# Characterization of an OxyR-Regulated Alkyl Hydroperoxide Reductase (*ahpC2D*) Operon in *Legionella pneumophila*

by

Jason J. LeBlanc

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## Abstract

*Legionella pneumophila* expresses two catalase-peroxidase enzymes that exhibit only weak hydrogen peroxide ($H_2O_2$)-scavenging activity, suggesting that other enzymes might decompose $H_2O_2$. Likely candidates included two alkyl hydroperoxide reductases (AhpC) that were identified in the *L. pneumophila* genome. AhpC1 and AhpC2/AhpD (AhpC2D) show similarity to the peroxide scavenging systems found in *Helicobacter pylori* and *Mycobacterium tuberculosis*, respectively. Our results indicated that: i) expression of *L. pneumophila ahpC1* and *ahpC2* restores $H_2O_2$ resistance in a catalase/peroxidase-deficient mutant of *Escherichia coli*; ii) both *L. pneumophila ahpC1*::*km* and *ahpC2D*::*km* mutants are more sensitive to various peroxides or reactive oxygen intermediate (ROI)-producing compounds than wild-type, a phenotype that could be alleviated by complementation; iii) *L. pneumophila ahpC* mutants were not affected in their intracellular growth in macrophage-like cells; iv) expression of *ahpC1* appeared post-exponentially in broth culture, whereas *ahpC2* was expressed during early exponential phase; v) *ahpC1* mRNA levels were consistently higher than those of *ahpC2D*; vi) *ahpC2D* expression is significantly increased upon loss of AhpC1 function. To address whether the *L. pneumophila* OxyR homologue (LpOxyR) could function as a regulator of the oxidative stress response as seen in other organisms, experiments were performed to demonstrate that: i) reduced and oxidized forms of LpOxyR can bind the promoter region of *ahpC2D* ($P_{ahpC2}$); defective LpOxyR binding resulted in loss of *ahpC2* transcriptional activity; iii) reduced LpOxyR displayed an extended DNA footprint that overlaps with the putative -35 region of *ahpC2* which was fully accessible to RNA polymerase with oxidized LpOxyR; iv) expression of LpOxyR was partially able to restore peroxide resistance in an *E. coli oxyR::km* mutant. However, unlike *E. coli* OxyR, LpOxyR was unable to bind to its own promoter. Since LpOxyR expression was growth phase-dependent, attempts were also made to determine possible regulators of *oxyR* expression. An acrylamide capture of DNA-bound complexes technique was used in an attempt to identify transcriptional regulators with no success. In summary, this study reports that AhpC1 or AhpC2D provide an essential peroxide-scavenging function to *L. pneumophila* and that LpOxyR functions as a peroxide sensor/transcriptional regulator capable of activating transcription of *ahpC2D* in response to oxidative stress.

# List of Abbreviations and Symbols Used

| | |
|---|---|
| ° | degree |
| μ | micro ($10^{-6}$) |
| Ω | Ohm |
| % | percent |
| ~ | approximately |
| ACES | 2-(2-amino-2-oxoethyl)-amino] ethanesulfonic acid |
| A | adenine |
| Ac | acrydite |
| ACDC | acrylamide capture of DNA-bound complexes |
| AhpC | alkyl hydroperoxide reductase |
| $Amp^R$ | ampicillin resistant |
| APS | ammonium persulfate |
| ATP | adenosine triphosphate |
| bp | base pair |
| BCYE | buffered charcoal yeast extract |
| BFA | brefeldin A |
| BSA | bovine serum albumin |
| BYE | buffered yeast extract |
| C | Celsius or cytosine |
| $Ca^{2+}$ | calcium |
| $Cat^S$ | chloramphenicol sensitive |
| c-di-GMP | Bis-(3'-5')-cyclic dimeric guanosine monophosphate |

| | |
|---|---|
| cDNA | complementary DNA |
| CHP | cumene hydroperoxide |
| CIP | calf intestinal phosphatase |
| COP | coatamer protein |
| cpm | counts per minute |
| Cys | cysteine |
| Cys-$S_P$H | peroxidatic cysteine |
| Cys-$S_R$H | resolving cysteine |
| CuZn | copper-zinc |
| Da | Dalton |
| DAG | diacylglycerol |
| dd$H_2O$ | double-distilled water |
| ddNTP | dideoxynucleoside triphosphate |
| DEPC | diethylpyrocarbonate |
| DMF | N, N,-dimethylformamide |
| DMSO | dimethylsulfoxide |
| DNA | deoxyribonucleic acid |
| DNaseI | deoxyribonuclease I |
| Dot | defect in organelle trafficking |
| dNTP | deoxynucleoside triphosphate |
| dsRNA | double-stranded RNA |
| DTT | dithiothreitol |
| EcOxyR | *E. coli* OxyR |

| | |
|---|---|
| EDTA | ethylenediaminetetraacetic acid |
| EHEC | enterohemorrhagic *E. coli* |
| EMSA | electrophoretic mobility shift assay |
| ER | endoplasmic reticulum |
| ERAD | endoplasmic reticulum associated degradation |
| ERGIC | endoplasmic reticulum intermediate compartment |
| f | femto ($10^{-15}$) |
| FBS | fetal bovine serum |
| FD | Farraday |
| Fe | iron |
| $Fe^{2+}$ | ferrous iron |
| $Fe^{3+}$ | ferric iron |
| fMLP | formyl-methionyl-leucyl-phenylalanine |
| Fur | ferric uptake regulator |
| g | gram |
| *g* | centrifugal force |
| G | guanine |
| $Gm^R$ | gentamicin resistant |
| GEF | guanine-nucleotide exchange factor |
| GFP | green fluorescence protein |
| Grx | glutaredoxin |
| GTP | guanosine triphosphate |
| h | hour |

| | |
|---|---|
| $H_2O_2$ | hydrogen peroxide |
| HEPES | 4-2-hydroxyethyl-1-piperazineethanesulfonic acid |
| $His_6$ | hexameric histidine tag |
| HOCl | hypochlorous acid |
| Icm | intracellular multiplication |
| IHF | integration host factor |
| IFN | interferon |
| IL | interleukin |
| iNOS | inducible nitric oxide synthase |
| $IP_3$ | inositol triphosphate or phosphatidylinositol triphosphate |
| IPTG | isopropyl-β-D-galactoside |
| k | kilo |
| $Km^R$ | kanamycin resistant |
| l | litre |
| LAM | lipoarabinomannan |
| LAMP | lysosome-associated membrane protein |
| LB | Luria-Bertani |
| LCV | *Legionella*-containing vacuole |
| LD | Legionnaires' disease |
| LLAP | *Legionella*-like amoebal pathogens |
| LpOxyR | *L. pneumophila* OxyR |
| LPS | lipopolysaccharide |
| LRR | leucine-rich repeat |

| | |
|---|---|
| m | meter or milli ($10^{-3}$) |
| M | molar |
| MAPK | mitogen-activated protein kinase |
| MEM | minimal essential media |
| MFS | major facilitator superfamily |
| Met | methionine |
| MH | Mueller-Hinton |
| MHC | major histocompatibility complex |
| MIF | mature intracellular form |
| min | minutes |
| Mip | macrophage infectivity potentiator |
| M-MuLV | Moloney Murine Leukemia Virus |
| Mn | manganese |
| MPO | myeloperoxidase |
| MOPS | 3-(N-morpholino) propanesulfonic acid |
| mRNA | messenger RNA |
| MsrA | methionine sufoxide reductase |
| $Mtz^R$ | metronidazol resistant |
| n | nano ($10^{-9}$) |
| N | normal |
| NADH | reduced nicotinamide adenine dinucleotide |
| NADPH | reduced nicotinamide adenine dinucleotide phosphate |
| NAD(P)H | NADH or NADPH |

| | |
|---|---|
| NBT | nitroblue tetrazolium |
| $Ni^{2+}$ | nickel |
| NO | nitric oxide |
| NLR | nucleotide-binding oligomerization domain (NOD)-LRR protein |
| NTA | nitriloacetic acid |
| $\bullet O_2^-$ | superoxide anion |
| OD | optical density |
| $OD_{600}$ | OD determined at 600 nm |
| $OD_{620}$ | OD determined at 620 nm |
| •OH | hydroxyl radical |
| $ONOO^-$ | peroxynitrite |
| $[^{32}P]$ | phosphor-32 |
| p | pico ($10^{-12}$) |
| $P_{ahpC1}$ | promoter region of *ahpC1* |
| $P_{ahpC2}$ | promoter region of *ahpC2* |
| $P_{OxyR}$ | promoter region of *oxyR* |
| PAMP | pathogen-associated molecular pattern |
| PBS | phosphate-buffered saline |
| PCR | polymerase chain reaction |
| PI | phosphatidylinositol |
| PI3K | phosphoinositide-3 kinase |
| PIP | phosphatidylinositol monophosphate |
| $PIP_2$ | phosphatidylinositol bisphosphate |

