. After 24 hours, numbers of intracellular bacteria increased by 2 orders of magnitude for all strains. All strains showed a slight increase in number during the next 48 hours as well, reaching their highest number at this point. At 72 hours, bacterial counts fell slightly for all strains tested. Statistical analysis revealed the slight difference between Lp02 and Lp02Δ*ihfAB* was not significant ($p = 0.0944$, student's t-test).

To determine if any attenuation had occurred during the construction of these strains, intracellular growth curves were performed using bacteria that had been passaged once through HeLa cells. The bacteria were collected from HeLa cells, plated on BCYEα and allowed to grow overnight. These strains were used to infect fresh monolayers in triplicate as described above. The results of these experiments are shown in figure 19. The strains showed the same ~ two fold increase in viable count after 48 hours of intracellular multiplication as observed in the previous experiment, thus indicating no substantial attenuation of the strains. The Lp02 strain did produce slightly higher, statistically significant ($p < 0.01$, student's t-test), numbers of intracellular bacteria at 48 and 72 hours. No differences were seen between the mutant and complementing strains, while the vector control did have a decrease in viability that was not statistically

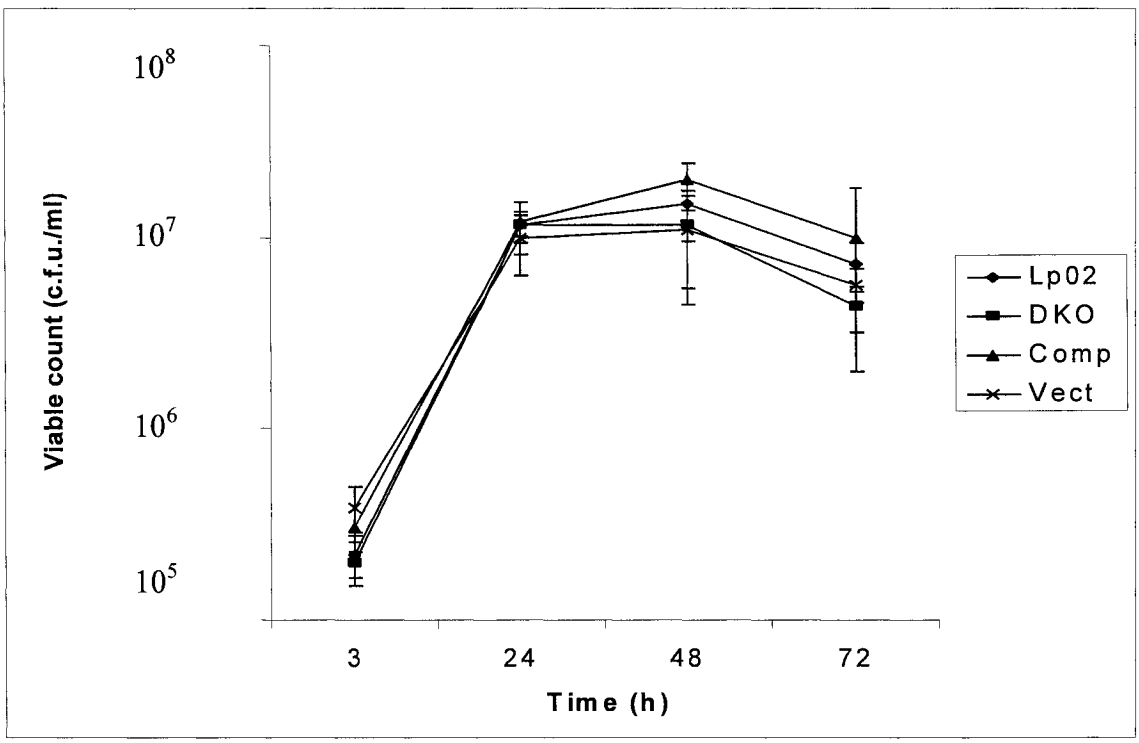


Figure 18. Intracellular growth experiments to determine the role of IHF in intracellular multiplication. Intracellular growth curves, performed in HeLa cells over 72 hours, using Lp02, Lp02Δ*ihfAB* (DKO), Lp02Δ*ihfAB*/pMMB206::*ihfAB* (Comp) and Lp02Δ*ihfAB*/pMMB206 (Vect) are shown above. HeLa cells were lysed with ddH_2O prior to serial ten-fold dilutions. Error bars represent the standard deviation of triplicate well counts. No significant differences were seen between the strains.

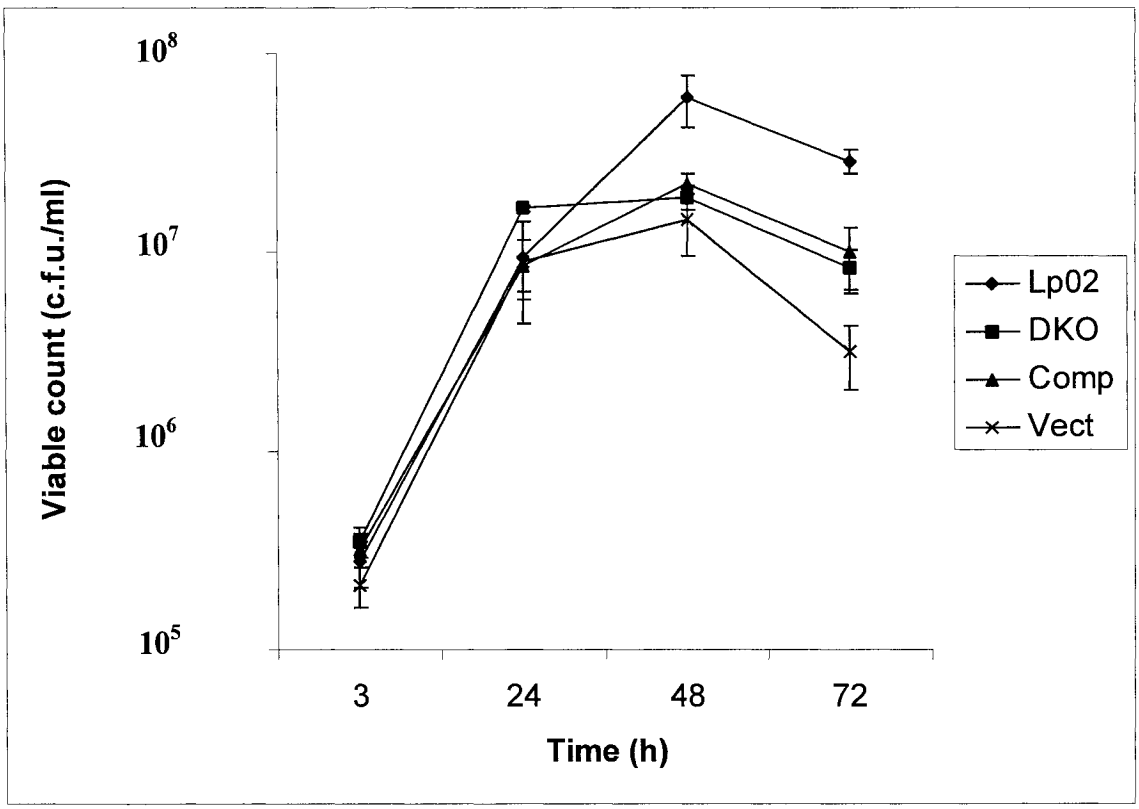


Figure 19. Intracellular growth experiments, using HeLa pasaged bacteria. Intracellular growth curves were performed in HeLa cells over 72 hours using Lp02, Lp02Δ*ihfAB* (DKO), Lp02Δ*ihfAB*/pMMB206::*ihfAB* (Comp), Lp02Δ*ihfAB*/pMMB206 (Vect). Lp02 was capable of reaching higher intracellular numbers after HeLa passage, when compared to the other three strains. HeLa cells were lysed with ddH_2O prior to serial ten-fold dilutions. Error bars represent the standard deviation of triplicate well counts.

significant ($p = 0.21$, student's t-test). These results, while showing a slight difference from agar grown bacteria, still illustrate the lack of any intracellular multiplication defects in the IHF mutant.

Similar experiments were performed to determine whether over expression of IHF resulted in any differences in intracellular multiplication. As the over expression construct also produced IHF during exponential phase, the improper temporal expression of IHF could also be examined. The experimental rationale for these tests was two-fold. First, IHF has been shown to induce chromosome condensation at high concentrations, and thus the effects of chromosome remodelling could be examined. Secondly, because IHF expression is generally confined to the post-exponential phase, it was possible that expression of IHF during exponential phase would result in premature arrestation of growth. To accomplish this, the over-expressing strain Lp02/pMMB206::*ihfAB*, the mock over expressing strain Lp02/pMMB206, the complementing strain Lp02Δ*ihfAB*/pMMB206::*ihfAB* and wild type were allowed to infect HeLa cells in the presence of 2 mM IPTG throughout the infection. The results of a representative experiment are shown in figure 20. Both the over expressing strain, and the Lp02/pMMB206 strain displayed slightly higher viability than the wild type and complementing strains, with an approximate 3-fold difference in viable count ($p<0.001$, student's t-test). All strains showed similar growth at 24 hours, and only the Lp02/pMMB206 had increased numbers at 48 hours. These experiments demonstrate that IHF has no significant effect on intracellular replication.

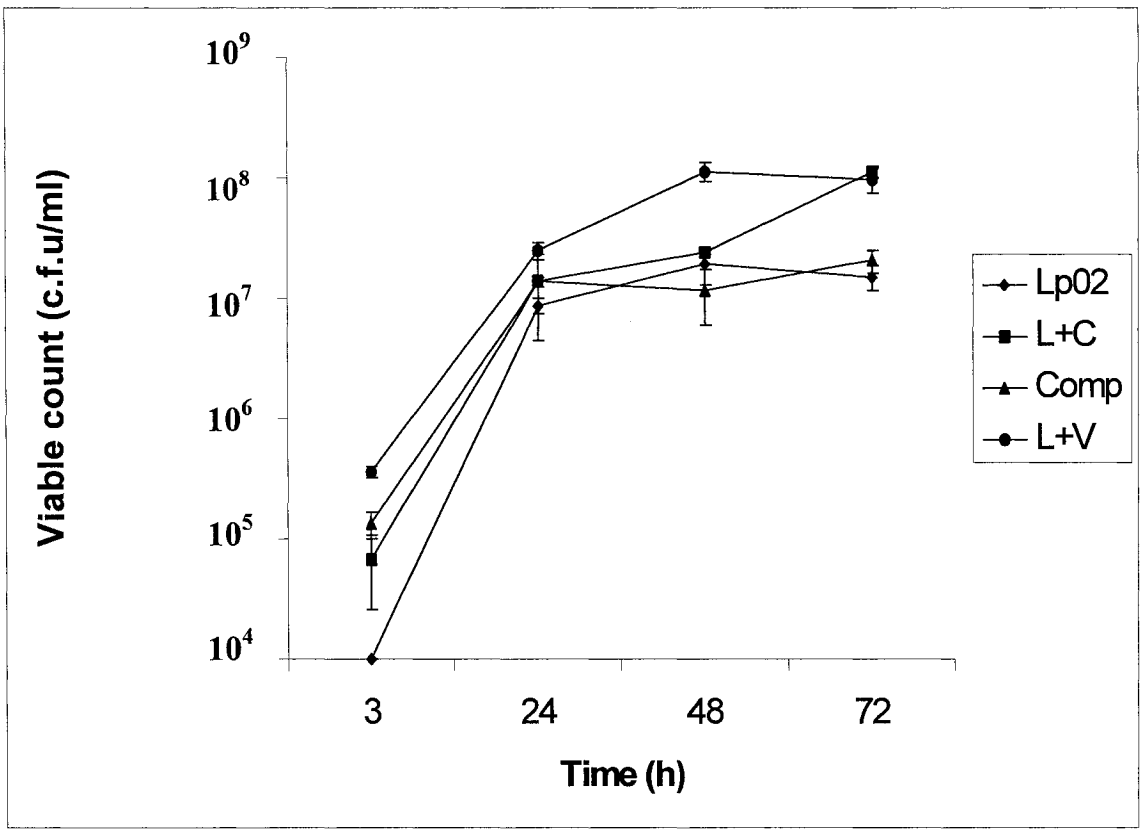


Figure 20. Effects IPTG induced over expression of IHF on intracellular growth in HeLa cells. Intracellular growth curves were performed over 72 hours in HeLa cells using Lp02, Lp02/pMMB206::*ihfAB* (L+C), Lp02Δ*ihfAB*/pMMB206::*ihfAB* (Comp), and Lp02/pMMB206 (L+V). HeLa cells were lysed with ddH_2O prior to serial ten-fold dilutions. Error bars represent the standard deviation of triplicate well counts. Both the Lp02/pMMB206::*ihfAB* (L+C) and Lp02/pMMB206 (L+V) strains had a small, but significant increase in final viable counts compared to the wild type (Lp02) and Lp02Δ*ihfAB*/pMMB206::*ihfAB* (Comp) strains.

3.17 Role of IHF in the Developmental Cycle of *Legionella pneumophila*

3.17.1 Gimenez staining

Previous work in our lab established that *L. pneumophila* undergoes maturation in HeLa cells, developing into a cyst-like mature intracellular form (MIF). This form is different from stationary phase, agar grown cells, and occurs late in the intracellular replicative cycle of the bacteria in HeLa cells. Because IHF is expressed late in the growth cycle and is most concentrated in MIFs, studies were conducted to determine if this transcriptional regulator was associated with MIF morphogenesis. Lp02Δ*ihfAB* mutants were examined for changes in cell wall structure by Gimenez stain during intracellular growth. Vegetative bacteria are unable to retain the Gimenez stain, whereas transition into stationary phase is associated with retention of the Gimenez stain (Garduno *et al.*, 2002). As is shown in figure 21, there were no apparent differences in Gimenez staining between Lp02 and Lp02Δ*ihfAB*, as both strains retained the Gimenez stain after 72 hours of intracellular growth. Similarly, both the complementation construct and the vector control showed no obvious defects in the ability to retain the Gimenez stain. All strains stained with similar intensity. Thus, IHF is not responsible for the as yet unidentified changes in membrane composition that accompany MIF formation and are responsible for retention of the Gimenez stain.