| | |
|---|---|
| PKC | protein kinase C |
| PLC | phospholipase C |
| PMA | phorbol 12-myristate 13-acetate |
| PMSF | phenylmethylsulphonylfluoride |
| $P_{oxyR}$ | promoter region of *oxyR* |
| PPIase | peptidyl-prolyl cis-trans isomerase |
| Prx | peroxiredoxin |
| qPCR | quantitative real-time PCR |
| RF | replicative form |
| rfu | relative fluorescence unit |
| RNA | ribonucleic acid |
| RNAP | RNA polymerase |
| RNase | ribonuclease |
| RNI | reactive nitrogen intermediate |
| ROI | reactive oxygen intermediate |
| rpm | revolutions per minute |
| RT | reverse transcriptase |
| RT-PCR | reverse transcriptase PCR |
| [$^{35}$S] | sulfur-35 |
| s | seconds |
| $Sac^R$ | sucrose resistant |
| SD | standard deviation |
| SDS | sodium dodecyl sulfate |

| | |
|---|---|
| SDS-PAGE | SDS-polyacrylamide gel electrophoresis |
| $Sm^R$ | streptomycin resistant |
| SNARE | soluble N-ethylmaleimide sensitive factor (NSF) attachment receptor |
| SOD | superoxide dismutase |
| -SOH | sulfenic acid |
| SPI | *Salmonella* pathogenecity island |
| T | thymine |
| T2SS | type II secretion system |
| T3SS | type III secretion system |
| T4SS | type IV secretion system |
| TAE | Tris acetate EDTA |
| TBE | Tris borate EDTA |
| tBOOH | *tert*-butyl hydroperoxide |
| TCA | tricarboxylic acid |
| TE | Tris EDTA |
| TEMED | N, N, N', N'-tetramethylethylenediamine |
| TLR | Toll-like receptor |
| TNF | tumor necrosis factor |
| TR | thioredoxin reductase |
| Trx | thioredoxin |
| U | units |
| V | volt |
| V-ATPase | vacuolar ATPase |

| | |
|---|---|
| VBNC | viable but non-culturable form |
| v/v | volume per volume |
| w/v | weight per volume |
| X-gal | 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside |

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## Chapter 1: Introduction

*Legionella pneumophila* is an intracellular pathogen responsible for most cases of Legionnaires' disease world wide (Frazer *et al.*, 1977; Horwitz, 1983 a and b). In natural environments, *L. pneumophila* multiplies in protozoa and acquires traits that increase its virulence (Cirillo *et al.*, 1994; Fields *et al.*, 2002; Greub and Raoult, 2004). In fact, *L. pneumophila* displays a developmental cycle in protozoa and some mammalian cell lines, where the organisms differentiate into metabolically dormant cyst-like forms that enable bacteria to survive for extended periods in a highly infectious state (Garduno *et al.*, 2002; Greub and Raoult, 2004). Transmission to susceptible humans occurs *via* inhalation of *Legionella*-contaminated aerosols where *L. pneumophila* infects alveolar macrophages (Fields *et al.*, 2002). By virtue of virulence factors such as the Dot/Icm type IVB secretion system, *L. pneumophila* avoids lysosomal degradation by generation of a replication-permissive endosome that fails to mature *via* the endocytic pathway (Berger and Isberg, 1993; Horwitz 1983 a and b; Marra *et al.*, 1992). Evasion of phagolysosomal fusion is a particularly apt strategy to avoid being consumed by protozoa or killed by human alveolar macrophages. However, the intracellular lifestyle of *L. pneumophila* likely exposes the organism to numerous stresses. Factors that enable *L. pneumophila* to respond to pH, nutrient starvation, osmotic shock, heat and oxidative stress are of particular interest to fully understand how the pathogen adapts to conditions faced in the intracellular milieu of protozoa or macrophages (Bachman and Swanson, 2001; Hales and Shuman, 1999; Hammer *et al.*, 2002; Lynch *et al.*, 2003).

Since some evidence suggests that *L. pneumophila* might be faced with an oxidative burst following ingestion by macrophages or protozoa (Davies *et al.*, 1991;

Halablab *et al.*, 1990; Jacobs *et al.*, 1994), protection against reactive oxygen intermediates (ROIs) might be crucial for pathogenesis. By preventing damage by ROIs during the oxidative burst, antioxidant enzymes like superoxide dismutases (SODs), catalases, and alkyl hydroperoxide reductases (AhpCs) confer protection to phagocytosed bacteria (Braunstein *et al.*, 2003; De Groote *et al.*, 1997; Gee *et al.*, 2005; Krishnakumar *et al.*, 2004; Master *et al.*, 2002; Manca *et al.*, 1999; Piddington *et al.*, 2001). It has been proposed that the *L. pneumophila* KatA and KatB catalase-peroxidases might provide catalase (hydroperoxidase) activity to detoxify the phagosomal milieu to promote intracellular growth (Bandyopadhyay and Steinman, 1998 and 2000). However, *katA*, *katB*, and *katA/katB* mutants are as sensitive as the wild-type strain when challenged with hydrogen peroxide ($H_2O_2$) (Bandyopadhyay and Steinman, 1998 and 2000). As AhpCs have been shown to be the primary scavengers of peroxides in *E. coli* (Seaver and Imlay, 2001), the two *ahpC* genes identified in the *L. pneumophila* genome are likely to encode proteins displaying similar functions. Previous studies indicated that AhpC1 levels are up-regulated during intracellular growth of *L. pneumophila* in macrophages (Rankin *et al.*, 2002) and *ahpC2* and *ahpD* mRNA levels are up-regulated during intracellular growth in protozoa (Brüggemann *et al.*, 2006). However, peroxide-scavenging functions of neither AhpC1 nor the AhpC2D system have been evaluated.

We initiated this study to characterize both *ahpC1* and the *ahpC2/ahpCD* system. The aims for this study were to determine if the AhpC enzymes are responsible for peroxide scavenging in *L. pneumophila* and to determine if they could detoxify ROIs in the phagosomal milieu of macrophages. Furthermore, since no regulators have yet been implicated in the resistance to oxidative stress in *L. pneumophila*, and OxyR has been

shown to activate transcription of *ahpC* in response to oxidative stress in other organisms (Charoenlap *et al.*, 2005; Ochsner *et al.*, 2000; Loprasert *et al.*, 2003; Mongkolsuk *et al.*, 2000), we investigated whether the *L. pneumophila* OxyR homolog identified in the *L. pneumophila* genome could play a functionally equivalent role.

## Chapter 2: Literature Review

*L. pneumophila*, the causative agent of Legionnaire's disease, has received much attention since the first documented outbreak in 1976. In natural environments, *L. pneumophila* survives and multiplies as an intracellular parasite of freshwater protozoa. When transmitted by aerosols to susceptible humans, this facultative intracellular parasite replicates in alveolar macrophages causing a potentially lethal pneumonia. Since the pioneering work by Marcus Horwitz in the early 1980s, the cell biology of the infection process has been mostly dissected. Numerous virulence factors have been associated with pathogenesis, yet the Dot/Icm type IV secretion system (T4SS) has been in the spotlight since the early 1990s. The sum of virulence traits eventually leads to lysis of alveolar macrophages and causes a destructive pulmonary inflammation where clinical manifestations ensue. Over the past 30 years, work undertaken in countless laboratories has enhanced our understanding of the ecology and pathogenesis of this respiratory pathogen.

### 2.1. Legionellosis

Legionellosis classically presents as one of two distinct clinical forms: a mild flu-like illness called Pontiac fever (Glick *et al.*, 1978), and Legionnaire's disease (LD), a more severe multi-system infection which includes pneumonia (Fraser *et al.*, 1977). Both are caused by Gram-negative intracellular pathogens of the *Legionella* family (Fields *et al.*, 2002). *L. pneumophila* is the predominant cause of legionellosis in humans, accounting for ~ 80-90% of all reported cases in the United States with a predominance for serogroup 1 (Fields *et al.*, 2002). Pontiac fever was first documented in 1968 in Pontiac

Michigan, but *L. pneumophila* was only identified as the etiological agent ten years after the fact (Glick *et al.*, 1978). Infected individuals displayed mild symptoms such as malaise and headaches, and usually recovered without hospitalization within five days (Glick *et al.*, 1978). In contrast, LD develops within 2 to 10 days after initial exposure to legionellae; with more severe symptoms that may include fever, muscle aches, chest pain, dry cough, abdominal pain, diarrhea, and neurological problems (Fraser *et al.*, 1977). The first documented outbreak of LD occurred in 1976 in and around the Bellevue Stratford Hotel in Philadelphia, which was hosting a convention of the Pennsylvania Division of the American Legion (Brenner *et al.*, 1979; Fraser *et al.*, 1977). Of attending members, numerous individuals contracted the disease and 34 people died (Fraser *et al.*, 1977).