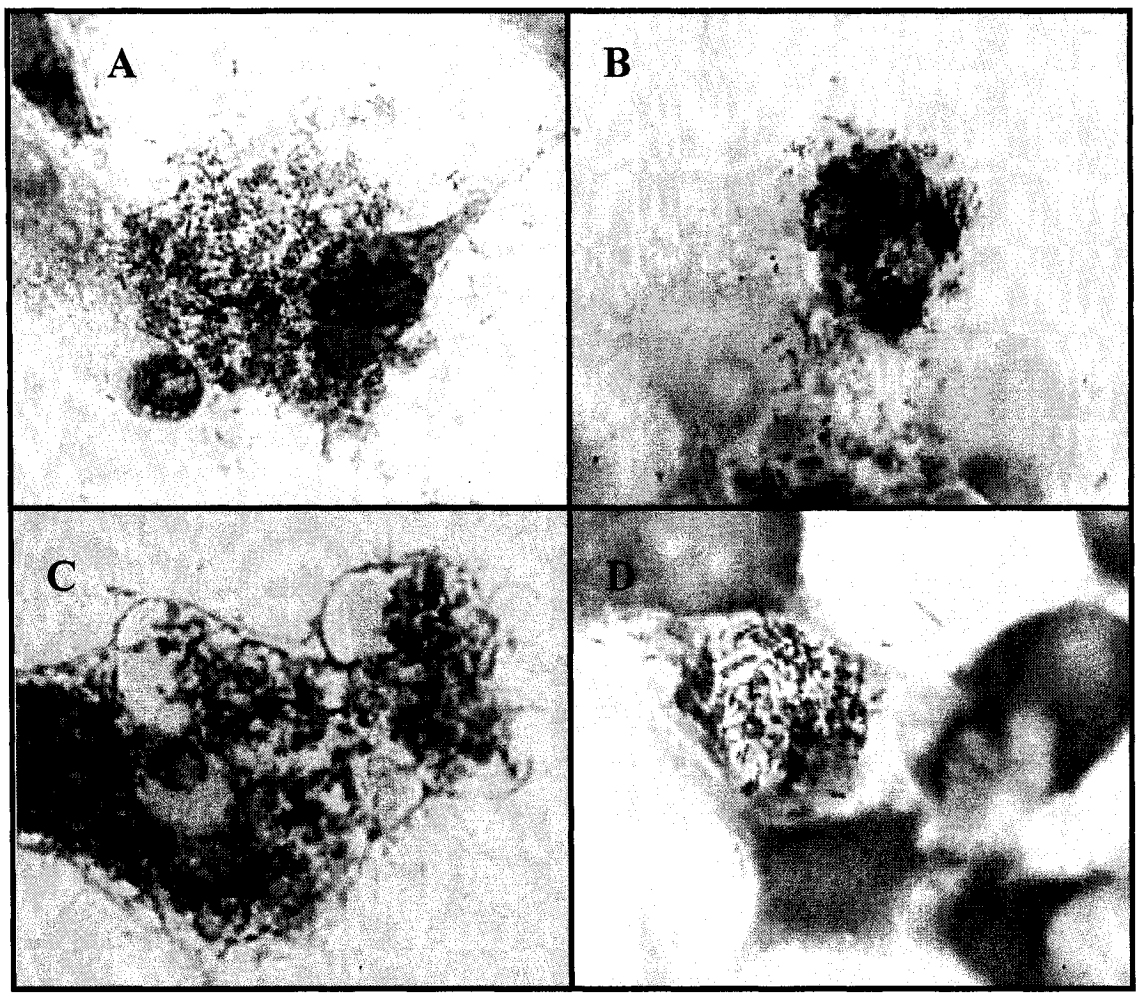


Figure 21. Gimenez staining of intracellular bacteria. Bacterial cells were grown for 72 hours in HeLa cell monolayers, and following three PBS washes to remove non-adherent HeLa cells, were assessed for their ability to retain the Gimenez stain. Lp02 (panel A), Lp02Δ*ihfAB* (panel B), Lp02Δ*ihfAB*/pMMB206::*ihfAB* (panel C), and Lp02Δ*ihfAB*/pMMB206 (panel D) showed no differences in ability to retain the stain, as judged by brightfield microscopy. All images were taken at 400 times magnification.

3.17.2 Analysis of vesicle formation using brightfield and phase contrast light microscopy.

It became apparent during the Gimenez staining that there are differences in vesicle morphology in the IHF mutant strains, and thus vesicles were examined using brightfield and phase contrast microscopy. The images in figure 22 are representative of these differences. Both types of microscopy revealed a difference in vesicle morphology. The wild type Lp02 strain is capable of producing large, bacteria-filled vesicles (indicated by arrows in panel A). Measurements of vesicle size in six separate fields, taken from independent wells, revealed an average vesicle size of 62.61 ± 20.56 μm (n = 22). The Lp02Δ*ihfAB* strain typically resides in smaller vesicles, sometimes in multiple vesicles per cell (indicated by arrows in panel B). Analysis of six separate fields, taken from independent wells, showed that the average vesicle size for IHF mutant-contianing vacuoles was 35.45 ± 8.23 μm (n = 30). This difference was statistically significant ($p < 0.001$, two-tailed student's t-test). Also apparent in this figure are the opaque areas commonly present in the mutant-strain infected cells. Visible in the brightfield images are the membranes surrounding the Lp02 vesicles. The Lp02 phase contrast image represents the large vesicles that are common to the Lp02 strain after 48 hours of infection (panel A). At equivalent time points, the Lp02Δ*ihfAB* strain did not produce vesicles of the same size as the wild type (panel B). Following an additional 24 hours of incubation, the mutant strain could occasionally produce larger vesicles that had cleared of the opaque areas typical of the cells infected with the mutant strain. These traits were observed in

more than 10 independent infections. Thus, vesicles containing the IHF deletion mutant are smaller than those containing the wild type Lp02 strain.

Phase contrast examination of the IHF complementing strains revealed that the defects in vesicle formation were not restored by in trans complementation, indicating IHF does not control vesicle size. Instead, both the complementing and mock complementing strains revealed even more substantial defects in vesicle formation when compared to the IHF deletion mutant (Figure 23). These small vesicles containing either the complementing or mock complementing strains were difficult to visualize using light microscopy. In addition, after 72 hours, most HeLa cells had rounded and detached from the well. Also, as exemplified in panel A, there was substantial clumping of the rounded cells that was typical of both the complementing and mock complementing strains (indicated by large arrow). To assess whether these phenotypes were caused by second site mutations acquired during the selection of the complementing clone, a second complementing construct, identified during the screening of the original complementing construct and shown to have similar IHF expression levels, was also analyzed. Similarly to the first complementing clone, the second clone produced an identical phenotype, strongly suggesting that the defects observed here were due to the pMMB206 vector. The fact that vesicle size was not restored upon in trans complementation of IHF indicates that the altered vesicle size of the IHF deletion mutant is not due solely to the deletion of IHF.

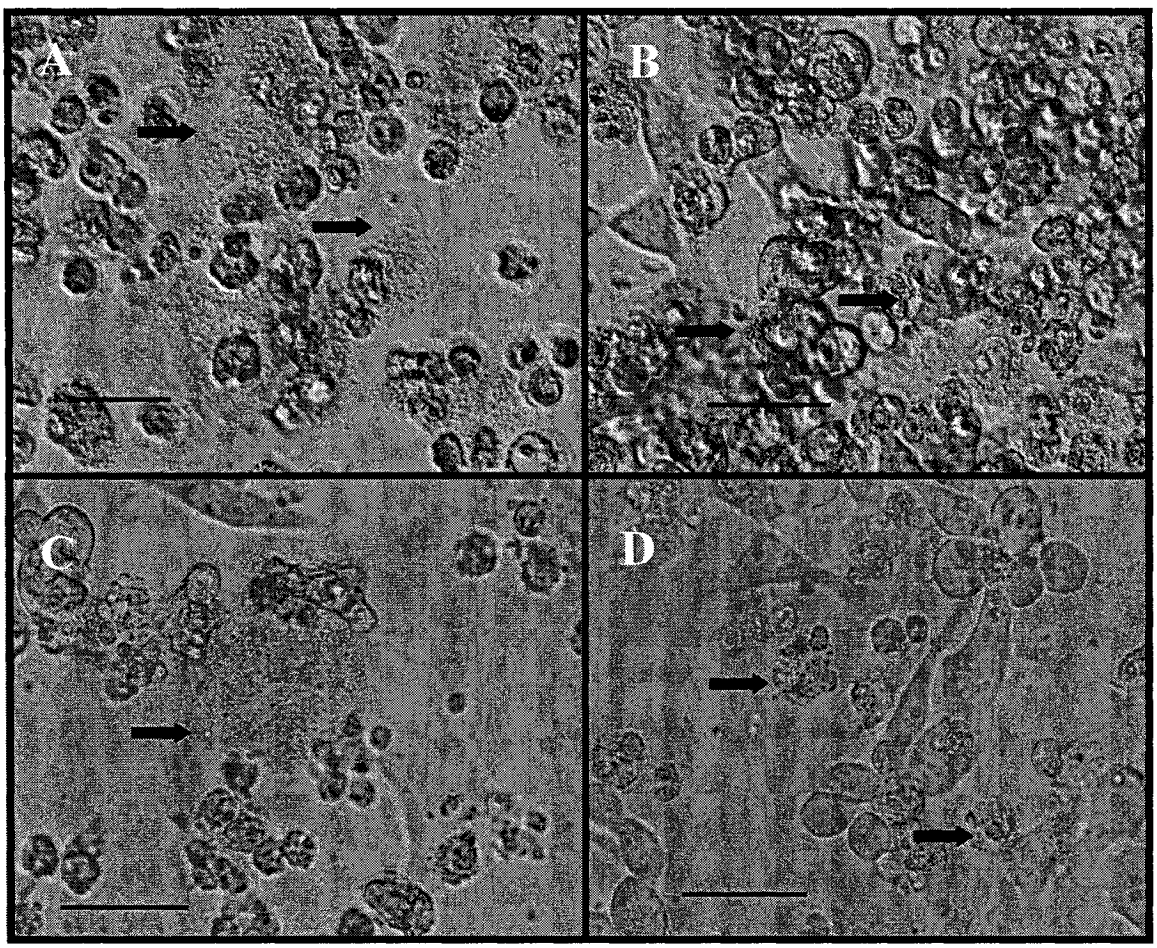


Figure 22. Brightfield and phase contrast microscopy of Lp02 and Lp02Δ*ihfAB* displaying altered vesicle morphology. HeLa cell monolayers were infected with Lp02 (panels A and C), or with Lp02Δ*ihfAB* (panels B and D) for 48 hours, and examined using phase contrast (panels A and B), and brightfield (panels C and D). Lp02 typically produces large, bacteria filled vesicles. Conversely, the Lp02Δ*ihfAB* mutant strain typically resides in smaller vesicles that are punctuated by opaque areas. Arrows identify vesicles in all panels. Scale bar represents 100 μm.

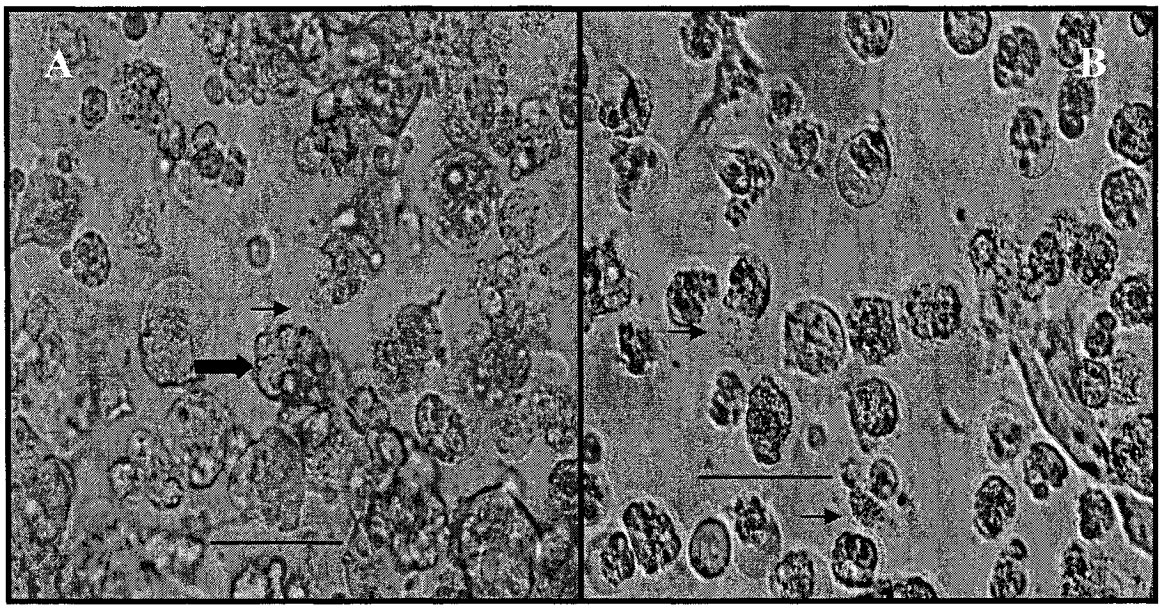


Figure 23. Altered vesicle morphology of the complementing and mock complementing strains. HeLa cell monolayers were infected with Lp02*ΔihfAB*/pMMB206::*ihfAB* (panel A), or with Lp02Δ*ihfAB*/pMMB206 (panel B) for 48 hours, and examined using phase contrast microscopy. Arrows identify the vesicles in all panels. Also note the large number of rounded cells in both panels, and the aggregated HeLa cells in panel A (large arrow). Scale bar represents 100 μm.

3.17.3 Electron microscopic examination of vesicle formation.

To further characterize the differences in vesicle morphology, 48-hour specimens were collected from all of the strains, and studied by electron microscopy (Figure 24). To enrich for vesicles during specimen preparation, after 48 hours of infection, flasks were gently shaken to free vesicles from the recently infected and uninfected HeLa cells in the monolayer. Supernatants were then harvested, fixed, and prepared for thin section electron microscopy. As was seen in the light microscopy experiments, the wild type strain generally produced large vesicles, free of cellular debris. These vesicles also displayed bacteria that contained several, resin impermeable PHB inclusions that appear white after exposure to the electron beam (panel A). Close examination of the Lp02Δ*ihfAB* infected cells revealed that the bacteria typically resided several smaller, well-separated vesicles (panel B). Also few bacteria of the mutant strain contained cytoplasmic inclusions, 26.8% (n = 628), compared to 88.7% (n = 469) for Lp02. The defects seen in the mutant strain were not restored by in trans complementation (panel C), or in the mock complemented strain (panel D). These strains also generally lacked cytoplasmic inclusions, with only 19.5% (n = 372) of complemented bacteria and 25.3% (n = 252) having inclusions. The diffuse vesicles seen in previous experiments with the Lp02/pMMB206::*ihfAB* were also observed in the electron microscopic examination (panel E). The vesicles seen in HeLa cells infected with the over-expressing strain were composed of multiple, small vesicles. Also of note, these small vesicles were generally spacious, containing only a few bacteria. Also, there were few bacteria containing cytoplasmic inclusions, as only 22.4% (n = 712) contained cytoplasmic inclusions.

Conversely, the mock over-expressing strain Lp02/pMMB206 strain was able to produce cytoplasmic inclusions at levels similar to wild type (84.0%, n = 356) (panel F). These vesicles more closely resemble those of the wild type strain, except they contain slightly more HeLa cell debris. It is also apparent that the vesicles in the Lp02 and Lp02/pMMB206 strains are larger, occupying more of the cytoplasm than those in the other four strains. In conclusion, premature induction of IHF expression negatively affects the production of PHB inclusions, as the IHF overexpressing strain produces less PHB than does the Lp02 or Lp02/pMMB206 strains. It is not possible to conclude whether deletion of IHF is the sole cause the PHB defects seen in the IHF mutant, as trans complementation failed to restore poly-β-hydroxybutyrate production.

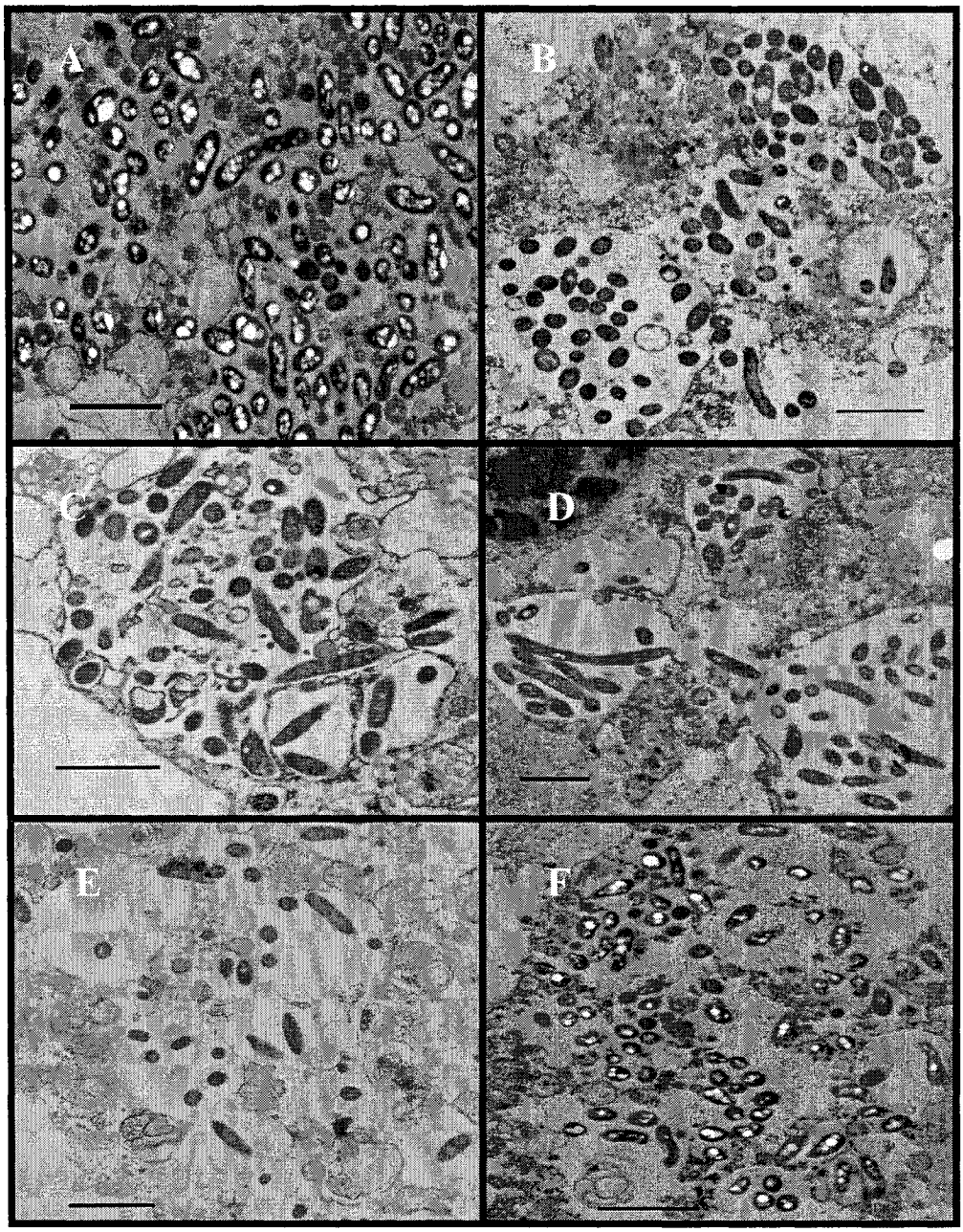


Figure 24. Thin-sectioned transmission electron micrographs of 48-hour infected HeLa cells. HeLa cell monolayers were infected with Lp02 (panel A), Lp02Δ*ihfAB* (panel B), Lp02Δ*ihfAB*/pMMB206::*ihfAB* (panel C), Lp02Δ*ihfAB*/pMMB206 (panel D), Lp02/pMMB206::*ihfAB* (panel E), and Lp02/pMMB206 (panel F). After 48 hours, infected, non-adherent cells were harvested, and stored in Cacodylate buffer prior to fixation. They were then assessed for their ability to form vesicles, and cytoplasmic inclusions. Scale bar represents 2 microns for all images.

3.17.4 Ultrastructural analysis of MIF morphogenesis

It became apparent after electron microscopic examination that the IHF deletion mutant, as well as the over-expressing strain, were defective for the production of poly-β-hydroxybutyrate (PHB) cytoplasmic inclusions. To further study this phenotype, ultrastructural analysis was done using electron microscopy at high magnification, the results of which are shown in figure 25. As was seen in the vesicle analysis, both the Lp02 strain (panel A), and the positive control Svir (panel B) did produce large, well-defined inclusions. Also, the Lp02 strain possessed the attributes previously described as being unique to MIFs. The Lp02 strain produced multilaminated, smooth cell envelopes, and formed internal membrane structures similar to the Svir strain. Also, both strains produced cells that were irregularly shaped. Conversely, the Lp02Δ*ihfAB* strain was generally unable to produce inclusions (panel C). Shown in panel D is one of the rare, inclusion-containing Lp02Δ*ihfAB* cells. Ultrastructural defects were also observed. The outer membrane had a ruffled appearance, with very little internal membrane formation. These bacteria also generally had a more typical, gram-negative appearance. These differences were observed in two independently infected monolayers for each strain, and in the > 100 grid sections analyzed.

Next the complementing and vector control strain were analyzed for their ability to form MIFs, based on PHB synthesis and membrane structure, shown in figure 26. Despite the inability of the complementing construct to restore wild type vesicle formation, defects in morphogenesis were complemented (panels A and C).

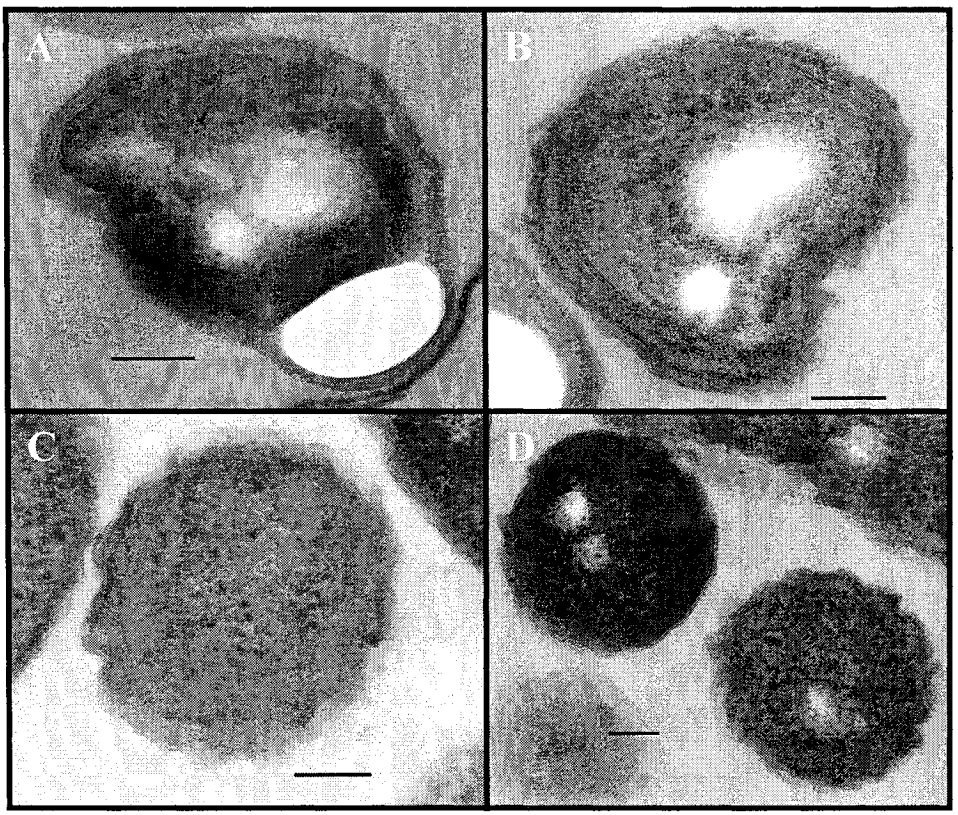


Figure 25. High magnification, thin-sectioned transmission electron micrographs of 48 hour, intracellularly grown Lp02, Svir and Lp02Δ*ihfAB*. After 48-hours, infected, non-adherent HeLa cells were harvested, and stored in Cacodylate buffer prior to fixation. Lp02 (panel A) and Svir (panel B) produced the typical MIF forms described previously. The Lp02Δ*ihfAB* strain (panels C and D) was generally unable to form cytoplasmic inclusions, and ultrastructuraly resembled typical gram-negative bacteria. Scale bar represents 100 nm for all images.

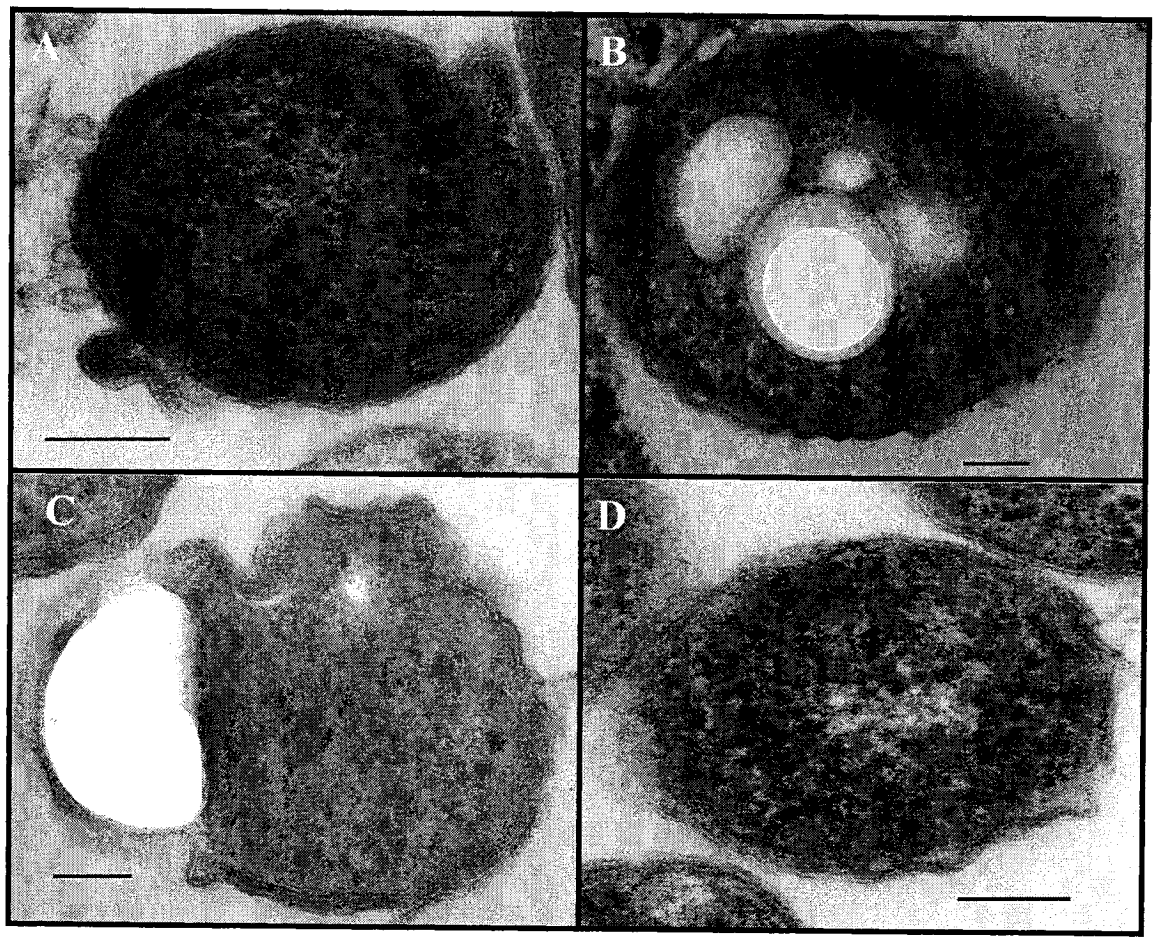


Figure 26. High magnification, thin-sectioned transmission electron micrographs of 48-hour, intracellularly grown Lp02Δ*ihfAB*/pMMB206::*ihfAB* and Lp02Δ*ihfAB*/pMMB206. After 48 hours, infected, non-adherent HeLa cells were harvested, and stored in Cacodylate buffer prior to fixation. In trans complementation of the Lp02Δ*ihfAB* strain was able to restore the MIF characteristics, including cytoplasmic inclusions, smooth envelope, and internal membranes (panels A and C). The Lp02Δ*ihfAB*/pMMB206 strain (panels B and D) resembled the IHF mutant strain. Scale bar represents 100 nm.

The Lp02Δ *ihfAB*/pMMB206::*ihfAB* complementing strain could produce the well defined cytoplasmic inclusions seen in the wild type, albeit infrequently (4.0 %, n = 372) panel C). As well, the complementing strain could also produce the internal membranes similar to wild type (panels A and C). The mock complemented strain Lp02Δ *ihfAB*/pMMB206 showed the typical gram-negative appearance, similar to Lp02Δ*ihfAB* (panels B and D), and produced well-defined inclusions with a similar frequency as the complementing construct (2.4 %, n = 252).

The Lp02/pMMB206::*ihfAB* over expressing strain and the mock over-expressing strain Lp02/pMMB206 were then analysed for their ability to form MIFs, and the results of this study are shown in figure 27. Lp02/pMMB206::*ihfAB* strains were also defective in MIF morphogenesis, containing few cytoplasmic inclusions, and lacking much of the internal membrane structure seen in MIFs (panels A and C). The over-expressing strain was only occasionally able to produce cells containing a multilaminated, smooth outer membrane, pictured in panel C. Conversely, the mock over expressing strain, Lp02/pMMB206 was capable of forming cysts, similarly to wild type strains.

Thus, integration host factor is required for MIF morphogenesis, as deletion mutants do not produce the characteristic thickened cell enevelopes and multilaminated membranes typical of MIFs. Additionally, the over-expressing strains are impaired for poly-β-hydroxybutyrate synthesis, indicating IHF plays a role in PHB inclusion formation.

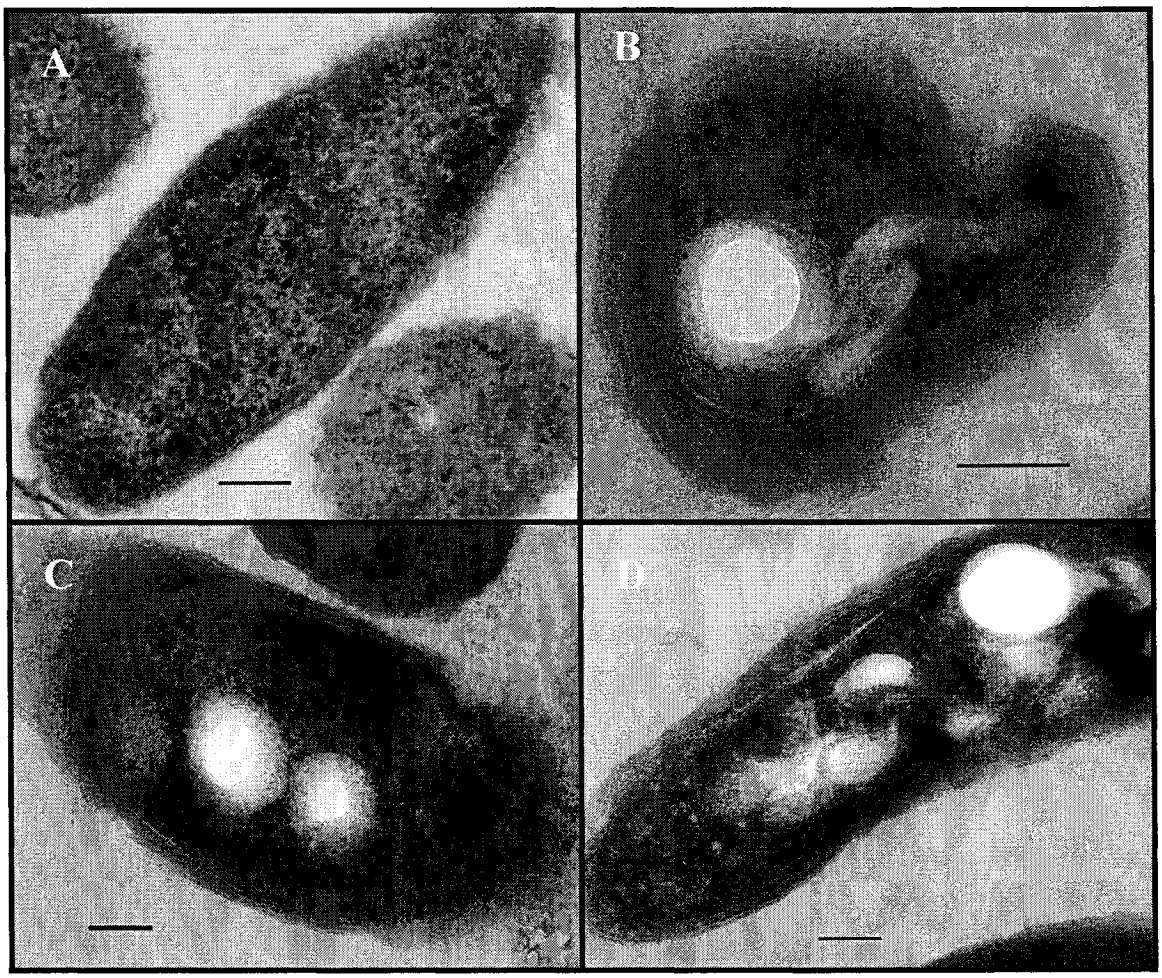
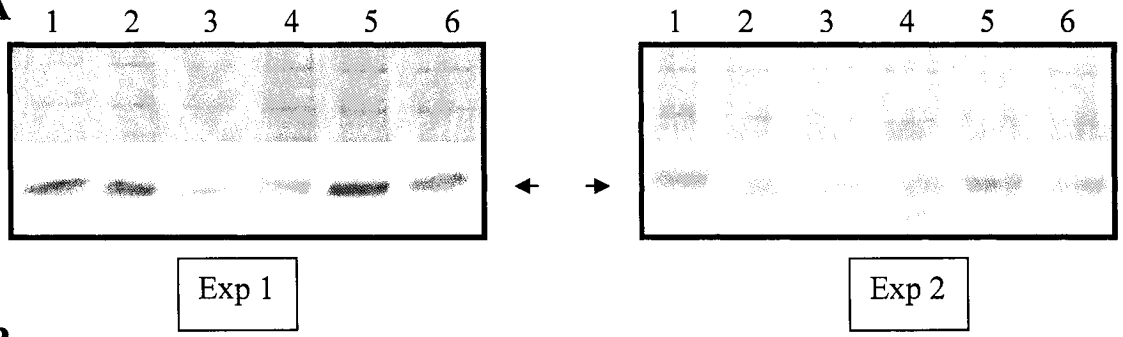


Figure 27. High magnification, thin-sectioned transmission electron micrographs of 48-hour, intracellularly grown Lp02/pMMB206::*ihfAB* and Lp02/pMMB206. After 48 hours, infected, non-adherent HeLa cells were harvested, and stored in Cacodylate buffer prior to fixation. The Lp02/pMMB206::*ihfAB* are somewhat defective in MIF morphogenesis (panel A and C). The Lp02/pMMB206 strain did produce cells with the typical MIF appearance (panels B and D). Scale bar represents 100 nm.