A connection between aerosols and human disease was not figured out until after an outbreak in Memphis, Tennessee in 1978 that involved a contaminated evaporative condensor that led to a hospital-wide infection. Legionella is ubiquitous in aquatic environments from which aerosolization and transmission by inhalation to susceptible humans is well established. Contaminated water sources include aerosol-generating devices such as cooling towers, showers and hospital ventilators (Fields *et al.*, 2002). No person-to-person transmission has been documented. Prevention strategies against LD include maintenance of aerosol-generating water systems under conditions that minimize the likelihood of transmission, such as the use of chemical biocides (such as monochloramine or chlorine dioxide), copper-silver ionization, or thermal control of the water (Fields *et al.*, 2002; Thomas *et al.*, 2004). However, association of legionellae with protozoa has been linked to failure in these measures.

The mortality rate for LD ranges from 5-30%, with a higher percentage occurring during nosocomial outbreaks among individuals diplaying risk factors for LD, including age (usually over 65), smoking, immunocompromising disease and immunosuppressive drugs (Fields *et al.*, 2002). In addition, individuals that exibit mutation in Toll-like receptor-five (TLR5), a receptor that recognizes flagellin, correlated with increased susceptibility to the disease (Hawn *et al.*, 2005). The estimated annual prevalence of LD is 8,000-18,000 cases in the United States each year, a figure that may be grossly underestimated since many infections go unreported. Accurate diagnosis requires primary islation of *L. pnumophila* on specialized media, serologic diagnosis by antigen-specific immune response, and nucleic acid amplification techniques that are not routinely performed on persons suffering from pneumonia (Fields *et al.*, 2002).

### 2.1.1. Diagnosis

Post-mortem human lung tissue was used to infect guinea pigs and spleen homogenates were inoculated into embryonated yolk sacs, resulting in the identification of *L. pneumophila* (Brenner *et al.*, 1979; McDade *et al.*, 1977). Early attempts to culture legionellae on common laboratory media such as Mueller-Hinton (MH) agar were unsuccessful (Feeley *et al.*, 1979). Addition of haemoglobin and IsoVitaleX permitted growth, resulting in the identification of soluble iron and L-cysteine as essential components of the Feeley-Gorman agar (Feeley *et al.*, 1979). Routine laboratory culture of virulent *L. pneumophila* was made possible using charcoal yeast extract agar in which starch was replaced with charcoal and essential amino acids were provided in yeast extract (Feeley *et al.*, 1979). Iron salts like ferric pyrophosphate establish equilibrium between cysteine and the oxidized dipeptide cystine, maintaining a steady-state level of

cysteine that promotes growth (Ewann and Hoffman, 2006). The α-ketoglutaric acid also serves as a primary carbon source for the organism (Tesh and Miller, 1981). Further refinements like addition of ACES buffer and charcoal led to the currently used buffered charcoal-yeast extract (BCYE) medium (Pascule *et al.*, 1980). Charcoal, α-ketoglutaric acid, and cysteine were shown to scavenge reactive oxygen intermediates (ROIs) generated in the medium during aerobic growth (Hoffman *et al.*, 1983; Pine *et al.*, 1986).

Legionellae can be identified by growth on BCYE from respiratory fluids by its unique requirement for L-cysteine (except for *L. oakridgensis* and *L. spiritensis*). Other confirmatory techniques must be implemented such as serological methods including direct fluorescent antibody (DFA), enzyme immunoassays (EIAs), and enzyme-linked immunosorbant assays (ELISAs) as well as genetic-based techniques including polymerase chain reaction (PCR), real-time PCR, and DNA sequencing (Fields *et al.*, 2002, Loens *et al.*, 2006). 16S rRNA analysis confirmed the *Legionellaceae* family as a monophyletic subgroup of γ-*Proteobacteria*, consisting of a single genus *Legionella* with 48 species (Fields *et al.*, 2002). Serotyping led to 70 serogroups in the *Legionellaceae* family, 15 of which were identified for *L. pneumophila* (Fields *et al.*, 2002). The nearest relative that shares distinct evolutionary relatedness with *Legionella* is *Coxiella burnetii*, an intracellular pathogen causing Q fever. Comparative genomics of their genomes revealed ~ 42% sequence similarity, including several known and putative virulence factors (Seshadri *et al.*, 2003; Seshadri and Samuel, 2005). A number of bacteria closely related to *Legionella* spp., the *Legionella*-like amoebal pathogens (LLAPs), have now been added to the *Legionellaceae* family. Though infrequent, these obligate intracellular organisms have been shown to cause pneumonia (Marrie *et al.*, 2001).

### 2.1.2. Immunity and Treatment

In the human lung, *L. pneumophila* grows within and kills alveolar macrophages and epithelial cells, resulting in a pneumonia that is highlighted by alveolar infiltration of erythrocytes, macrophages, and neutrophils and subsequent edema by capillary leakage (Winn *et al.*, 1982). Innate and adaptive immune responses play a role in clearance of *L. pneumophila*. First, Toll-like recptors (TLRs) on the surface of host cells are able to recognize *L. pneumophila* to stimulate pro-inflammatory cytokine release by neutrophils, macrophages, and dendritic cells, which in turn recruit lymphocytes and activate macrophages (Neild and Roy, 2004). For example, recognition of *L. pneumophila* flagellin by TLR5 on the surface of natural killer cells leads to downstream activation of NF-κB and production of the pro-inflammatory cytokines like gamma interferon (IFN-γ) (Spörri *et al.*, 2006). IFN-γ is known to activate macrophages to restrict *L. pneumophila* growth by increasing ROI production and iron starvation (Byrd and Horwitz, 2000), resulting in a *Legionella*-containing vacuole (LCV) that fails to evade the endocytic pathway (Santic *et al.*, 2005). Secondly, dendritic cells are antigen-processing cells that restrict *L. pneumophila* growth and are able to prime naïve T cells through peptides presented on their major histocompability complex (MHC) class II molecules. Stimulation of lymphocytes by class II MHC leads to antibody production (Neild and Roy, 2004). Individuals who seroconvert (produce anti-*Legionella* antibodies) will overcome *L. pneumophila* infection, presumably due to enhanced uptake and destruction in neutrophils or activated macrophages (Neild and Roy, 2004). However, antibody production may not be protective in all cases (Weeratne *et al.*, 1994). For patients displaying risk factors, delay of appropriate therapy can result in prolonged

hospitalization, complications, and death. Erythromycin, the former drug of choice, has now been replaced by another macrolide (azithromycin) and/or a quinolone (Pedro-Botet and Yu, 2006).

## 2.2. The Microbial Ecology of *L. pneumophila*

With the exception of *L. longbeachae* which has been found in potting soil, legionellae are ubiquitous aquatic organisms (Fields *et al.*, 2002). Legionellae are able to survive for extended periods of time in aquatic biofilms (Rogers *et al.*, 1994) where they fall prey to grazing amoebae (Greub and Raoult, 2004). Bacterial-protozoan interactions not only represent a shelter against stresses, but serve as a niche for replication and selection of virulence traits that prime the pathogen for human infection (Greub and Raoult, 2004).

### 2.2.1. Association with Biofilms

Biofilms provide nutrients and shelter from environmental stresses and thus could provide a suitable environment for persistence (Fields *et al.*, 2002). *L. pneumophila* can survive for extended periods of time as a viable but non-culturable (VBNC) form; however, it is less clear whether the bacteria replicate in biofilms without protozoa (Steinert *et al.*, 1997). Recent data by Temmerman *et al.* (2006) suggested that *L. pneumophila* was able to feed (by necrotrophy) on heat-killed microbial cells present in biofilms or water systems. However, these findings should be interpreted with caution, since most legionellae have an absolute requirement for cysteine (Pine *et al.*, 1979). In the aerobic biofilms cysteine would be oxidized to cystine, which can not be assimilated by the organism (Ewann and Hoffman, 2006). Cysteine auxotrophy likely prevents cyst

germination in natural environments, whereas this essential amino acid would be fully available in protozoa (Ewann and Hoffman, 2006).