3.17.5 Expression of MagA in the IHF mutant backgrounds

Due to the defects in MIF morphogenesis, other MIF specific characteristics were examined. Previous studies in our laboratory had identified MagA as a protein whose expression is upregulated in MIFs, and thus serves as a marker for morphogenesis. To determine whether the defects in morphogenesis correlate with alterations in MagA levels, western blots using MIFs from HeLa cells were run, and levels of MagA were analysed by densitometry (Figure 28). Densitometric values were obtained using Image Pro software and calculated as integrated optical density. Densitometry of bands was standardized using bands present after the Ponceau S staining, and the results of two experiments are shown. While the Lp02Δ*ihfAB* strain did show a slight decrease in MagA expression, it was not statistically significant and the defect was not restored by complementation. In addition, the restoration of IHF expression had the greatest inhibitory effect on MagA levels, resulting in a 3 to 3.5-fold decrease in expression. Thus, the presence of the pMMB206 vector in the mutant background resulted in a substantial inhibition of MagA expression. The over-expressing strain Lp02/pMMB206::*ihfAB* did show an increase over the levels seen in the mock over expressing strain Lp02/pMMB206. The presence of the vector in the wild type strains also negatively impacted MagA expression; however, this decrease was less dramatic than that seen in the IHF mutant background. Thus, IHF is not an important regulator of MagA production.

A



B

Strain	Integrated OD Exp 1	Integrated OD Exp 2
1 - Lp02	6.19 ± 0.04	19.47 ± 14.11
2 - Lp02Δ*ihfAB*	5.08 ± 0.31	7.01 ± 1.61
3 - Lp02Δ*ihfAB*/pMMB206::*ihfAB*	2.74 ± 1.04	4.72 ± 1.58
4 - Lp02Δ*ihfAB*/pMMB206	2.32 ± 0.09	9.97 ± 1.43
5 - Lp02/pMMB206::*ihfAB*	7.89 ± 0.75	20.33 ± 15.96
6 - Lp02/pMMB206	3.93 ± 1.28	12.22 ± 2.18

Figure 28. Expression of MagA in intracellularly grown bacteria, as judged by densitometry. HeLa cells where infected for 96 hours with various strains. Monolayers and supernatants were harvested, and HeLa cells lysed with ddH_2O. Bacteria were then enumerated, and viable counts used to standardize the number of bacterial cells used for Western blotting. The resulting western blots are shown in panel A (arrow indicates the MagA protein), along with the Ponceau S stained bands used for standardizing (immediately above). The corrected densitometric values are shown in panel B. Numbers next to strain name indicate the lanes containing that strain in panel A. Lp02 demonstrated a slight increase in MagA expression when compared to Lp02Δ*ihfAB* strain. This defect was not restored by complementation. Additionally, the IHF over expressing strain (Lp02/pMMB206::*ihfAB*) also showed greater expression of MagA than the mock over expressing strain (Lp02/pMMB206).

3.17.6 Infectivity of Mature forms

As MIFs have been shown to be more infectious than stationary phase bacteria, IHF mutants were examined for their ability to infect L929 cells. Intracellularly grown Lp02Δ*ihfAB* bacteria were compared to wild type MIFs and to their respective agar-grown counterparts. This technique uses an agar overlay to allow the formation of plaques in order to quantify infectivity. The results of these assays are shown in Table 6 below. Plaquing efficiency is calculated (# plaque forming units/ total inoculum) x 100. No statistically significant differences were seen in plaquing efficiency between the wild type and mutant strains ($p > 0.1$, student's t-test). There were, however, differences in the plaquing efficiencies of the Lp02Δ*ihfAB* strains containing the plasmid pMMB206. These strains produced extremely small plaques on the monolayer, making it impossible to accurately distinguish the plaques from imperfections in the monolayer. Again, these findings further demonstrate complications caused by the pMMB206 vector in MIF morphogenesis in the mutant background. Thus, the terminally differentiated IHF mutant is more infectious than its agar-grown form, and displays similar infectivity to the Lp02 strain.

	In Vitro		In Vivo	
Strain	Lp02	Lp02Δ*ihfAB*	Lp02	Lp02Δ*ihfAB*
Plaquing Efficiency	0.158 ± 0.067	0.137 ± 0.090	6.47 ± 6.41	5.18 ± 1.67

Table 6. L929 plaque assays using both agar grown, and HeLa cell grown Lp02 and Lp02Δ*ihfAB*. L929 monolayers were infected with both agar-grown (in vitro) bacteria, and bacteria isolated from HeLa cells (in vivo). Enumeration of plaques occurred after 96 hours. Plaquing efficiency = (number of plaques per 1 ml of inoculum / c.f.u. per 1 ml of inoculum) x 100. No significant differences were seen in the IHF mutant strains when compared to Lp02.

3.17.7 Two-dimensional SDS polyacrylamide gel electrophoresis of intracellular Lp02 and Lp02Δ*ihfAB*.

Because ultrastructural differences were seen between the wild type Lp02 and the IHF mutant strain Lp02Δ*ihfAB*, two-dimensional gels were employed to determine the proteins responsible for these differences. Lp02 MIFs, and the terminally differentiated Lp02Δ*ihfAB* cells were harvested from HeLa cells using Percoll density gradients and disrupted by sonication. Duplicate 2D gels were then run on non-linear pH 3-10 isoelectric focusing strips. Two representative gels are shown in figure 29. Proteins were then identified and are listed in table 7. All proteins identified were upregulated in the wild type, except for protein 10. Protein 25 was identified as a mitochondrial ATPase precursor, and not of bacterial origin. No other proteins identified were of eukaryotic origin. Some of the identified proteins were also identified in the in vitro experiments discussed above, including GspA, and GreA. Statistical significance was calculated using duplicate gels (student's t-test). Interestingly, the quartet of MagA spots seen in the in vitro gels resolved to two predominant spots in both the MIF and Lp02Δ*ihfAB* terminally differentiated forms. Again, as in the in vitro gels, multiple MagA spots and substantial background made densitometric analysis impossible. These experiments demonstrate there are protein expression differences between MIFs and the intracellularly grown Lp02Δ*ihfAB* forms, although it is not clear whether any of these proteins affect MIF morphogenesis.

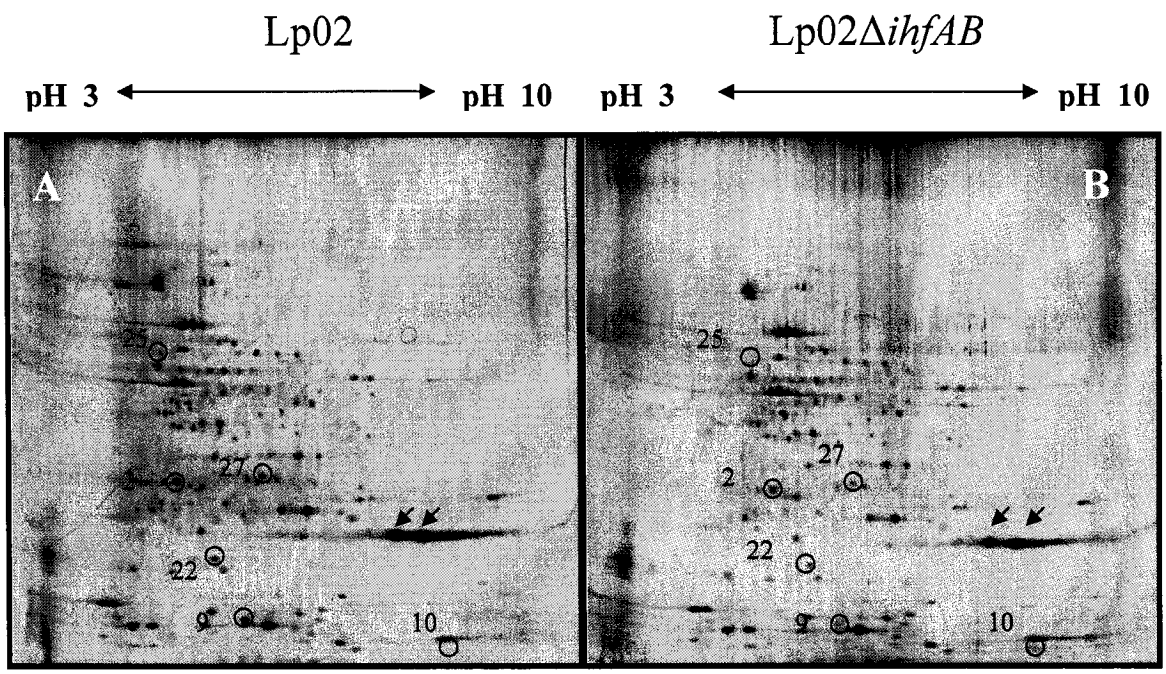


Figure 29. Identification of proteins whose expression is altered in the Lp02Δ*ihfAB* purified intracellular forms. Two-dimensional SDS PAGE gels of crude lysates of Lp02 MIFs (panel A), and the mature form of Lp02Δ*ihfAB* (panel B) were focused on pH 3-10 IEF strips, and run on 10-20% gradient polyacrylamide gel. Protein differences between both gels are circled. Arrows are used to identify the MagA protein.

Spot number	ID	MW (kDa)	Calc pI	% Difference	P Value
2	Hypothetical protein lpg0294	25.5	5.22	37.4	0.1506
9	Universal Stress Protein A UspA	15.1	5.5	81.9	0.0143
10	hypothetical protein lpp1177	9.2	7.88	94.6 (IHF)	0.0562
22	Transcription Elongation Factor GreA	17.7	5.40	43.8	0.0492
25	ATP synthase beta chain, mitochondrial precursor	56.5	5.26	67.5	0.0788
27	Hypothetical Protein lpg1551	28.8	5.94	48.8	0.0112

Table 7: LC-MS identification of proteins whose expression is altered in the Lp02Δ*ihfAB* mature form. Proteins identified as being differentially expressed were excised, digested with trypsin, and the resulting polypeptide fragments were sequenced by LC-MS. % coverage = percentage of total amino acid sequence identified by in trypsonized fragments. MW = Molecular weight. Calc pI – Predicted isolectric point from sequence data. % Difference = % difference in expression of the protein. Proteins whose expression is increased in the mutant background are marked in brackets (IHF).

3.17.8 Resilience testing of the IHF mutant and over-expressing strains.

One of the characteristics of the *Legionella pneumophila* mature intracellular form is its resilience to a variety of challenges including pH 11 and Triton X-100. Based on the ultrastructural differences observed between the Lp02 MIFs and the mature Lp02Δ*ihfAB*, it was hypothesized that these defects might correlate with decreased resistance to a variety of stresses. Intracellular bacteria were harvested from HeLa cells, and subjected to a detergent, and high pH challenges. Garduno *et al.* (2002) had classified bacteria as resistant to detergent if the $O.D._{620}$ decreased by more then 50%. Using this measure, it was determined that MIFs harvested at 72 hours for all of the strains tested were resistant to 0.1x Triton X-100. There were, however, differences in the final remaining percentages of bacteria after detergent exposure, and these are listed in table 8. The optical density of all strains decreased more than that of the Lp02 strain, with the complementing and mock complementing strains demonstrating the greatest decrease. The second challenge involved exposing ~10^5 bacteria to Tris base, pH 11 for 16 hours. The ability of the bacteria to form a pellet after 1 minute at 16,000 x *g* was determined, the results of which are shown in table 8. In these experiments, the Lp02, Lp02/pMMB206::*ihfAB* and Lp02/pMMB206 strains were able to form pellets after pH 11 exposure and retained minimal viability on BCYEα media. Conversely, none of the Lp02Δ*ihfAB*, Lp02Δ*ihfAB*/pMMB206::*ihfAB* or Lp02Δ*ihfAB*/pMMB206 strains were capable of forming pellets.

Also, it has been previously shown that stationary phase bacteria acquire the ability to resist heat, and to remain viable in ddH_2O for prolonged periods. To determine

whether IHF was involved in these stationary phase phenotypes, bacteria were assessed for their sensitivity to heat challenge (50˚C, 30 min), and for their ability to maintain viability after being incubated in ddH_2O. The results of these experiments are shown in table 8. After suspension in ddH_2O for 8 days at 37°C, no differences were seen viability between the Lp02, Lp02Δ*ihfAB* and Lp02Δ*ihfAB*/pMMB206::*ihfAB* strains. The remaining strains showed slightly higher viability, with Lp02/pMMB206 displaying the highest percent viability (38.4 ± 14.68). The results of 50˚C testing displayed variability, but generally, the Lp02Δ*ihfAB* and Lp02Δ*ihfAB*/pMMB206 strains were less susceptible to 50˚C than the Lp02 and Lp02Δ*ihfAB*/pMMB206::*ihfAB*. Thus, despite the ultrastructural differences that occur in the IHF mutant, these mutants do not show any significant changes in resistance to heat, detergent or survival in ddH_2O when compared to wild type, and defects in pH 11 resistance were not restored by trans complementation. These data demonstrate that IHF is not required for any of the stress resistance traits previously shown to be stationary phase or MIF related.

	Mature Forms		Stationary phase bacteria	
Strain	Triton X-100	pH 11	% viability in ddH_2O	% viability 50°C
Lp02	76.25 ± 2.02	+	6.05 ± 2.73	5.73 ± 4.46
Lp02Δ*ihfAB*	58.42 ± 6.77	-	5.74 ± 4.17	22.87 ± 13.28
Lp02Δ*ihfAB*/pMMB206 ::*ihfAB*	57.65	-	7.14 ± 3.71	1.14 ± 0.499
Lp02Δ*ihfAB*/pMMB206	63.64	-	25.4 ± 9.36	42.74 ± 32.83
Lp02/pMMB206::*ihfAB*	66.70 ± 3.49	+	20.55 ± 6.38	10.58 ± 4.89
Lp02/pMMB206	63.88 ± 4.71	+	38.4 ± 14.68	65.87 ± 35.53

Table 8. Resilience testing on intracellularly grown, and agar-grown stationary phase bacteria. Detergent resistance was determined by measuring the decline in optical density of a 1.0 O.D. suspension of intracellularly grown bacteria during exposure to 0.1x Triton X-100 at 620 nm. Values above reflect the final percentage of the initial optical density after 96 hours exposure. pH 11 resistance was determined by resuspending ~ 10^5 of intracellularly grown bacteria in Tris base, pH 11, and determining their ability to form pellets (+) or not (-) after centrifugation at 16,060 x *g*. Data for water survival experiments using stationary phase bacteria is presented as the percent of the original 0.1 O.D. suspension that remained viable in ddH_2O at 37°C for 8 days. Data for heat survival experiments using stationary phase bacteria is presented as the percent of the original 0.1 O.D. suspension that remained viable after 30 minutes at 50°C. Bacterial counts were performed by 10-fold serial dilution in ddH_2O, and enumerated on BCYEα media with appropriate selection.