### 2.2.2. *L. pneumophila*, Protozoa, and Human Transmission

Aquatic protozoa feed by phagocytosis, where bacteria are engulfed and digested in phagolysosomes yet amoeba-resistant bacteria (ARB), like *L. pneumophila* and others, have evolved mechanisms to survive and replicate in host cells (Greub and Raoult, 2004). To date, *L. pneumophila* has been found to replicate in numerous amoebae, and ciliates, and slime mold (Abu Kwaik *et al.*, 1998; Fields *et al.*, 2002; Steinert *et al.*, 2002). *Acanthameobae castellanii* has been shown to resuscitate *L. pneumophila* from its environmental VBNC state, revealing important ecological significance (Steinert *et al.*, 1997). Human disease is thought to arise from alterations in the aquatic environment that favor protozoa grazing, leading to rapid multiplication of legionellae (Fields, 1996). Growth within protozoa protects *L. pneumophila* from chlorination, which may explain why elimination of legionellae from water systems is so difficult. Indeed, continuous treatment with chlorine dioxide was identified as the most efficient method for controlling *L. pneumophila* in water systems; however, presence of protozoa resulted in quick re-colonization by *L. pneumophila* (Thomas *et al.*, 2004). Rowbotham (1986) also suggested that *Legionella*-infected amoebae may represent an important vehicle for human transmission (see Figure 1a). This hypothesis was confirmed in a murine model where co-infection of *L. pneumophila* and protozoa resulted in a more severe pneumonia than with either organism alone (Brieland *et al.*, 1996). Rowbotham (1986) also suggested that *Legionella*-filled vacuoles could represent a transmissible form (see Figure 1b). In *A. castellanii* or *polyphaga*, *L. pneumophila* were expelled in vesicles that could

easily be inhaled ("respirable") to deliver a substantial dose of organisms (Berk *et al.*, 1998). The release of vesicles from protozoa was recently attributed to two proteins, LepA and LepB (Chen *et al.*, 2004), that were translocated into host cells by the Dot/Icm system. Finally, intracellular growth in environmental protozoa is known to select for traits promoting environmental survival (Greub and Raoult, 2004; Rowbotham, 1986) such as resistance to biocides, cold, antibiotics, or traits required for the intracellular lifestyle within human macrophages (Brüggemann *et al.*, 2006; Greub and Raoult, 2004). *L. pneumophila* is now known to differentiate into a highly infectious cyst-like form following intracellular growth in protozoa (Cirillo *et al.*, 1994), which may also represent a transmissible form (see Figure 1c). Human transmission is thought to arise from the "accidental" encounter with alveolar macrophages following inhalation of contaminated aerosols (Swansson and Hammer, 2000).

### 2.3. Developmental Cycle of *L. pneumophila*

Developmental cycles have been observed in numerous organisms. For example, *C. burnetii* alternates between a replicative large-cell variant and the environmentally resistant small-cell variant (Samuel *et al.*, 2003). Similarily, chlamydiae differentiate from the replicative reticulate bodies to the environmentally resilient infectious elementary bodies (Hammerschlag, 2002). The differentiation of *L. pneumophila* in protozoa and mammalian cells has been well documented. The first hints of a developmental cycle for *L. pneumophila* were morphological differences reported by Rodgers (1979), where media-grown bacteria were predominently bacilli and intracellular bacteria spanned a spectrum of forms ranging from bacilli to cocci. The pioneering work of Rowbotham (1986) established that during transit in protozoa, *L. pneumophila*

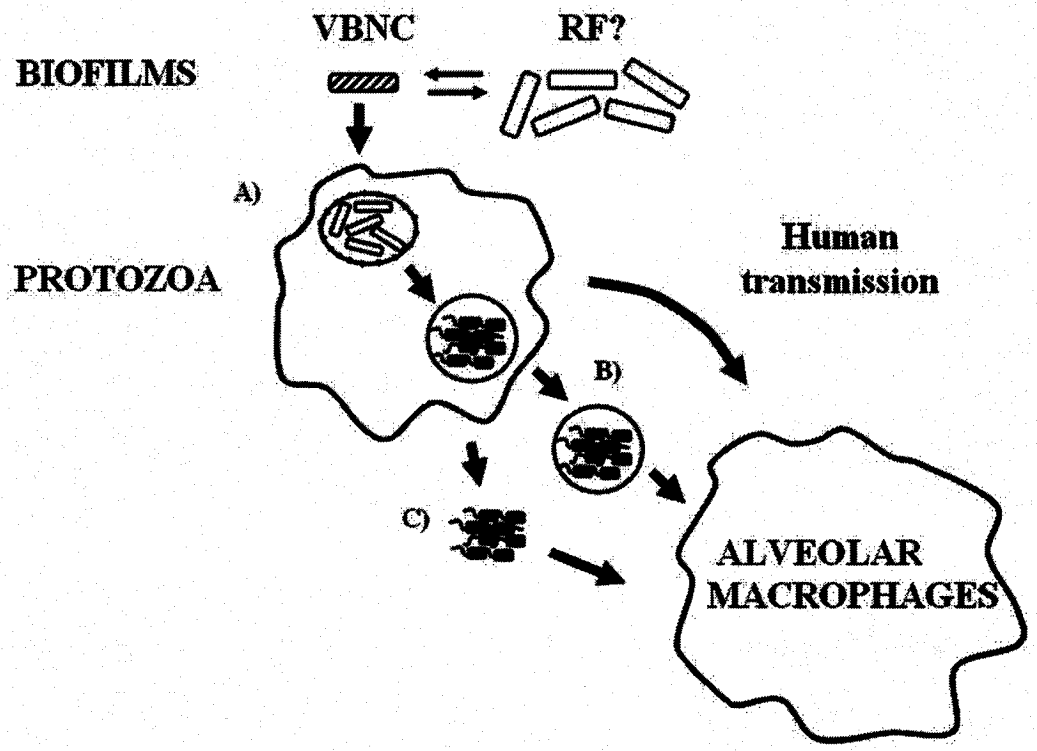



**Figure 1** Transmission of *L. pneumophila*. In biofilms, *L. pneumophila* resides as a viable but non-culturable (VBNC) form which may or may not be able to transform into a replicative form (RF). Grazing by protozoa is known to revive the VBNC form of *L. pneumophila*. Transmission to humans could be mediated by inhalation of aerosols containing: A) intra-protozoan *L. pneumophila*; B) vesicles containing *L. pneumophila* that have been shed by protozoa; or C) a highly infectous transmissible form of the organism that arises following intracellular growth in protozoa.

alternates between two morphologically distinct forms differing in shape, motility, surface-protein expression, and storage of energy-rich polymers. Cirillo *et al.* (1994) reported that amoebae-grown bacteria accumulate inclusions of poly β-hydroxybutyric acid (PHBA) and display a thicker cell wall than bacteria grown in vitro. A significant finding by this group was that amoebae-grown *L. pneumophila* were more invasive for epithelial cells and macrophages than were laboratory media-grown bacteria. Direct correlation of morphological changes to a developmental cycle was finally revealed by ultrastructural analysis using a HeLa cell model (Faulkner and Garduño, 2002; Garduño *et al.*, 1998 and 2002a and b). Throughout the infection of host cells, *L. pneumophila* was shown to alternate between a replicative form (RF) and a metabolically dormant, highly infectious cyst-like form termed the mature intracellular form (MIF) (Garduño *et al.*, 2002). The stage-specific transition was emphasized by the various intermediate (RF-to-MIF or MIF-to-RF) morphological forms (Faulkner and Garduño, 1998). RFs and MIFs could be distinguished by Giménez staining, where MIFs retained the carbol fuschin red color (Giménez-positive) whereas RFs, being unable to retain the stain, stained green by the malachite green (Giménez-negative) counterstain (Garduño *et al.*, 2002). BCYE-grown *L. pneumophila* also displayed some phenotypic characteristics of differentiation during stationary phase such as Giménez-positive staining (Garduño *et al.*, 2002); however, stationary-phase bacteria never fully differentiated to MIFs (Garduño *et al.*, 2002). Compared to RFs, MIFs were metabolically dormant and displayed numerous ultrastructural differences, including PHBA inclusions, a thickened cell-wall architecture, and multi-laminated intracytoplasmic membranes, which were all reflected by increased resistance to various stresses (Garduño *et al.*, 2002). It should be noted that due to early

demise of the host, *L. pneumophila* does not differentiate into MIF-like forms in infected macrophages, which may partly explain why LD is not a communicable disease (Garduño *et al.*, 2002). Nonetheless, *L. pneumophila* progeny arising from intracellular growth in macrophages express numerous transmission traits that could facilitate their uptake by other phagocytes (Molofsky and Swanson, 2004).