CHAPTER 4: DISCUSSION

As an intracellular parasite of fresh water protozoa, *Legionella pneumophila* cycles between periods of intracellular multiplication and periods between hosts in which bacteria exist as planktonic cysts. One of the observations on which the present studies are based is that virulent *L. pneumophila* is sodium sensitive and challenge with NaCl alters the activity of a putative DNA binding protein, termed OmpT, which binds the *ompS* promoter. In this investigation, the promoter region of *ompS* was used as a tool for the identification and characterization of regulatory factors. In keeping with the concept of a bacterial developmental cycle in which regulatory factors are expected to function in a stage-specific manner, IHF was identified as one of several factors controlling expression of late genes associated with differentiation of vegetative bacteria into cyst like MIFs.

4.1 Sodium and gene expression

4.1.1 The effects of sodium on gene expression

The *Legionella pneumophila* MOMP, and the gene that encodes it, *ompS*, were first cloned and sequenced in this laboratory (Hoffman *et al.*, 1992). Studies by Weeratna (PhD thesis, Dalhousie University, 1995) found that *ompS* was not expressed from its own promoter when cloned and introduced into *E. coli*, implying that a *Legionella*-specific factor activates the *ompS* promoter. The work of Weeratna identified a low molecular weight *L. pneumophila* DNA binding protein that bound the *ompS* promoter.

However, this protein could neither be purified, nor its encoding gene identified. Nonetheless, this putative protein was named OmpT (Weeratna, PhD thesis, Dalhousie University, 1995). When *L. pneumophila* bacteria were challenged with ~ 0.15 M NaCl, the DNA binding activity of OmpT was abolished. The work presented in this thesis determined that OmpT binding to the *ompS* promoter occurs in exponentially growing bacteria and that this activity was absent in bacteria that had been grown into stationary phase. These data suggest that OmpT-mediated regulation may be limited to exponential phase. The fact that IHF mutants show no alteration in OmpT binding strongly supports the assertion that IHF is not OmpT. In addition, stationary phase extracts display different mobility shift profiles than exponential phase extracts, suggesting that different proteins occupy the *ompS* promoter during stationary phase. It is tempting to speculate that these proteins, including IHF, displace OmpT from the promoter region as the bacteria enter stationary phase. Additionally, it is possible that IHF causes repression of OmpT synthesis, or potential protein kinases that may activate it.

There have been several reports indicating differences between *L. pneumophila* strains, including large genomic deletions in the Lp02 variant of the Philadelphia-1 strain (Brassinga *et al.*, 2003). To this end, experiments were conducted to ensure that the OmpT binding phenomenon occurred in the thymidine auxotrophic strain Lp02. Similarly to the Philadelphia-1 strain Svir used by Weeratna, crude extracts from late exponential phase Lp02 cultures challenged with 145 mM sodium chloride also lost the ability to bind to the *ompS* promoter region. These results confirmed that Lp02 is a suitable strain for studying sodium regulation.

There is a difference between the concentrations of sodium used to elicit the sodium response of *L. pneumophila*, and those required to induce osmotic effects. While there have not been any published studies to determine the exact concentration of salts required to induce osmotic-mediated decreases in viable count, reports describing osmotically-induced changes in protein profile used 0.5 M sodium chloride (Abu Kwaik *et al.*, 1993), or 0.3 M potassium chloride (Byrne and Swanson, 1998). The *L. pneumophila* cultures challenged with ~0.15 M sodium or potassium did not produce the 19-protein profile differences previously seen during osmotic challenge (Abu Kwaik *et al.*, 1993), suggesting that the sodium and potassium challenges used herein did not cause a significant osmotic stress. The sodium response is produced between concentrations of 0.1 and 0.15 M sodium chloride. Thus, the gene expression differences noted in this study are representative of the physiological changes that occur during the sodium response of *L. pneumophila*, despite the fact that OmpT was not identified. It is possible that these salt concentrations do not affect the synthesis or levels of OmpT, but instead alter its active state. This would make identification of OmpT by this approach extremely difficult

The first evidence that sodium may play a signalling role in *L. pneumophila* came from the preliminary attempts to produce an effective growth medium, where sodium was shown to inhibit the replication of virulent *Legionella pneumophila* (Feeley *et al.*, 1979). The inability to replicate in the presence of sodium is not merely a physiological effect, as avirulent mutants are capable of replicating in its presence (Catrenich and Johnson,

1989). While virulent bacteria do not replicate in the presence of sodium in laboratory growth media, they remain viable, essentially in a suspended growth state. Immediately upon entry into host cells and prior to the commencement of bacterial replication, *L. pneumophila* enters a lag period of 6-10 hours in mice bone marrow derived macrophages (Sturgill-Koszycki and Swanson, 2000). During this period, the expression of several proteins, including OmpS, is decreased (Fernandez *et al.*, 1996). The inhibition of growth caused by sodium, coupled with the decrease in *ompS* expression, suggest that *ompS* expression might be tightly regulated and coordinated with cell division. Interestingly, while sodium does inhibit the growth of *L. pneumophila*, it does not do so through the premature induction of stationary phase, as the mobility shift profiles of the *ompS* promoter region in sodium challenged extracts is different from that of stationary phase extracts. Thus, this offers insight into the sodium repression of growth, and demonstrates that the growth repression is not merely due to entry into stationary phase.

4.1.2 Effects of salts on global gene expression as determined by two-dimensional SDS PAGE.

Two-dimensional SDS PAGE was employed not only in an attempt to identify OmpT, but also to assess whether there are global changes that occur after osmotic challenge. Both KCl and NaCl challenges produced the same changes in protein expression profiles when compared to wild type, and thus this technique was not successful in identifying sodium-specific changes in gene expression. While a number of proteins were identified, it was difficult to obtain statistically significant densitometric

data. Due to the low expression of many of these proteins, they where not amenable to densitometric analysis, specifically spots 1, 2, 4, 5, 7 and 8 of Fig. 3. The following is a general discussion of the sodium-regulated *L. pneumophila* proteins listed in Table 3.

One of the most robust of the identified proteins, the general stress protein (GspA), has also been previously identified as a protein whose expression is increased upon osmotic challenge (Abu Kwaik and Engleberg, 1994). Northern blot analysis has revealed that upon osmotic stress, GspA mRNA levels increase 6.7-fold, and it is a member of the heat shock regulon. Null mutations in *gspA* result in increased susceptibility to in vitro stresses, including osmotic challenge (Abu Kwaik *et al.*, 1997). Despite the protein's role in the in vitro stress response, *gspA* null mutants are not defective for intracellular multiplication. Interestingly, both wild type legionellae and the *gspA* null mutant bacteria harvested from macrophages and amoebae displayed increased resistance to in vitro stress challenges, a concept that will be discussed later in this work. The fact that GspA, a protein previously shown to be induced upon osmotic stress was seen during the experiments herein serves to validate the approach.

Of particular interest is protein 1 (10 on challenged gels shown in Fig. 3, panels B and C). Upon osmotic challenge, this protein displayed a shift towards a more basic pI, while the levels of expression remained unchanged. The protein was identified as lpg0406, an uncharacterized *L. pneumophila* protein that bears considerable sequence identity and similarity to two major sets of proteins; alkylhydroperoxidase (*ahpD*) cores, and carboxymuconolactone decarboxylases (CAD). Domain database searches suggest

that this protein is more closely related to the CAD family, and this is supported by the fact that a large number of the blast hits for this protein are in the CAD family (11 of 20 with > 7e-11. Carboxymuconolactone decarboxylases are members of the protocatechuate degradation pathway, in which aromatic compounds are degraded for detoxification and nutritional supplements (Iwagami *et al.*, 2000). Of particular interest are the CAD enzymes that are capable of eventually converting aromatic compounds to succinate and acetyl coenzyme A (Lorite *et al.*, 1998). Succinate is a mild stimulator of oxygen uptake and can be used as a carbon source by *L. pneumophila* (Tesh *et al.*, 1983). Blast analysis of the MagA protein also revealed the presence of a CMD domain in the N-terminal region of the protein. The MagA protein also undergoes extensive post-translational modification. Alignment of the MagA and lpg0406 protein sequences did not reveal any significantly similar regions or alignments. While there are no described functions for the lpg0406 or MagA proteins, it is possible that they function together in catechol metabolism though other enzymes of this pathway are absent in *L. pneumophila*.

Database searches for homologues of lpg0406 did not reveal any possible explanations for the observed shift towards a more basic pI. Post-translational modification of proteins can occur through a variety of ways including methylation, phosphorylation and acetylation. In addition, the oxidation of cysteines in various proteins can cause shifts in the isoelectric focusing point, as has been shown for AhpC and GapA (Hochgrafe *et al.*, 2005). From the mass spectral data, unsuccessful attempts were made to identify the modification that caused the shift in pI in lpg0406, and reviews

of the literature did not reveal any reports of post translational modification for this protein.

One of the proteins whose expression was decreased upon osmotic shift was a 27-kDa outer membrane protein, putatively identified as DsbA, a disulfide-isomerase. Blast searches reveal the closest homologue exists in *Coxiella*, a bacterial species closely related to *Legionella* sp. This identification is perhaps questionable, as the disulfide bond isomerases have been shown to be upregulated in response to extracytoplasmic stresses, including osmotic stress (Alba *et al.*, 2001), yet were downregulated in *L. pneumophila*.

Another protein, lpg1585, was also identified as being differencially expressed in the IHF mutant. lpg1585 has no significant blast hits to any proteins sequenced to date, and contains no putative domains, thus it is impossible to speculate as to its possible role in osmotic stress.

Spot 6, identified as the *L. pneumophila* ATP transporter, ABC binding cassette, ATP-binding protein contains extensive homology to an uncharacterized *P. aeruginosa* ABC multidrug transporter (expected probability = 7e-99). Correlations exist between osmolarity and the activity of these multidrug transporters, an example being the OpuC system of *Listeria monocytogenes* (Sleator *et al.*, 2001). While the effects of osmolarity increases on expression of the transporter have not been studied, it does establish a link between the two. There are also examples of multidrug transporters whose expression is modulated by sodium. The *Bacillus subtilis natB* gene, which is the membrane-spanning

component of the ABC transporter, is upregulated in response to sodium and potassium challenge (Cheng *et al.*, 1997). Again, while the *L. pneumophila* transporter homologue appears to be downregulated, the fact that osmotic levels can modulate expression of these systems in other bacteria lends credence to the observation that levels become altered by salt challenge in *L. pneumophila*.

It should be noted these lysates were prepared by French pressure cell in the absence of detergent, and thus these gels most likely under represent the protein content of the cellular envelope. Therefore, if any of the proteins whose expression is decreased in response to osmotic challenge, it may be only the cytoplasmic pools that have decreased, and the membrane content increased.

Of the final two proteins identified, the ribosome recycling factor gave only slight decreases, and the definition of the spot in the gel made densitometric analysis particularly difficult. The final protein, succinyl CoA synthetase beta chain, was located in a region of the gel that contained significant background staining from neighbouring proteins, again making analysis difficult.

While 2D gel electrophoresis is a powerful tool for monitoring global changes in protein expression, and for monitoring protein modification states based on pI's, there are limitations that should be considered. One of the major drawbacks is the propensity of silver stained proteins to become saturated, making analysis difficult (Chevalier *et al.*, 2004). Thus differences in expression must be great enough to cause a change in spot

intensity, but the total levels of expression must be non-saturating. Additionally, proteins that contain relatively few trypsin cleavage sites, or large numbers of cleavage sites are not always amenable to mass spectral identification. Large protein fragments are more difficult to separate by chromatography and spectral data are too complex for identification, while small fragments can lead to ambiguous spectra. Because densitometric analysis reflects spot intensity differences, and thus not specifically protein expression differences, additional experiments including quantitative PCR, or northern blot would be helpful in validating the proteomic results obtained above, and determining the exact expression level differences.

4.1.3 Binding proteins of the *ompS* promoter

Since the identity of OmpT was not revealed from either the column chromatography, or from the two-dimensional gel electrophoresis, the *ompS* promoter was analyzed for previously described binding motifs. The analysis revealed the presence of three Integration Host Factor (IHF) binding sites within the promoter. PCR was used to create both 3' and 5' deletions of the *ompS* promoter region. Neither of the two truncated fragments displayed any retardation using Lp02 unchallenged lysate. This suggests that the site recognized by OmpT spans both fragments and that multiple sites are required in both fragments, or that additional, non-specific DNA is required on either side of the binding site for OmpT to bind correctly. It should also be considered that multiple proteins might be required for the OmpT mobility shift, and occupy different regions on the *ompS* promoter.

The relationship between *L. pneumophila* and sodium had been documented long before any genes responsible for this phenotype had been described. The work by Catrenich and Johnson (1989) allowed them to formulate a number of possible mechanisms by which sodium may exert its effect. One possibility is general inhibition of protein synthesis, which accounts for the fact that sodium inhibits bacterial division, but cells remain viable. The 2D gels presented in this work do not show global changes in protein expression, and are evidence that global repression of protein synthesis is not the cause of the sodium effect. An additional theory offered by Catrenich and Johnson was that sodium levels antagonize intracellular levels of magnesium and potassium, and may affect enzymes or ribosomes, as these ions are required for growth of *L. pneumophila* (Tesh and Miller, 1982). While this possibility was not addressed in this work, a possible experiment to test this theory would be to attempt to reverse the sodium-mediated inhibition by adding increasing amounts of potassium to the growth media.

4.1.4 Sodium

Other authors have examined the issue of other ions being able to induce the sodium-like effects. Previous work in our lab has shown that lysates from late exponential phase *L. pneumophila* cultures challenged with either calcium chloride or magnesium chloride decrease binding of OmpT to the *ompS* promoter, although microscopic examination revealed irregularly shaped cells upon treatment. Additionally, the experiments done previously using NaCl and KCl were conducted using ~ 0.12 M potassium chloride, not

equal molarities of the two salts, as was performed in this work. As the 2D gels presented above showed no difference in expression of any proteins between the two challenges, the KCl challenged mobility shifts previously described should be performed using equal molarities. Also, it has been shown that LiCl can substitute for sodium chloride in distinguishing between virulent and avirulent bacteria, although the decrease in viability in virulent cells is less pronounced (Sadosky *et al.*, 1993). The fact that protein profiles of both sodium and potassium challenged cultures are identical, and that lithium can substitute for sodium in differentiating between virulent and avirulent strains demonstrates the need for further investigation of this issue.

While data presented in this work also implicate IHF as another stationary phase regulator involved in sodium sensing, it is not clear how they might affect the exponential phase sodium sensitive regulator OmpT. The results of the mobility shift experiments using exponential and stationary phase lysates of Lp02 and Lp02Δ*ihfAB* do offer a possible explanation. Deletion of IHF has no effect on the OmpT mediated mobility shift seen in exponential phase, yet, there are differences in mobility shift profile between wild type and IHF mutant stationary phase cell extracts. These confirm the bioinformatic predictions and purified protein mobility shifts, and demonstrates that IHF is not OmpT. In *L. pneumophila*, like in most other bacteria, IHF is growth cycle regulated, with expression increasing in stationary phase, and reaching a maximum in the MIFs. This allows for the formation of a model, in which during exponential phase, OmpT binds the ompS promoter. During entry into stationary phase, and thus concomitant with the increase in IHF production, IHF either replaces, or in addition to OmpT, binds the OmpS

promoter. The model of IHF replacing OmpT would allow for temporal influence of sodium on the *ompS* promoter, as the OmpT mediated effects could only occur during exponential phase, and increased IHF expression in stationary phase would abolish this activity. In addition, the decrease in MIF IHF levels upon entry into exponential phase would also allow for OmpT activity to occur after the commencement of replication. Because OmpT has not been identified, it is not possible to prove this theory simply based on EMSA competition experiments. A possible approach would be to express IHF during exponential phase, and determine whether OmpT binding to the *ompS* promoter occurs.

In order to test these various hypotheses regarding OmpT function and regulation, it will be necessary to identify this binding protein. A possible approach would be to excise OmpT shifted bands from and electrophoretic mobility shift experiment, and determine its sequence using mass scpectroscopy. It may also be possible to create a random transposon mutant library and screen these mutants for decreased activity of the *ompS* reporter using a GFP fusion. Finally, to aid in the identification of the other protein(s) that bind the *ompS* promoter during stationary phase, including possibly OmpT, anti-IHF antibodies could be used in immunoprecipitation assays using IHF and the *ompS* promoter. These types of experiments will be necessary in order to determine the identity of OmpT.

There is evidence that supports this theory, as IHF has been shown to bind to the promoters of outer membrane proteins in other bacteria. In *E. coli*, IHF binds a 35 base

pair region upstream of the *ompC* gene, repressing expression from two of the three promoters (Huang *et al.*, 1990). Additionally, IHF mutant strains also overexpress OmpC. IHF has also been shown to inhibit OmpR mediated expression of *ompF* in an in vitro system (Ramani *et al.*, 1992). Non-porin outer membrane proteins are also regulated in part by IHF, including the cysteine-rich protein (Crp) operon (Zhong *et al.*, 2001). Thus, the results obtained above are supported by multiple examples of other outer membrane proteins whose promoters are bound by IHF.

While there are no homologous proteins in any of the genomes sequenced thus far, there is some experimental evidence supporting the notion that OmpS may be a porin, thus offering a possible explanation for its role in sodium expression (Gabay *et al.*, 1985). Attempts to use paradigms to identify OmpT have also failed. In *E. coli*, regulation of outer membrane proteins in response to osmotic stress is mediated by the transcriptional regulator OmpR and its cognate sensor EnvZ (Matsuyama *et al.*, 1986). Regulation also involves the high osmolarity porin OmpF, and its negative regulator, the mRNA *micF* (Aiba *et al.*, 1987). No homologues of these proteins exist in *L. pneumophila*, with the closest similar proteins being another two-component regulator, the CpxAR system (CpxA 28% identity to EnvZ, CpxR 39% identity to OmpR and no homologous DNA region to *micF*), that has been shown not to be involved in virulence (Gal-Mor and Segal, 2003). Bioinformatic analysis therefore suggests that the mechanism of sodium modulation of gene expression in *L. pneumophila* is different from that of *E. coli*.

4.2 In vitro analysis of Integration Host Factor

4.2.1 The novel suicide vector pBOCRdxA

Construction of the IHF mutants was performed using the suicide vector pBOCRdxA and a procedure created in this lab. The suicide vector uses a novel suicide negative selection gene, the oxygen-insensitive NADPH nitroreductase *rdxA* from *Helicobacter pylori*, first described in this lab (Goodwin *et al.*, 1998), which confers sensitivity to metronidazole. By incorporating the *rdxA* gene into the allelic replacement delivery vector, it was possible to discriminate between colonies that harboured the suicide plasmid (failed to grow on media containing metronidazole), and those that had undergone recombination (grew in the presence of metronidazole). While it would be possible to directly plate transformants onto media containing metronidazole and thus eliminate a replica-plating step, creation of the mutants was not performed in this way. The basis behind this decision was that by replica plating bacteria onto selective media with and without metronidazole, the colonies selected would never have been in the presence of potentially mutagenic compounds, and limits the possibility of second site mutations. This new vector should also be useful in creation of chromosomal deletion mutants in most aerobic bacteria, and those anaerobes with low-level nitroreductase activity. Thus, the suicide vector created in this lab should prove to be useful not only for the creation of allelic replacement mutants in *L. pneumophila,* but also in other bacterial species.

The relative ease in creating each mutant strongly supports the notion that no second site mutations occurred which allowed for the successful chromosomal deletion. The lack of detectable phenotypes in the single mutants backgrounds is not surprising, as it has been reported in other bacteria as well. Studies done on *E. coli* IHF single gene mutants demonstrated no physiological, subunit-specific differences (Bykowski and Sirko, 1998). The lack of sodium sensitivity differences in the single mutants seen in the experiments presented above is thus not surprising. The lack of phenotypes in the single mutants may be due to homodimer formation by the individual subunits. It has been suggested that IhfB subunits can form functional homodimers, as T4 phage growth can occur in an IhfA single deletion mutant (Zablewska and Kur, 1995). Deletion of both IHF subunits to form double deletion mutants has also been described in *E. coli* (Hiszczynska-Sawicka and Kur, 1997 and Spira and Yagil, 1999).

4.2.2 Complementation of the IHF double deletion mutant

Complementation of the IHF double deletion mutant was not easily accomplished. Initial attempts to complement the chromosomal mutations relied on expression of each subunit from its own promoter. While expression of the α subunit was sufficient from its own promoter, despite the presence of ~ 800 base pairs of upstream DNA and a putative promoter sequence, no expression of the IhfB protein occurred. While the subunit is expressed in the chromosome, the reasons for the lack of expression remain unknown, mainly because little is known about the regulators of IHF gene expression. Ditto *et al.* demonstrated that RelA, SpoT, HU and Fis mutants of *E. coli* show no difference in IHF

gene expression (Ditto *et al.*, 1994). In *E. coli*, transcription of the *ihfB* gene occurs mainly from a series of promoters that lie ~ 2.5 kilobases upstream from the coding sequence of *ihfB*, in the coding sequence of the *cmk* gene (Weglenska *et al.*, 1996). During stationary phase, northern blot analysis revealed expression of a transcript originating only 100 base pairs from the coding sequence. The fact that the chromosomal location of the *ihfB* gene is different in *L. pneumophila* indicates the upstream promoters may not function here. It has also been shown that while levels of IhfA are dramatically decreased in an *rpoS* mutant, IhfB levels remain essentially the same (Aviv *et al.*, 1994). There is evidence that IHF has a negative regulatory role in the expression of its own subunits, but this does not explain the lack of expression of *ihfB* seen in the initial complementing attempts (Bykowski and Sirko, 1998). It therefore remains unclear as to why there was no expression from the IhfB complementing construct, and suggests there may be deviations in the mechanisms by which the *ihfB* gene are regulated in *E. coli* and *L. pneumophila*. It will be necessary to perform primer extension experiments in order to determine the location of the *ihfB* promoter in *L. pneumophila*.

4.2.3 IHF binding to the *ompS* promoter

The results presented above revealed that IHF was not OmpT and not involved in binding to the *ompS* promoter during late exponential phase, but does bind the promoter during stationary phase, in agreement with the bioinformatic predictions. The fact that Lp02Δ*ihfAB* lysates are still capable of retarding the *ompS* amplicon indicates that at least one other protein binds this region in stationary phase, possibly even OmpT. IHF has

been shown to bind to the promoters of outer membrane proteins in other bacteria. As discussed above, in *E. coli*, IHF binds a 35 base pair region upstream of the *ompC* gene, repressing expression from two of the three promoters (Huang *et al.*, 1990). Additionally, IHF mutant strains also overexpress OmpC. IHF has also been shown to inhibit OmpR mediated expression of *ompF* in an in vitro system (Ramani *et al.*, 1992). Thus, the results obtained above are supported by multiple examples of other outer membrane proteins whose promoters are bound by IHF during stationary phase.

4.2.4 Developmental regulation of IHF

There are multiple possible explanations for the fact that IHF binds to the *ompS* promoter only during stationary phase. It is possible that IHF was expressed constitutively, but another developmentally regulated regulator with greater affinity remained bound to the *ompS* promoter fragment. Another, more likely possibility was that IHF was only expressed later in the growth cycle, as IHF is developmentally regulated in most bacteria. The analysis of IHF expression throughout the growth cycle confirms the second possibility. The fact that the Lp02Δ*ihfAB* mutant lacks one of the proteins that binds the *ompS* promoter will make it easier to identify the other regulatory components of the MOMP gene during stationary phase. This could be accomplished using magnetic bead protein capture assay. Similarly, immunoprecipitation experiments using anti-IHF antibodies and wild type cell lysates may also help identify these proteins.

The developmental expression of IHF is by no means unique, as most bacteria express this protein during stationary phase. The increase in expression of IHF upon entry into stationary phase in *E. coli* has been well documented (see literature review). There are, however, bacteria that display different regulation of IHF. As discussed previously, in *C. trachomatis*, IHF levels also increase in the late stages of intracellular growth, but contrary to *E. coli*, levels declined after 30 hours, and were undetectable in elementary bodies (Zhong *et al.*, 2001). In *N. gonorrhoeae*, *ihf* mRNA levels decrease upon entry into stationary phase, although the regulators of IHF in this system are not known (Hill *et al.*, 1998). Despite the abnormal systems listed above, the general theme throughout gram-negative bacteria is that expression of IHF, like in *E. coli*, increases upon entry into stationary phase. As virulence trait expression in *L. pneumophila* is growth phase regulated (Hammer and Swanson, 1999), with expression coinciding with the rise in IHF levels, the role of IHF was further studied.

4.2.5 Growth rate of the Lp02Δ*ihfAB* deletion mutant

The lack of any differences in growth rate in BYEα broth between the wild type and Lp02Δ*ihfAB* deletion mutants is not unexpected. IHF double deletion mutants in *E. coli* show no difference in exponential growth rate in laboratory media (Hiszczynska and Kur, 1997). The experiments described above used late exponential phase cultures as starter cultures for the growth experiment, and so it would be interesting to determine whether any differences in lag time or growth rate would occur using stationary phase starter cultures. While IHF has never been previously deleted in *L. pneumophila*, deletion of

other stationary phase regulators, such as RpoS, did not produce any difference in growth rate (Hales and Shuman, 1999).

4.2.6 Sodium sensitivity assays

The results of the sodium sensitivity assays are similar to results obtained for other *L. pneumophila* post-exponential phase regulators. The stationary phase sigma factor, RpoS, which is required for efficient intracellular replication of *L. pneumophila,* is required for sodium sensitivity (Bachman and Swanson, 2001). Similarly, sodium sensitivity is also regulated by the LetA/S two component regulatory system (Hammer *et al.*, 2002). In both cases, while expression of the transmission phenotype requires these two systems, it was suggested by the authors that there are other factors that may also play a role, and it is tempting to speculate a role for IHF as well. Despite the identification of these proteins that are responsible for the expression of the sodium sensitivity trait, the factor or factors responsible for mediating sodium sensitivity remain unknown. The fact that the IHF double knockout also shows a smaller degree of sodium resistance than other regulators furthers the notion that this protein may be linked through the others to expression of the sodium sensitivity mediator. The *nudA* gene has been implicated with increased resistance to sodium in the growth media (Edelstein *et al.*, 2005). While showing growth defects on laboratory media, the *nudA* mutant strain also demonstrated a significant increase in viability on media containing sodium chloride. While the *nudA* gene was not identified as one of the genes differentially expressed in the IHF mutant background (see below), it will be of interest to determine whether IHF plays a role in the expression of

this gene. The fact that the IHF overexpressing strain displayed much more resistance to sodium chloride was unexpected, but indicates the importance of proper temporal expression and total levels of IHF in the sodium sensitivity phenotype.

While several genes, including most of the *dot/icm* genes, have been implicated in resistance, one protein has been implicated in hypersensitivity to sodium chloride. Mutants in the *dotL* gene, a member of the *dot/icm* virulence gene system, displayed increased sensitivity to sodium chloride (discussed above), similar to the mock complemented IHF mutant strain. While the effect of plasmids on *dotL* gene expression in the IHF mutant was not examined in this study, it will be interesting to determine whether there are any differences in expression of this gene in the Lp02*ihfAB*/pMMB206 strain.

4.2.7 IHF-mediated changes in protein expression as determined by two-dimensional SDS-PAGE

Two-dimensional SDS PAGE identified a number of proteins that were differentially expressed between in vitro grown Lp02 and the Lp02*ihfAB* mutants (refer to Fig. 14 and Table 5). Although IHF is expressed at this point in the growth cycle, it was not identifed in the two-dimensional gels.

The first protein identified was the uncharacterized *L. pneumophila* homologue of GreA, a transcription elongation factor. The role of these factors is to cleave nascent

transcripts that are held by arrested elongation complexes (Sen *et al.*, 2001). The GreA homologue in *S. mutans* has been identified in two-dimensional gels as a protein whose expression is upregulated in response to acid stress (Len *et al.*, 2004). Interestingly, in *Sinorhizobium meliloti*, the GreA protein has been identified, along with RelA, as a factor required for resistance to sodium in the growth medium (Wei *et al.*, 2004). While it is suggested that these proteins may represent a general physiological response to stress, the role of this protein in sodium sensing is of obvious interest for this work.

Also identified during proteomic analysis was the nucleoside diphosphate kinase, *ndk*. This protein is responsible for the phosphorylation of nucleoside diphosphates to the corresponding triphosphate (Rodriguez and Ingraham, 1983). During this work, it was difficult to measure the exact intensity of this spot due to the smearing seen in the IHF mutant strain, and so the 11.8% increase calculated may be an underestimate. Correlations between virulence and Ndk activity have been made in *P. aeruginosa*. AlgR2, a regulator required for alginate production, is involved in Ndk expression, although this decrease is compensated by another kinase (Schlictman *et al.*, 1995). The role of this protein in alginate production is in the synthesis of GDP-mannose, where Ndk complexed to pyruvate kinase, transfers phosphate from ATP to GDP, an essential step in GDP-mannose synthesis (Sundin *et al.*, 1996). The *P. aeruginosa* Ndk also prevents early bacterial cell death in stationary phase when overexpressed in an AlgR2 mutant background (Kim *et al.*, 1998). There is also evidence that Ndk may be secreted in *V. cholera*, as it is found in fractionated growth media, and addition of ATP to these fractions induces macrophage and mast cell death, through a P2Z receptor-mediated

process (Punj *et al.*, 2000). The *M. tuberculosis* Ndk is also secreted and induces P2Z mediated macrophage cell death (Chopra *et al.*, 2003). While it is not known whether the *L. pneumophila* homologue of this protein is secreted, it is tempting to speculate a host cell interaction, and also a role in the general physiology of the IHF mutant strain.

Expression of the cold shock protein CspE was substantially increased in the IHF mutant strain. The cold shock proteins are a conserved set of proteins involved in DNA supercoiling, transcription and translation (Jones and Inouye, 1994). RNA-protein crosslinking studies revealed that CspE is present in the transcription initiation complex, and interacts with the normal release of the sigma factor from the complex (Hanna and Liu, 1998). The exact function of the CspE protein appears to act as an anti-terminator, RNA chaperone, which keeps mRNA molecules in a transcription accessible form at low temperatures (Bae *et al.*, 2000). In *E. coli*, CspE is a negative regulator of CspA, another cold shock protein (Bae *et al.*, 1999). 2D gel electrophoresis revealed a number of proteins whose expression was altered in the *E. coli cspE* deletion mutant, including UspA, shown in this study to be reduced in the IHF mutant background (see below). Overexpression of CspE has also been shown to increase the levels of supercoiling in plasmids (Sand *et al.*, 2003), and provides a possible explanation for some of the negative plasmid effects seen in the IHF mutant.