Growth cycle-dependent phenotypic changes are thought to occur by radical changes in protein expression (Abu Kwaik *et al.*, 1998a and b). For example, the MIF-associated gene A (MagA) has been identified to be predominantly expressed during MIF morphogenesis and serves as a marker for development (Hiltz *et al.*, 2004). Further evidence supporting the developmental cycle of *L. pneumophila*, Brüggemann *et al.* (2006) used a microarray comprised of all genes found in the *L. pneumophila* genome to monitor the transcriptome of *L. pneumophila* during an infection of *A. castellanii*. These data showed that the expression of several hundred *L. pneumophila* genes were up- or down-regulated following the intracellular life cycle in protozoa (Brüggemann *et al.*, 2006). Genes associated with the replicative phase included those encoding factors that promote replication such as putative amino acid transporters, enzymes of the tricarboxylic acid (TCA) cycle, components of the electron transport chain, and some virulence associated factors like CsrA (Brüggemann *et al.*, 2006). Eventually, as nutrients and oxygen became scarce, genes involved in the replicative phase were down-regulated and those involved in the transmissive phase were up-regulated, including genes encoding Dot/Icm-secreted effector proteins, EnhABC, RtxA, LetE, FliA, integration host factor (IHF), several uncharacterized transcriptional regulators, and two-component systems (Brüggemann *et al.*, 2006). A family of regulators possessing a

GGDEF or EAL motif, termed GGDEF/EAL was also up-regulated exclusively during the transmission phase in vivo (Brüggemann *et al.*, 2006). Members of this family are known to regulate the transition between motile and sessile bacteria found in biofilms when nutrients are scarce (Römling and Amikam, 2006). This process is regulated through diguanylate cyclase and phosphodiesterase activities of GGDEF/EAL proteins, which control intracellular concentrations of bis-(3', 5')-cyclic dimeric guanosine monophosphate (c-di-GMP) (Römling and Amikam, 2006). In *S. typhimurium* mutation in an EAL-domain protein influencing c-di-GMP levels has been linked to the ability of the organism to kill macrophages and resist to peroxides (Hisert *et al.*, 2005). Though showing some promising avenues, no further work has yet been performed on GGDEF/EAL proteins in *L. pneumophila*. Overall, the *L. pneumophila* microarray has revealed differential regulation between the replicative and transmissive phases of growth in protozoa (Brüggemann *et al.*, 2006). However, these data should be interpreted with caution, since protein levels may not always correlate with the transcriptome due to post-trancriptional regulation known to occur in *L. pneumophila*. A more logical approach to identify proteins involved in *L. pneumophila* differentiation includes proteomics, which has previously been used (Morash, 2006) and is the subject of current studies.

### 2.3.1. Genetic Control of Differentiation

Over the years, a model has been established where cellular differentiation of *L. pneumophila* is dictated by nutrient supply (Molofsky and Swanson, 2004). *L. pneumophila* expresses transmission traits when nutrients are limiting and replication traits when growth conditions are favorable (Byrne and Swanson, 1998). By analogy to the stringent response in *E. coli*, when *L. pneumophila* enters post-exponential phase, the

limiting amino acids lead to uncharged transfer RNAs (tRNAs) that are detected by the ribosome-associated RelA synthase, converting guanosine triphosphate (GTP) to guanosine 3'-5'-bipyrophosphate (ppGpp) (Hammer and Swanson, 1999). Accumulation of the ppGpp alarmone activates a regulatory cascade involving the stationary-phase sigma factor (RpoS, $\sigma^S$ or $\sigma^{38}$), the flagellar sigma factor FliA ($\sigma^{28}$), the two-component regulatory system LetA/S, and its co-activator LetE (Molofsky and Swanson, 2004). The current model suggests that the role of LetA/S is to de-repress transmission traits that are repressed by CsrA (Molofsky and Swanson, 2004). Altogether, the stringent response leads to the expression of transmission traits such as motility, cytotoxicity, stress resistance, and surface composition modification (Molofsky and Swanson, 2004). Though the stringent response model was initially proposed on data obtained using broth culture (Byrne and Swanson, 1998), similar traits have been documented during intracellular growth in protozoa and macrophages (Hammer and Swanson, 1999; Molofsky and Swanson, 2004). If nutrient supply is adequate, the organisms exit lag phase, down-regulate transmission traits, and convert to replicative forms (Molofsky and Swanson, 2004). In fact, the repressor of transmission traits CsrA is expressed exclusively during replication (Molofsky and Swanson, 2003). In contrast, *L. pneumophila* transmission traits such as Mip, the T4SS components/effectors (DotO, DotH, RalF, LidA, and SidC), and contact-dependent cytotoxicity are repressed during intracellular replication, but are expressed during entry and exit phases (Molofsky and Swanson, 2004). Therefore, once the nutrient supply is consumed, *L. pneumophila* coordinates expression of transmission traits that promote escape from its host, stress

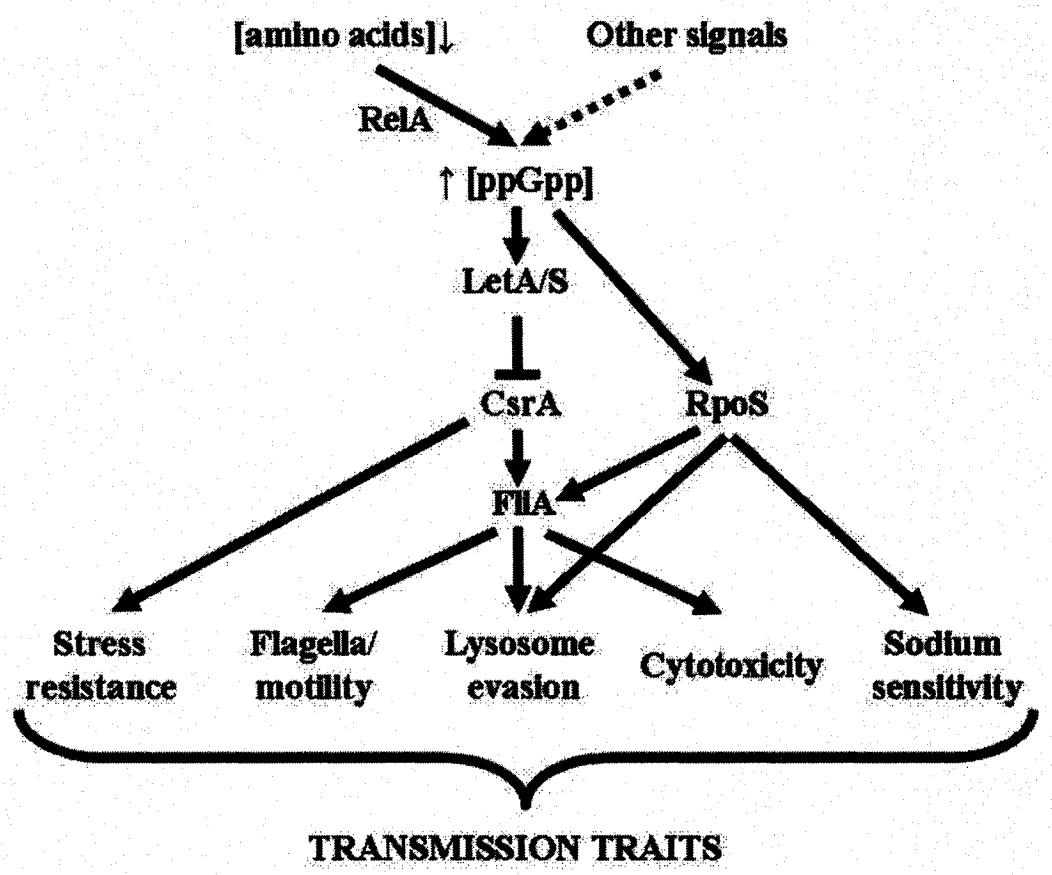



**Figure 2** Schematic represention of the stringent response model. Under starvation conditions, a panel of transmission traits is expressed. Limiting nutrients trigger RelA to produce the alarmone ppGpp, which in turn stimulates LetA/S, RpoS, and FliA. Active LetA is thought to relieve post-transcriptional repression by CsrA, presumably by producing a small *csrB*-like regulatory RNA to sequester CsrA (Molofsky and Swanson, 2004).

resistance, and factors that would enable infection of a new phagocyte (Molfsky and Swanson, 2004).

In *E. coli*, ppGpp regulates transcription by biasing the competition between sigma factors for RNA polymerase (RNAP) (Jishage *et al.*, 2002). Indeed, ppGpp seems to destabilize interactions between RNAP and the "housekeeping" sigma factor $\sigma^{70}$ (RpoD or $\sigma^{D}$), which are replaced with the alternative sigma factors. Differentiation of *L. pneumophila* to a transmissible form is thought to require both RpoS and FliA sigma factors (Molofsky and Swanson, 2004). Expression of transmission traits by *L. pneumophila* has previously been correlated with an accumulation of ppGpp and expression of RpoS (Byrne and Swanson, 1998; Hales and Shuman, 1999a and b). RelA and RpoS appear to be required for flagellin expression and intracellular growth in protozoa, but not in macrophages (Bachman and Swanson, 2001; Zusman *et al.*, 2002). Unlike the situation in *E. coli*, *L. pneumophila rpoS* transcripts are abundant in exponential phase (Bachman *et al.* 2004), suggesting that RpoS may play a more important role during replication. *E. coli* RpoS mediates the expression of catalase (KatE) in stationary-phase, yet this type of response was not attributed to *L. pneumophila* RpoS (Bachman and Swanson, 2001; Hales and Shuman, 1999). FliA is required not only for motility (Fettes *et al.*, 2001; Heuner *et al.*, 2002), but for additional transmission traits including contact-dependent cytotoxicity and lysosome evasion (Hammer *et al.*, 2002; Heuner *et al.*, 2002; Molofsky *et al.*, 2005). By analogy to what is seen for *E. coli* where flagellation is co-regulated with lipopolysaccharide (LPS) modification, FliA could induce lysosome avoidance by alteration of surface components, such as the modification of *L. pneumophila* LPS (Fernandez-Moreira *et al.*, 2006).

Post-transcriptional control by LetA/S and CsrA is thought to play a pivital role in virulence (Molofsky and Swanson, 2004). The "*Legionella* transmission activator and sensor" proteins (LetA and LetS, respectively) form a two-component regulatory system that functions with the co-activator LetE to induce many transmission traits in response to the alarmone ppGpp (Bachman and Swanson, 2004b; Hammer *et al.*, 2002; Lynch *et al.*, 2003). Indeed, *letA* mutant strains are unable to express transmission traits (Molofsky and Swanson, 2004). LetA has recently been implicated in the regulation of Mip (probably MagA), DotA and RalF (Shi *et al.*, 2006) and factors that could promote egress from the host cell (Broich *et al.*, 2006). Recent models suggest that the role of ppGpp-activated LetA is to relieve the post-transcriptional repression of the transmission phenotypes mediated by the carbon-storage regulator A (CsrA) (Molofsky and Swanson, 2003). CsrA is an RNA-binding protein that is generally necessary for post-transcriptional control of stationary-phase physiology (Romeo, 1998). In *E. coli*, CsrA binds the Shine-Dalgarno sequences (ribosome binding sites) to stabilize mRNA transcripts and prevent translation (Romeo, 1998). In *L. pneumophila*, CsrA is expressed during exponential growth phase and was shown to repress a panel of transmission traits such as stress resistance, flagellation, contact-dependent cytotoxicity, coccoid morphology, lysosome evasion, intracellular growth, and increased expression of *rpoS*, *letE*, and *fliA* (Fettes *et al.*, 2001; Forsbach-Birk *et al.*, 2004; Hammer *et al.*, 2002; Heuner *et al.*, 2002; Molofsky and Swanson, 2003 and 2004). Therefore, CsrA-mediated repression must be alleviated during transmission phase and restored during the replicative phase (Molofsky and Swanson, 2003 and 2004). At the end of replication,

accumulation of ppGpp is thought to activate LetA/S and mediate de-repression of CsrA, thereby promoting expression of transmission traits (Molofsky and Swanson, 2004).

### 2.3.2. Downfalls of the Stringent Response Model

There is an obvious disparity between involvement of the stringent response and the RpoS signalling cascade triggering *L. pneumophila* virulence traits. RelA, RpoS and LetA have all been shown to be required for intracellular growth in amoebae, but are completely dispensable for growth in macrophages (Bachman and Swanson, 2001; Gal-Mor and Segal, 2003; Hales and Shuman, 1999a and b). Furthermore, a *relA letA* double mutant was shown to display similar growth kinetics to that of wild-type *L. pneumophila* in macrophages, suggesting that transmission traits may be induced by signals that bypass both RelA- and LetA-dependent pathways (Hammer *et al.*, 2002; Zusman *et al.*, 2002). Abu-Zant *et al.* (2006) highlighted discrepancies in the stringent response model by demonstrating that RpoS, but not RelA, was required for intracellular growth in human macrophages and in *A. polyphaga*, and for pore-forming activity, phagosome biogenesis, and phagosomal adaptation. Other evidence suggests flaws in the stringent response model, including the lack of *L. pneumophila csrB* and *csrC* RNAs which are needed to inhibit the activity of the CsrA in *E. coli* (Weilbacher *et al.*, 2003). It is possible that effective monitoring of the *L. pneumophila* microenvironment and coordination of differentiation are not solely governed by limiting amino acids.

## 2.4. *L. pneumophila* Pathogenesis

After dissemination from environmental protozoa, *L. pneumophila* invades and replicates within human alveolar macrophages, monocytes, and alveolar epithelial cells

(Goa *et al.*, 1998). After phagocytosis, the facultative intracellular pathogen *L. pneumophila* is found within a membrane-bound compartment, termed the *Legionella*-containing vacuole (LCV), which rapidly diverges from the endosomal-lysosomal pathway (Horwitz, 1983). The LCV undergoes neither phagosome-lysosomal fusion nor acidification (Horwitz and Maxfield, 1984; Sturgill-Koszycki and Swanson, 2000). Instead, organelles like mitochondria and small vesicles derived from the endoplasmic reticulum (ER) are recuited to the LCV (Horwitz, 1983; Swanson and Isberg, 1995; Tilney *et al.*, 2001). Biogenesis of the LCV involves interception of secretory vesicles from the ER (Kagan and Roy, 2002; Robinson and Roy, 2006), which allows the organisms to remodel the LCV to resemble an ER-like vacuole (Horwitz, 1983; Swanson and Isberg, 1995; Tilney *et al.*, 2001; Kagan and Roy, 2002). The LCV becomes studded with ribosomes, where replicating bacteria can be found (Katz and Hashemi, 1982; Li *et al.*, 2005; Lu and Clark, 2005). *L. pneumophila* eventually escapes from the LCV and continues to replicate in the cytoplasm (Molmoret *et al.*, 2004). Egress from host macrophages is thought to occur by apoptosis (Gao and Abu Kwaik, 1999) and necrosis induced by pore formation in the host plasma membrane (Alli *et al.*, 2000; Gao and Abu Kwaik, 2000). Current views from *L. pneumophila* uptake to egress are addressed below after describing the most studied virulence determinant, the Dot/Icm type IV secretion system (T4SS).

### 2.4.1. The *L. pneumophila* Type IV Secretion System

*L. pneumophila* contains two T4SSs, yet only the Dot/Icm system is known to play a crucial role in virulence (Berger and Isberg, 1993; Marra *et al.*, 1992; Segal *et al.*, 1999). The Dot/Icm T4SS of *L. pneumophila* was discovered simultaneously by two

independent laboratories (Berger and Isberg, 1993; Marra *et al.*, 1992) and is now known to be encoded by 26 genes designated *dot* for "defect in organelle trafficking" or *icm* for "intracellular multiplication" (Sexton and Vogel, 2002). Phagosomes containing *dot/icm* mutants fail to evade the endocytic pathway and quickly acquire endosome markers such as the lysosomal-associated membrane protein (LAMP)-1, the vacuolar $H^+$-ATPase (V-ATPase), and the small GTPase Rab5 (Clemens *et al.*, 2000; Coers *et al.*, 1999; Lu and Clarke, 2005). Though the global architecture of the Dot/Icm system has not yet been elucidated, the macromolecular complex is thought to act as molecular syringe to inject effectors into the host cell to modulate host cell functions normally occurring within minutes of bacterial internalization (Coers *et al.*, 1999; Roy *et al.*, 1998; Wiater *et al.*, 1998). T4SSs have been used to mediate transfer of proteins into host cells, such as the export of the pertussis toxin by *B. pertussis* and CagA by *H. pylori*, respectively (Christie, 2001). Over fifty proteins have now been identified as putative effectors for the *L. pneumophila* Dot/Icm system.

The "recruitment of ARF to *Legionella*-phagosome factor (RalF)" was the first characterized Dot/Icm substrate (Nagai *et al.*, 2002) and functions as a guanine-nucleotide exchange factor (GEF), exchanging GDP for GTP to activate the small GTPase Arf1 (Amor *et al.*, 2005; Nagai *et al.*, 2002). Indeed, Arf1 was shown to be recruited to the LCV by Dot/Icm-dependent secretion of RalF (Nagai *et al.*, 2002), and interfering with Arf1 activity abrogates LCV biogenesis (Kagan and Roy, 2002; Derré and Isberg, 2004; Kagan *et al.*, 2004). Overexpression of RalF in a yeast model caused drastic growth defects (Campodonico *et al.*, 2005). Surprisingly, *ralF* mutants are still

capable of evading the endocytic pathway to generate the LCV in protozoa and macrophages, despite absence of RalF-mediated recruitment of Arf1 (Nagai *et al.*, 2002).

Like RalF, other Dot/Icm-secreted proteins also localize to the cytoplasmic face of the LCV in macrophages, including the SidC and LidA (Luo and Isberg, 2004). Weber *et al.* (2006) recently reported that SidC is able to specifically bind to a specific phosphoinositide (PI) found on the LCV, phosphatidylinositol-4-phosphate [PI(4)P] (Weber *et al.*, 2006). SidC and LidA both contain coiled-coil motifs likely acting as synaptosomal-associated protein receptors (SNAREs) to mediate protein-protein interactions involved during uptake and LCV biogenesis (Conover *et al.*, 2003; Derré and Isberg, 2005; Kagan and Roy, 2002; Weber *et al.*, 2006). Overexpression of LidA resulted in redistribution of the ER-Golgi intermediate compartment (ERGIC) (Derré and Isberg, 2005). More recently, Machner and Isberg (2006) demonstrated that two translocated substrates, SidM and LidA, synergistically recruit the small GTPase Rab1 to the LCV fot the recruitment of early secretory vesicles from the ER that are necessary for LCV biogenesis. Like the Arf1-activating effector RalF, SidM was shown to possess GEF activity that could stimulate Rab1 (Machner and Isberg, 2006). Since Rab1 is known to be crucial for LCV biogenesis, the lack of intracellular growth defects in macrophages infected with *sidM* mutants was surprising (Machner and Isberg, 2006).