The DNA binding stress protein Dps is upregulated in the IHF deletion mutant. Dps was originally characterized in *E. coli* as a starvation-induced protein that binds to and protects DNA (Almiron *et al.*, 1992). In *E. coli*, while IHF is required for full expression

of DPS, along with the stationary phase sigma factor (Altuvia *et al.*, 1994), the *L. pneumophila* IHF deletion mutant displays increased expression of this stress protein. The role of Dps-like proteins has been sparsely studied in intracellular multiplication. Dps mutants of *L. monocytogenes* show decreased ability to replicate within J774.A1 mouse macrophages, due to a defect in resistance to oxidative stress (Olsen *et al.*, 2005).

The 50S ribosomal protein L9, also termed RplI, was also identified as being differentially expressed in the IHF mutant strain. The protein has been implicated in a process known as hopping, where ribosomes "hop" over a coding gap in mRNA during translation due to the presence of hairpin structures (Herbst *et al.*, 1994). Little is known regarding any possible roles for this protein other than in translation, although the gene is essential in *B. subtilis* (Ohashi *et al.*, 2003).

The presence of lipase activity in supernatants of *L. pneumophila* cultures has been known for some time (Thorpe and Miller, 1981). The lipase is secreted via the type-two secretion system shown to be required for full virulence in tissue culture models (Aragon *et al.*, 2000). While mutations in LipA and LipB, two other *L. pneumophila* lipases, have been studied and determined not to play a significant role in intracellular multiplication (Aragon *et al.*, 2002), the role of lpg1889 is not known. The lpg1889 lipase bears strong sequence identity to several other putative lipases, including a cold-resistant lipase from *Pseudomonas* B11-1 (Choo *et al.*, 1998). The fact that this protein is uncharacterized in *L. pneumophila*, and poorly characterized in other bacteria makes it difficult to speculate as to any possible roles it may have.

UspA was first described in 1992 as a protein induced upon starvation and whose expression was independent of several of the known stress-condition regulators, including RelA/SpoT, OmpR and H-NS (Nystrom and Neidhardt, 1992). The synthesis of UspA is upregulated in response to declines in growth rate, and mutants in this gene are unable to survive growth arrest in the presence of various stresses, indicating UspA may have a general protective function (Nystrom and Neidhardt, 1994).

Thioredoxin reductase, a flavoprotein, transfers electrons between thioredoxin and NADPH (Ronchi and Williams, 1972). While no studies have been conducted on the *L. pneumophila* thioredoxin reductase, it may function in some respect with the MagA protein, which also contains the CXXC motif common to the thioredoxin reductase family (Hiltz *et al.*, 2004). Thioredoxin reductases have been implicated in the virulence of *M. tuberculosis*, as they allow for an alternative reducing pathway in KatG mutants, which are resistant to Isoniazid (Jaeger *et al.*, 2004). Thioredoxin reductase has also been implicated in the intracellular survival of *M. smegmatis*, as strains overexpressing the *M. leprae* thioredoxin-thioredoxin reductase hybrid protein resist killing by macrophages (Wieles *et al.*, 1997).

Enoyl Reductase, a FabI homologue, was identified as being differentially regulated, although statistical analysis revealed this difference not to be significant. Enoyl reductase reduces enoyl-ACP during fatty acid chain elongation and transfers reducing equivalents to either NADH or NADPH (Bergler *et al.*, 1996).

Also upregulated in the IHF mutant background was the 27-kDa outer membrane protein, that was also identified in the two-dimensional gels of salt challenged lysates (see above). This protein was putatively identified as a DsbA homologue, and may suggest an altered extracytoplasmic stress response in the IHF mutant.

The increased expression of several stress related proteins, including CspE, Dps, GreA and UspA indicate that the IHF deletion mutant faces increased stress. This may be due to a decrease in a particular defence mechanism, or a global increase in stress sensitivity, or disregulation of the stress response.

4.2.8 *dot/icm* gene expression

There were no major differences in gene expression of any of the reporter constructs with *dot/icm* genes in the IHF-negative background. This is not unprecedented, as mutations in several other regulatory proteins do not result in changes of *dot/icm* gene expression. The CpxAR system, involved in mediating responses to extracellular stress in *E. coli*, plays a small role in the expression of the *icmWX* operon, *icmV-dotA*, and *icmR*, although no defects in intracellular growth were observed CpxAR mutants (Gal-Mor and Segal, 2003). In the same study, the extracytoplasmic sigma factor RpoE was shown to have no effect on *dot/icm* gene expression. RpoS mutants show only a slight decrease in *icmP* transcription, similar to RelA mutants (Zusman *et al.*, 2002). While it is apparent that IHF does not play a significant role in the expression of the genes analyzed in this study, it is possible that other genes in the system undergo some regulation by IHF

and the possibility should be examined. Also of interest, it was apparent that none of the genes studied showed any significant growth phase regulation upon entry into stationary phase, as others have examined growth phase regulation of these genes only briefly.

The relationship between the *dot/icm* system and sodium remain a mystery. It has been suggested that DotL acts as a plug to block the influx of sodium through the Dot/Icm complex (Buscher *et al.*, 2005). It is tempting to think of the Dot/Icm system as a "straw", sampling the environment for sodium in order to regulate virulence gene expression once the bacterium is internalized. While intriguing, further structural analysis of the Dot/Icm secretion system will be required before such theories can be truly tested.

4.3 IHF role in intracellular multiplication and developmental cycle

4.3.1 Attachment, Invasion and Infectivity

The primary protein responsible for invasion and one of the proteins involved in attachment, is the chaperonin Hsp60, as it has been demonstrated that anti-Hsp60 antibodies prevent attachment and entry of virulent *L. pneumophila* into HeLa cells, and Hsp60 coated latex beads are internalized efficiently (Garduno *et al.*, 1998b). While there was considerable variability in the invasion and attachment experiments conducted herein, there was no statistical difference in attachment or invasion. At this point it should be noted that this experiment is actually measuring bacterial host cell-association, not just attachment, since the three-hour time point represents both external and internalized bacteria. In order to test attachment directly, host cells would have had to be treated with

actin inhibitors, such as cytochalasin D, to prevent bacterial uptake. In support of the lack of differences in the attachment-invasion data, there were no differences in any of the three-hour time points taken during the intracellular growth curve experiments. The invasion numbers obtained for the IHF mutant strain may also be slightly over estimated, as the mutants do show limited survival upon upon exposure to 100 μg/μl of gentamicin in culture media, and thus some of the extracellular bacteria may have survived. Use of another antibiotic may remove some of the variability from future experiments. These results also suggest that there are no significant differences in Hsp60 expression, as one would predict these would correlate with defects in invasion. In support of this, no obvious differences were seen in the Hsp60 spot in any of the two-dimensional gels performed.

L929 plaque assays have been successfully used to not only distinguish between virulent and avirulent bacteria, but also to quantify the infectivity of *L. pneumophila* (Fernandez *et al.*, 1989). The results obtained from the plaque assay experiments confirmed the attachment and invasion results obtained above for the in vitro grown bacteria. Previous studies have shown that MIFs are approximately 10-fold more infectious than stationary phase, agar-grown bacteria (Garduno *et al.*, 2002). The results obtained in this study are similar to those previously described, and thus the IHF mutant is not defective for infectivity. Plaque assays performed using either agar-grown or mature forms of the complementing and mock complementing constructs did produce plaques, but they were very small and difficult to enumerate. This is more evidence that

the presence of the pMMB206 plasmid in the IHF mutant background has some inhibitory role of the intracellular growth of *L. pneumophila*.

4.3.2 Intracellular multiplication

While the sodium sensitivity assays suggested there may be defects in virulence, all strains grew equally well intracellularly in HeLa cells. Additionally, the fact that there were no defects for attachment and invasion demonstrate that IHF does not play a role in the infection process. Since IHF expression occurs later in growth cycle, and not during exponential growth, it is not surprising that the bacteria replicate efficiently in host cells. While the CsrA protein, expressed during exponential phase is required for intracellular multiplication (Molofsky *et al.*, 2003), many stationary phase regulators have been described that do not significantly affect intracellular growth. The stationary phase sigma factor RpoS, while being required for efficient replication in protozoan cells, is dispensable for intracellular replication within HL-60 and THP-1 derived macrophages (Hales and Shuman, 1999). Similarly, the RelA ppGpp synthetase is also dispensable for replication in macrophages (Zusman *et al.*, 2002). The LetA/S two-component regulatory system, also involved in sodium sensitivity and motility, is only partially required for infection of macrophages (Hammer *et al.*, 2002). It is apparent that there are several levels of regulation that are responsible for controlling the stationary phase genes required for intracellular multiplication, and deletion mutants in many of these genes, including possibly IHF, may be required before mutants completely defective for intracellular multiplication in non-protozoan cells are obtained.

While IHF was not required for intracellular multiplication of *L. pneumophila*, it is required in at least one other bacterial species. As discussed above, *B. abortus* requires IHF for full expression of the *virB* operon and for intracellular multiplication (Sieira *et al.*, 2004).

4.3.3 Vesicle morphology

During the intracellular growth curve experiments discussed above, it became apparent that the IHF mutant had altered vesicle morphology. The defects were not restored by trans-complementation as the IHF mutants harbouring the pMMB206 vector showed further alterations in vesicle morphology, and increased aggregation of HeLa cells. To address the possibility that the lack of complementation was due to a second site mutation in the complementing construct, a second clone was examined and produced similar results. This experiment may not be conclusive, as the complementing clones were selected from the same initial transformation, and thus there is a small chance they may be clonal. Definitive proof that the defect is not restored by in trans complementation will require isolating a clone from a separate transformation. These results also further the notion that the pMMB206 vector has a deleterious effect on Lp02*ihfAB* physiology, as vesicle morphology was further altered in IHF mutant strains containing the pMMB206 vector.

While there have been many reports of mutants that are defective for avoiding phagosome-lysosome fusion, and thus defective for formation of the *Legionella*-

containing vacuole, there have been only a few reports of *L. pneumophila* proteins that are responsible for vesicle structure. Mutants in the *pmiA* protein are partially defective for the recruitment of ER derived membrane, possibly in a *dot/icm* related mechanism (Miyake *et al.*, 2005). IcmR mutants have been shown to replicate in primary derived macrophage cells, but form small, isolated vacuoles, and are defective for pore-formation (Coers *et al.*, 2000). Because these experiments were performed in primary derived cells, it makes direct comparison of results difficult, but the effects of IHF deletion on IcmR expression should be examined. Similarly, formation of the replicative vacuole has also been shown to require the activation of caspase-3 through a Dot/Icm manner that is also required for phagosome biogenesis (Molmoret *et al.*, 2004b). MagA mutants also display altered vesicle morphology, although the physiological basis for this observation remains unknown. There are also large variations in vesicle morphology among non *L. pneumophila* strains, further exemplifying the lack of understanding of the genes required for maintenance and formation of the phagosome (Ogawa *et al.*, 2001).

It will be interesting to determine whether the IHF mutant strains reside in phagosomes that possess all of the characteristic markers of the *Legionella*-containing phagosome, including acquisition of late-endosomal markers and RER derived membrane. The two-dimensional gel analysis performed in this work did not identify any proteins that have been previously found to be involved in host-cell trafficking.

4.3.4 Role of IHF in *L. pneumophila* development

Gimenez staining, originally used for the identification of *Rickettsiae*, was first employed to identify *L. pneumophila* in lung tissue of infected patients (McDade *et al.*, 1977). The intense staining seen with Gimenez stain occurs only in intracellular bacteria, while stationary phase bacteria appear dull red (Garduno *et al.*, 2002). It is hypothesized that the changes in cell envelope architecture typical of MIFs is responsible for the ability to retain the Gimenez stain, although the fact that the IHF mutant and the vector control, which do not form the multi-laminated membranes, stain with equal intensity when compared to the wild type and complemented strains. These results suggest that some other differences, including possible changes in protein expression or membrane lipid composition not visible by electron microscopy are responsible for the Gimenez staining phenotype. Two-dimensional gel electrophoresis has revealed several protein differences between stationary phase and MIF cells, and one or more of these may be responsible for the ultrastructural changes that occur during MIF morphogenesis.

L. pneumophila produces large amounts of poly-beta hydroxybutyrate (PHB), a reserve compound that can be used as an energy source when nutrients become scarce (James *et al.*, 1999). The PHB stores can be seen by electron microscopy as large, cytoplasmic, resin-impermeable inclusions. Ultrastructural analysis has revealed that these inclusions are especially prominent in MIF forms, and to a much lesser extent in bacteria grown to stationary phase (Garduno *et al.*, 2002). While PHB synthesis appears to be growth phase regulated (i.e. present only in stationary phase and MIFs), the regulators of PHB synthesis in *L. pneumophila* are unknown, and no regulatory genes have been identified that reduce PHB inclusion levels. While the process of PHB

synthesis and accumulation have not been studied in *L. pneumophila*, the *L. pneumophila* Philadelphia-1 genome sequence reveals the presence of at least nine homologs of the PHB biosynthetic pathway, based on simple text searches. Interestingly, only low scoring blast hits were obtained for the PhaP homologues, with the closest hit being from *B. thuringiensis* (expected probability = e-05), and thus probably not significant. PhaP is a phasin, a protein that is the major constituent of the membrane which surrounds the cytoplasmic PHB inclusions (Potter and Steinbuchel, 2005). The closest homologue to the PHB regulatory protein, PhaR, is the PhaR from *Burkholderia mallei* (expected probability = 2e-13). In *Rhizobium etli*, PHB synthesis is regulated by the Rsh protein, a member of the RelA/SpoT protein family (Calderon-Flores *et al.*, 2005). There are no reports of IHF being involved in PHB synthesis in other bacterial species. Given the large number of potential genes involved in PHB synthesis in the *L. pneumophila* genome, it is difficult to speculate as to the reason for the observed defects in PHB synthesis, although it is apparent that total IHF levels and temporal expression are critical for the proper synthesis of PHB, as the mutants and overexpressing strains show marked defects in the formation of cytoplasmic inclusions.

The formation of MIFs also involves changes in the cell envelope structure, including a thickening of the membranes, resulting in a relatively uniform, electron dense structure, as well as the appearance of cytoplasmic laminations (Garduno *et al.*, 2002). Cyst formation has been described for a number of bacteria, and in some instances, the regulators responsible for this phenotype have been at least partially described. In *A. vinelandii*, encystment is induced by nutrient deprivation, and is characterized by the

production of PHB, as well as production of extracellular structures. The formation of these extracellular structures, termed intine and exine, is dependent on a functional AlgR, a regulator of alginate biosynthesis with homologues in a variety of bacteria including *P. aeruginosa* (Nunez *et al.*, 1999). The protein with the highest percent identity to AlgR in the *L. pneumophila* genome is the response regulator GacA (LetA) (expected probability = 5e- 16, 35% identity). The function of LetA has been described extensively, and while electron microscopic examination of this mutant has not been performed to assess its role in development, it has been implicated in stress resistance, sodium sensitivity, and is required for proficient infection of macrophages (Hammer *et al.*, 2002). The relationship between LetA and growth phase related virulence, as well as the fact that IHF mutants are defective for maturation, indicates that the role of IHF in LetA expression should be examined.

4.3.5 Two-dimensional gel electrophoresis of wild-type MIFs and late forms from Lp02*ihfAB* (refer to Fig 27 and Table 8)

It is interesting that the MagA protein, present as a quartet of spots in 2D protein gels of in vitro grown bacteria, is present essentially as a doublet of spots after in vivo growth. This may represent an accumulation of specific post-translationally modified versions that play a specific role late in infection. However, no function has been attributed to MagA, and therefore this functional argument is merely speculation.

Lpg0294 is a hypothetical protein with no significant homology to any described protein, the closest being a protein from *Shewanella baltica*, e-value = 0.038. The protein does contain considerable putative helical regions, but no putative domains were revealed during BLAST searches, and thus it is not possible to make any assertions regarding its function.

Only one protein was identified as being downregulated in the IHF mutant both during stationary phase and in MIFs, UspA. The functions of the universal stress protein are discussed above.

As was the case with Lpg0294, Lpp1177 is not homologous to any known proteins as judged by BLAST results. In addition, the protein is unique to *L. pneumophila* Paris strain and thus absent in Philadelphia-1. While it is possible that it may be a misidentified trypsin cleavage product from the LC-MS, the fact that there was 38% sequence coverage argues against this. The lack of homologues and any conserved domains also makes inferences about its function impossible.