It should be noted that Dot/Icm effectors usually have numerous paralogs. This apparent redundancy of effectors has hampered efforts to identify function, since *L. pneumophila* strains lacking even multiple effector proteins show no discernable defects in inhibition of the LCV maturation or in intracellular multiplication (Luo and Isberg, 2004; Ninio *et al.*, 2005; Campodinico *et al.*, 2005). Current models suggest that

multiple effectors may act in concert to promote formation and trafficking of the LCV (Dorer *et al.*, 2006). The exact Dot/Icm secreted proteins or combinations involved in LCV biogenesis remain to be determined.

### 2.4.2. Factors Promoting Uptake of *L. pneumophila*

Other than the Dot/Icm system, numerous other factors have been linked to *L. pneumophila* virulence. Theses include bacterial factors involved in the uptake of *L. pneumophila* in the host cell such as the type IV pilus, Mip, Hsp60, MOMP, EnhC and RtxA (Cianciotto, 2001). The initial interaction of *L. pneumophila* and host cells may involve pili (Stone and Abu Kwaik, 1998). Mutant strains defective in *pilE*, encoding the pilin for the type IV pilus, have reduced attachment to both protozoa and human macrophages, and defects in DNA transformation (Stone and Abu Kwaik, 1999). Unlike the *pilE* mutant, mutation of *pilD* impaired growth in amoeba, human macrophages, and epithelial cells, suggesting that PilD may have additional roles (Liles *et al.*, 1998 and 1999). Indeed, it was later found that PilD processes many proteins destined for the type II secretion system (T2SS) (Liles *et al.*, 1998 and 1999; Rossier and Cianciotto, 2001; Rossier *et al.*, 2004). Though PilD and the T2SS are both essential for virulence, none of the identified substrates was required for virulence, suggesting that additional T2SS-secreted factors contribute to *L. pneumophila* pathogenesis.

The macrophage infectivity potentiator (Mip) family of chaperones displaying peptidyl-prolyl-*cis/trans* isomerase (PPIase) activity are known to play an important role in virulence (Horne *et al.*, 1997). In *L. pneumophila*, Mip was shown to be a surface-expressed protein involved in entry into macrophage and protozoa, but is not necessary for intracellular growth (Cianciotto *et al.*, 2001; Wintermeyer *et al.*, 1995). Though the

mechanism remains elusive, inhibition of Mip PPIase activity abolishes early establishment of infection in *A. castellanii* and human macrophages (Helbig *et al.*, 2003). More recently, Wagner *et al.* (2006) demonstrated that Mip binds collagen and promotes epithelial transmigration, which may play an important role in pathogenesis and tissue destruction.

Heat shock protein 60 (Hsp60) has been localized in the periplasmic space and on the surface of *L. pneumophila* (Garduño *et al.*, 1998). Purified *L. pneumophila* Hsp60-coated latex beads were rapidly taken up by non-phagocytic HeLa cells and anti-Hsp60 antibodies prevented invasion by *L. pneumophila*, together suggesting that Hsp60 functions as an invasin (Hoffman *et al.*, 1989 and 1990; Garduño *et al.*, 1998a, 1998b, and 2002). *L. pneumophila* Hsp60-coated beads were also shown to activate both PKC and IL-1 production in human macrophages, even in presence cytochalasin D (preventing uptake) (Retzlaff *et al.*, 1996). Therefore, host cell receptors may recognize Hsp60 and mediate signalling cascades promoting uptake and production of pro-inflammatory cytokines. In addition, Hsp60 levels have also been shown to increase during infection of human monocytes and this protein is released into the phagosomal milieu (Fernandez *et al.*, 1996). The exact mechanism behind Hsp60 secretion and its role during intracellular growth remain unclear.

The product of *L. pneumophila ompS*, the major outer membrane protein (MOMP or OmpS) is a homotrimeric porin (Butler and Hoffman, 1990; Hoffman *et al.*, 1992a and b; Gabay *et al.*, 1985). Earlier work demonstrated that complement may bind to OmpS to promote uptake by complement receptors; however, this work could not be reproduced by others (Weissgerber *et al.*, 2003). OmpS did promote attachment (not invasion) in a

HeLa cell model (Garduño *et al.*, 1998c). Purified OmpS was shown to be a major protective antigen of cellular immunity in guinea pigs, and lymphocyte proliferation in response to OmpS could be observed in humans up to seven years post-infection (Weeratna *et al.*, 1994). In fact, guinea pigs vaccinated with purified OmpS survived lethal challenges with *L. pneumophila*, suggesting that OmpS is an important antigen associated with the development of a protective cellular immunity (Weeratna *et al.*, 1994).

Cirillo *et al.* (2000) identified factors required for efficient adherence and entry of *L. pneumophila* into human epithelial cells and macrophages. The product of the "enhanced entry" gene *enhC* may be mediated through its Sel-1 domain, known to promote protein-protein interactions with host cells (Brüggemann *et al.*, 2006). More recently, an EnhC homolog termed LpnE, which contains a Sel-1 domain and was shown to be required for efficient entry into host cells (Bandyopadhyay *et al.*, 2004; Newton *et al.*, 2006). The *rtxA* gene encodes a pore-forming cytotoxin (Kirby *et al.*, 1998) that has been implicated in adherence, entry and survival in both macrophage and protozoa (Cirillo *et al.*, 2001 and 2002). The mechanisms by which RtxA affects adherence and entry are not known.

### 2.4.3. Uptake of *L. pneumophila* into Host Cells

Uptake of legionellae by *H. veriformis* has been shown to be mediated by the galactose/N-acetylglucosamine (Gal/GalNAc) lectin (Venkataraman *et al.*, 1997). In contrast to induction of receptor phosphorylation in macrophages (Coxon *et al.*, 1998), binding to the Gal/GalNAc receptor of protozoa induces tyrosine dephosphorylation (Venkataraman *et al.*, 1997). The resulting disruption of the protozoan cytoskeleton

facilitates entry of the bacterium by a sort of receptor-mediated endocytosis (King *et al.*, 1991). Uptake of *L. pneumophila* in macrophages has been shown to occur through coiling phagocytosis (Horwitz, 1984), conventional phagocytosis (Horwitz and Silverstein, 1981; Payne and Horwitz, 1987; Steinert *et al.*, 2002) and macropinocytosis (Watarai *et al.*, 2001). Phagocytosis of *L. pneumophila* into macrophages is enhanced by serum antibodies and complement, yet replication was not affected (Horwitz and Silverstein, 1981; Payne and Horwitz, 1987).

Surprisingly, Cirillo *et al* (1999) showed that previous growth in *A. castellanii* enhanced uptake of *L. pneumophila* by a wide variety of cells, including protozoa, macrophages, and even non-phagocytic epithelial cells (Cirillo *et al.*, 1994). Furthermore, the discovery of the *dot/icm* mutants suggested that bacterial factors are involved in LCV biogenesis (Berger and Isberg, 1993; Marra *et al.*, 1992). This phenotype was eventually linked to the Dot/Icm system where secreted factors were thought to bypass signals triggered during uptake that would normally target the phagosome for degradation by the endocytic pathway (Hilbi *et al.*, 2001; Molofsky and Swanson, 2004; Segal *et al.*, 1999; Watarai *et al.*, 2001). The current hypothesis suggests that the Dot/Icm system plays a critical role in the intracellular establishment of the LCV and may work during uptake (Coers *et al.*, 1999; Hilbi *et al.*, 2001; Roy *et al.*, 1998; Watarai *et al.*, 2001). In fact, by placing *dotA* under the control of an inducible promoter, the Dot/Icm system was shown to be required for invasion and early establishment of the LCV, but is dispensable during replication (Roy *et al.*, 1998). Hilbi *et al.* (2001) demonstrated that wild-type *L. pneumophila* but not *dot/icm* mutants were able to induce phagocytosis in macrophages and protozoa. Moreover, efficient uptake

was restored in *dot/icm* mutants by co-infection with wild-type cells, suggesting that the Dot/Icm system may stimulate initial bacterial uptake by secretion of effectors into the host (Coers *et al.*, 1999; Hilbi *et al.*, 2001). Nagai *et al.* (2005) used cytochalasin D to block internalization of *L. pneumophila* and showed that attachment is sufficient to mediate Dot/Icm-dependent translocation of RalF into the host cell. In summary, Dot/Icm seems to play a role in uptake of *L. pneumophila* and formation of the LCV.