GreA, the elongation factor was also previously identified in the in vitro gels as being decreased in its expression. As the role of these proteins is to allow for elongation of arrested transcription complexes, the decreased expression of this gene may influence general protein synthesis, and be partially responsible for the observed developmental defects (Symersky *et al.*, 2005). There are no reports of this protein being directly implicated in intracellular growth or developmental regulation.

One of the proteins identified was the eukaryotic ATP synthase beta chain. The 28% sequence coverage over the 529 amino acids of the human ATP synthase was considerably higher than any of the bacterial hits. It is difficult to determine whether this protein is a contaminant, although this is unlikely because no other HeLa proteins were identified. In addition, ATP synthase mitochondrial precursor has been isolated from *F. tularensis*-containing phagosomes derived from macrophages containing mutations in the innate immunity-associated protein Bcg/Nramp1 (Kovarova *et al.*, 2002). The ATP synthase has also been isolated from purified phagosomes containing latex beads, along with other mitochondrial proteins, despite the absence of mitochondria from the preparations, indicating the ATP synthase identified in this work is not a contaminant (Garin *et al.*, 2001). During purification of the intracellular bacteria using Percoll gradients, it is probable that phagosome material co-localized with the bacterial fraction. As mitochondria become closely associated with the phagosomes of wild type *L. pneumophila*, it is tempting to speculate that the enrichment of ATP synthase in the wild type preparations represents an active recruitment process by the bacteria.

Lpg1551, while having identical homologues in both of the other sequenced *Legionella pneumophila* strains, is not homologous to any other proteins in the NCBI database. Analysis of the 28.7 kDa protein revealed the presence of a coiled-coil domain in the N-terminal region comprising 9% of the total protein, and substantial helical structure. As coiled-coil containing proteins are often related to DNA binding, it is possible that this is a DNA binding protein. Interestingly, this protein is located

approximately 2 kilobases upstream of nucleotide diphosphate kinase, which was identified during analysis the in vitro 2D gels described above.

4.3.6 MagA

The MagA protein, previously described in our lab, is upregulated post-exponentially. While the function of this protein remains to be determined, there are homologues in several species of bacteria, and it contains a motif resembling that found in alkyl hydroperoxide reductases, including a CXXC motif (Hiltz *et al.*, 2004). It was apparent from the two-dimensional gels that the MagA protein undergoes extensive post translational modifications, as two and four isoforms could be identified. While it was not possible to determine what modification occurred, the homology to oxidative stress proteins makes it tempting to speculate that they are oxidations. These pI shifts have been documented for the AhpC proteins upon exposure to oxidizing agents such as peroxide (Hochgrafe *et al.*, 2005, McAtee *et al.*, 2001). In the mature forms of both the Lp02 and Lp02Δ*ihfAB* strains, the multiple isoforms of MagA were mostly replaced by two major more basic pI isoforms. It will be interesting to see whether the modifications that result in these pI shifts can be inhibited by making amino acid substitutions in MagA once the modified bases have been identified, and to see whether these residues are required for the phenotypes attributed to MagA, including Gimenez staining and HeLa cytopathology (Hiltz *et al.*, 2004).

Bioinformatic analysis of the MagA promoter revealed the presence of two perfect consensus sequences, within 182 and 885 bp of the coding sequence. Allowing for one mismatch, three additional binding sites became apparent, within 185, 283 and 789 bp of the coding sequence. The presence of IHF binding sites in the *magA* promoter suggested a possible role for IHF in *magA* regulation. Examination of MagA levels in intracellular bacteria revealed that the presence of the pMMB206::*ihfAB* vector in the IHF mutant strain resulted in a significant decrease in MagA levels when compared to both the wild type and IHF mutant strain. The Lp02/pMMB206 strain gave inconsistent results in the two independent experiments performed. The overexpressing strain consistently yielded the most expression of MagA. As MagA is upregulated post exponentially, coinciding with increased IHF expression, it is tempting to speculate a role for IHF in the regulation of MagA, even though there is no difference in Gimenez staining in the IHF mutant. It is apparent, however, from this experiment that both the presence of IHF, as well as temporal regulation is important for proper expression of MagA. These results lend credence to the idea that the IHF mutant does not tolerate the pMMB206 vector well. The difficulties in complementation of intracellular phenotypes, and experimental variability also make conclusions difficult in this aspect of the project.

4.3.7 Stress resistance assays

L. pneumophila becomes increasingly resistant to environmental stress as it enters stationary phase (Hales and Shuman, 1999), and in some cases, such as sensitivity to detergents and basic pH, even further resistance is developed when *L. pneumophila* fully

differentiates into MIFs (Garduno *et al.*, 2002). The GspA protein has been implicated in the *L. pneumophila* stress response, as mutants are more susceptible to multiple stresses including heat and osmotic shocks (Abu Kwaik *et al.*, 1997). The stationary phase sigma factor RpoS is not required for the increased stress resistance of stationary phase cultures (Hales and Shuman, 1999).

Of the two stationary phase related stress responses studied in this work, IHF played no role in survivability in water, and only a minor role in heat resistance. While the decrease in PHB seen in the mutant backgrounds suggested there may be a decrease in survival, none was seen after eight days. It is possible that much longer times are required before water survivability differences are seen, as *L pneumophila* has been shown to survive upwards of 500 days in water suspensions, using PHB as a carbon source (James *et al.*, 1999). In *L. dumoffii*, the DjlA protein, which is related to DnaJ, is required for resistance to heat in stationary phase, as well as other challenges (Ohnishi *et al.*, 2004). While this protein was not identified in the two-dimensional SDS gels, it is possible that IHF plays some role in its regulation.

The resistance of MIFs to stresses such as detergent and low pH has been examined (Garduno *et al.*, 2002). The increased stress resistance of intracellular bacteria has also been described for macrophage and amoebae-grown bacteria (Abu Kwaik *et al.*, 1997). While the genes responsible for the increased resistance to these stresses is unknown, it is tempting to speculate that the changes in cell envelope architecture mediate this phenotype. The results obtained in this work support this notion, as the IHF mutant and

vector control, which are defective for morphogenesis, and the complementing construct, which is slightly defective for MIF formation, are susceptible to highly alkaline conditions (pH 11). Based on the scoring system used by Garduno *et al.* (2002), where bacteria are considered sensitive when their optical density decreases by more than 50%, all strains tested were resistant to detergent. While all strains were resistant to detergent, all strains did show some decrease in optical density over 96 hours, with the IHF mutant strain, and the complementing and mock complementing strains showing the greatest decrease.

The results obtained for the Lp02 MIFs in this study demonstrate similar responses to detergent and alkaline stresses to those described for Svir and 2064 strains by Garduno *et al.* (2002). While the ultrastructural studies performed in this work demonstrated defects in the intracellular maturation of the IHF mutant, they did not fully correlate with phenotypic differences. While remaining resistant to detergents, the IHF mutant was not able to resist high alkalinity, and thus the IHF mutant may be defective for only a subset of stress resistance traits attributed to MIFs. This suggests that MIF formation might require a number of factors, and perturbations in any one of which can alter the final product, including pMMB206 and IHF. It is difficult to speculate as to why phenotypic differences were not more obvious, as the proteins responsible for conferring these phenotypes have not been identified. Additionally, while the role of other stationary phase regulators in MIF formation has not been studied, they may provide insight into the results obtained here.

4.4 Integrated model for IHF regulation in stationary phase

The data presented in this work has revealed that integration host factor plays a role in sodium sensitivity, and in the developmental cycle, as it is required for MIF morphogenesis. The fact that IHF functions during the post-exponential phase, suggests that it may interact with the other described stationary phase regulators. Both the LetA/S system and the RpoS protein are also required for sodium sensitivity, suggesting a possible relationship. Similarly, both the RpoS and LetA/S proteins, as well as IHF, are not absolutely required for intracellular multiplication. Conversely, both of those genes are also required for expression of the flagellar operon, while the IHF mutants show no obvious defects in motility, as judged by light microscopy. The LetA/S/E system has been implicated in stress resistance and motility, while the signals sensed by this system remain unknown. While the role of RpoS and the LetA/S proteins in IHF expression in *L. pneumophila* was not examined in this study, the *ihfA* promoter does contain an RpoS binding site, suggesting a possible regulatory role for RpoS in IHF expression. The preceding data allows for the formation of a model, where IHF is at least in part regulated by RpoS, although it is not known whether both subunits are affected equally. It is reasonable to assume that IHF functions in concert with RpoS to mediate sodium sensitivity. The mechanism by which sodium sensitivity is mediated is unknown, and may involve multiple genes, thus RpoS and IHF may regulate the same or different genes. It has recently been demonstrated that while RpoS is partially required for intracellular growth, the ppGpp synthetase RelA is not (Abu-Zant *et al.*, 2006). Thus, while there is a role for RpoS in phagosome biogenesis, it is not in response to nutrient deprivation-mediated RelA activity, but some other unidentified factor. Another ppGpp

synthetase, SpoT, has been deemed essential, and thus it is possible that this is the source of ppGpp required for RpoS induction (Zusman *et al.*, 2002). Although ppGpp levels in a *relA* deletion mutant are substantially lowered in stationary phase, the levels of ppGpp in intracellular bacteria was not examined, and thus it cannot be ruled out that SpoT compensates in some way for RelA mutants. In addition, it is reasonable to assume that SpoT functions as more than just a ppGpp synthetase, as RelA mediated ppGpp synthetase activity is not enough to support mutations in SpoT. Therefore, it is possible that SpoT plays a significant role in the induction of RpoS, both as a ppGpp synthetase, and in an as yet unidentified capacity. A proposed model for the position of IHF in the previously described model for stationary phase regulators is shown in figure 30.

While the literature on the role of IHF in cyst morphogenesis in other bacteria is extremely scarce, there are reports of encystment in response to environmental stresses, including *B. burgdorferi*, suggesting the possibility that RpoS may be involved (Murgia and Cinco, 2004). Additionally, encystment in *A. vinelandii* occurs in response to stress conditions, and is partially regulated by the response regulator AlgR (Nunez *et al.*, 1999). The formation of the MIF by *L. pneumophila* is thought to be due to nutrient deprivation in the spent host cell, leading to a stress response (Garduno *et al.*, 2002). While the roles of the other stationary phase regulators in MIF formation has not been determined, the two-dimensional gels used to compare expression differences between wild type and IHF mutant strains revealed altered levels in several stress proteins. While it is unknown whether these stress related proteins are responsible for the defect in MIF formation, it is tempting to speculate that the altered stress response, possibly involving RpoS, is

responsible for the observed defects. Determination of the roles of RpoS and LetA/S in MIF formation will help to confirm the validity of this hypothesis.

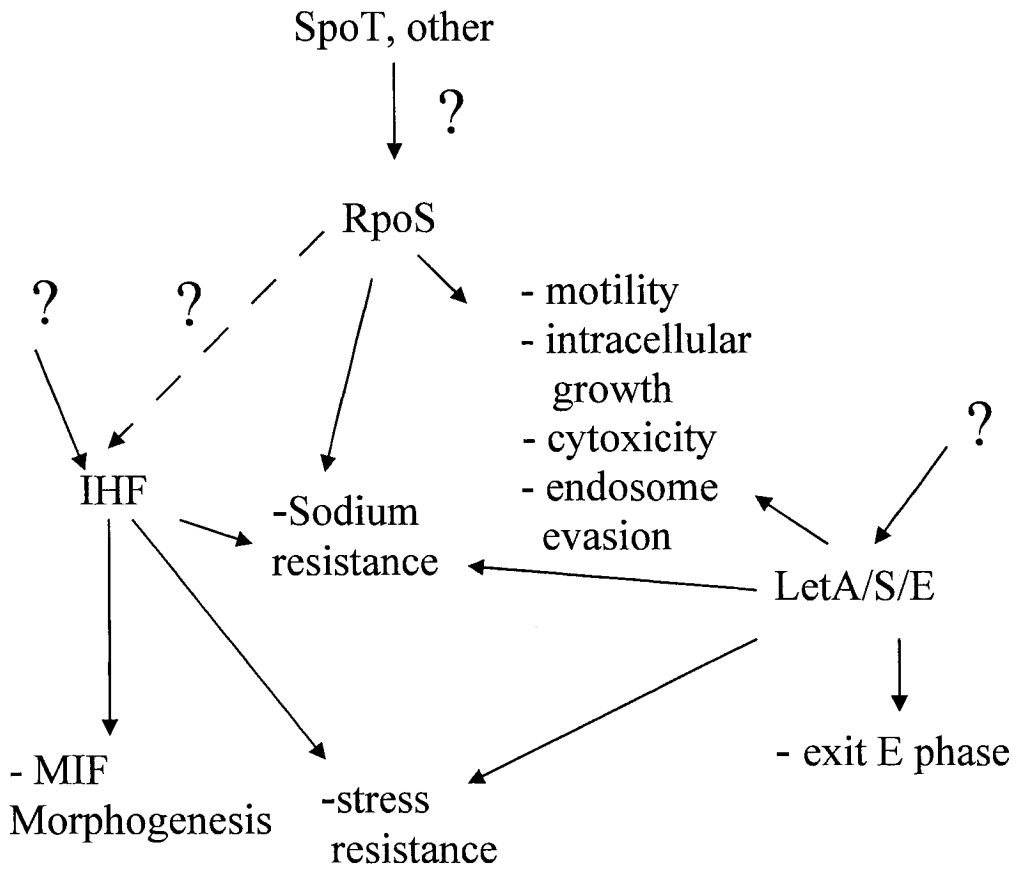


Figure 30. Proposed model for the position of IHF in the previously described model for stationary phase regulators. Unknown factors, possibly including SpoT, induce the expression of RpoS. Additionally, several RpoS regulated traits are also affected by the LetA/S two-component regulatory system. A possible role for RpoS in IHF expression is denoted by a question mark, as are other unknown regulators. IHF co-operates in sodium sensitivity, and potentially stress resistance, as determined by two-dimensional gel electrophoresis, and is the only factor described thus far responsible for MIF morphogenesis. Adapted from Bachman and Swanson, 2001.

4.5 Conclusions and future directions

The work within this dissertation has clarified somewhat the role of sodium in modulation of gene expression in *L. pneumophila*. The binding of OmpT to the *ompS* promoter, and subsequent abolishing of binding by sodium challenge also occurs in the Lp02 strain. Additionally, column chromatography suggests that more than one protein may be responsible for the mobility shift of the *ompS* promoter. Finally, the two-dimensional SDS gels reveal that at equal molarities, both sodium and potassium exert the same effects on global gene expression. Thus, while it will require further research to elucidate the identity of OmpT, the *ompS* promoter will continue to be a powerful tool in its elucidation. Additionally, further work is needed to clarify the role of not only sodium, but also other ions in gene expression and virulence.

The experiments presented in this thesis also describe the role of IHF in the sodium sensitivity and developmental cycle of *L. pneumophila*. Both IHF mutants, and the over expressing strains displayed increased resistance to the presence of sodium in the growth media. Integration host factor mutants are partially defective in MIF formation, as these strains lack the thickened cell envelopes, and multilaminated, internal membranes. While PHB synthesis was affected in the IHF mutant, this defect was even more pronounced in IHF mutant strains carrying the pMMB206 plasmid. Despite the differences in cell envelope ultrastructure, few changes to environmental stress resistance were observed, and those identified were not restored upon in trans complementation, so they cannot be considered IHF based. Two-dimensional SDS PAGE revealed differences between wild

type and IHF mutant strains both in vitro and in vivo. Future experiments on the IHF mutants should include attempts to complement these mutants using chromosomal insertions to negate the negative effects of plasmids in the mutant background. Additionally, the role of IHF in the expression of the proteins identified by two-dimensional gel experiments should be confirmed. Finally, the role of IHF in the expression of RpoS and LetA/S, and vice versa, will define the position of IHF in the cascade of previously described stationary phase regulators.

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