Watarai *et al.* (2001) demonstrated that the Dot/Icm system is required for formation of a macropinosome-like vacuole for uptake of *L. pneumophila* into A/J-derived murine macrophages. Macropinocytosis appears, at least superficially, comparable to phagocytosis; however, the two can be distinguished by phosphoinositide metabolism (Cardelli, 2001). PI metabolism is crucial during endocytosis and exocytosis since phosphorylation of the inositol ring governs the recruitment of specific membrane coat proteins, allowing spatio-temporal coordination of membrane dynamics, actin remodeling, and intracellular trafficking (Cardelli, 2001). $PI(4,5)P_2$ plays a central role in both phagocytosis and macropinocytosis (Cardelli, 2001). The phagocytic pathway is activated by function of phospholipase C (PLC) leading to diacylglycerol (DAG) and inositol triphosphate ($IP_3$) production from $PI(4,5)P_2$, whereas activation of macropinocytosis requires action of PI3-kinases (PI3K) to generate $PI(3,4,5)P_3$ from $PI(4,5)P_2$ (Cardelli, 2001). Weber *et al.* (2006) used a *Dictostelium discoideum* mutant lacking PI3Ks and an inhibitor of PI3K (wortmannin) to show that PI3Ks are dispensable for *L. pneumophila* intracellular growth, a concept previously established in macrophages. In contrast, phagocytosis of *dot/icm* mutants and *E. coli* was abolished with wortmannin, signifying that the Dot/Icm system bypasses the requirement for PI3Ks

during phagocytosis (Khelef *et al.*, 2001; Weber *et al.*, 2006). Other PI kinases may play important roles in formation of the LCV, since PI(4)P has been found exclusively in membranes of the LCV and is capable of binding the the Dot/Icm-secreted effectors SidC and SdcA (Weber *et al.*, 2006).

Unlike *M. tuberculosis* which inhibits $Ca^{2+}$ signalling to generate its replication niche (Chua *et al.*, 2004), Fajardo *et al.* (2004) demonstrated that *L. pneumophila* is internalized by a phagocytic process involving activation of the PLC pathway via heterotrimeric G-proteins (Figure 3), modulation of intracellular $Ca^{2+}$ levels through action of ER-derived $Ca^{2+}$-binding proteins, and the overall reorganization of the actin network by specific cytoskeleton-associated proteins. The PLC-dependent $Ca^{2+}$ signaling cascade may be particularly important for *L. pneumophila* since $Ca^{2+}$ is known to be required for activation of protein kinase C, which controls the production of pro-inflammatory cytokines and activation of the oxidative burst (Coxon *et al.*, 1998; Wieland *et al.*, 2006).

### 2.4.4. Dot/Icm-Independent Inhibition of Phagolysosome Fusion

*L. pneumophila* has the ability to prevent transport of the LCV to the endocytic pathway, a phenotype often atributed to the Dot/Icm system. Though the Dot/Icm T4SS may interfere with host traffic to modulate traffic of ER-derived vesicles to the LCV, several lines of evidence suggest that other factors may be responsible for initial isolation of the LCV from the endosomal network (Joshi *et al.*, 2001). For example, *dot/icm* mutants remain viable without replicating for several hours in vacuoles possessing LAMP-1, which fail to acquire additional lysosomal markers (Joshi *et al.*, 2001). Similar characteristics were decribed for phagosomes harboring formalin-killed wild-type

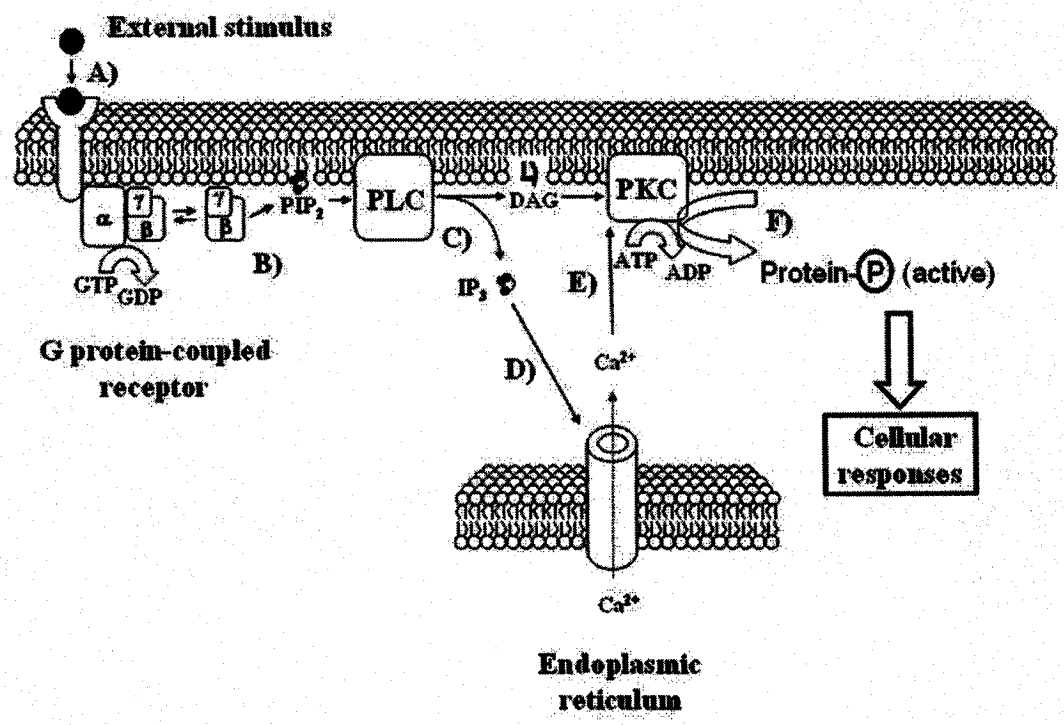



**Figure 3** Activation cascade of protein kinase C. A) External stimuli such as the bacterial attachment stimulate G protein-coupled receptors B) produce $PIP_2$ from PI found in the host cell plasma membrane. C) $PIP_2$ serves as a substrate for PLC, generating DAG and $IP_3$. D) $IP_3$ activates $Ca^{2+}$ channels in the ER membrane, activation the release of $Ca^{2+}$ into the intracellular milieu (E). F) At the expense of ATP, activated PKC can phosphorylate a number of host proteins, leading to cellular responses. Abbreviations: diacylglycerol (DAG), inositol triphosphate ($IP_3$); phospholipase C (PLC), protein kinase C (PKC).

*L. pneumophila* (Joshi *et al.*, 2001). Dot/Icm-dependent factors seem to promote the generation of the LCV whereas a Dot-independent formalin-resistant activity seems to be required to prevent lysosomal degradation (Joshi *et al.*, 2001).

Fernandez-Moreira *et al.* (2006) proposed a model where transmissive *L. pneumophila* regulates its surface composition and sheds vesicles into the LCV to inhibit phagosome-lysosome fusion. Organisms like *E. coli* and *S. typhimurium* are known to shed LPS and vesicles derived from their outer membranes (Beveridge, 1999), a strategy used for virulence in some organisms. Curiously for *L. pneumophila*, beads coated with post-exponential-phase *L. pneumophila* and *dotA* mutant membranes were able to avoid lysosomes, but this phenomenon was not observed with exponential-phase wild-type, *dotA*, and *letA*, post-exponential *letA*, or *E. coli*-derived membranes (Fernandez-Moreira *et al.*, 2006). This ability was correlated with developmentally regulated changes in surface glycoconjugates, where transmissive-phase *L. pneumophila* specifically express distinct high-molecular-weight LPS (Fernandez-Moreira *et al.*, 2006). Interestingly, phase-variable expression of LPS had been shown to contribute to the virulence of *L. pneumophila* in a guinea pig model (Lüneberg *et al.*, 1998). Mutation of several LPS biosynthetic or modification genes failed to alter *L. pneumophila* virulence (Luck *et al.*, 2001). Unmentioned by Fernandez-Moreira *et al.* (2006) was that a *L. pneumophila rcp* mutant is not only susceptible to cationic peptides but was enfeebled for intracellular growth (Robey *et al.*, 2001), presumably due to homology to a lipid A-modifying protein. Altogether, *L. pneumophila* seems to evade the endocytic pathway by a Dot/Icm-independent, developmentally regulated process; however, additional studies are required

to determine whether LPS modifications, a particular outer membrane protein, and/or a Dot/Icm-independent secreted factor that inhibits phagosome-lysosome fusion.

### 2.4.5. Phagosome Maturation

After attachment of bacteria to specific host cell receptors and subsequent induction of signal transduction cascades, the actin-dependent processes mediating membrane dynamics during phagocytosis ultimately lead to phagosome formation (Scott *et al.*, 2003). Once the phagosome is formed, maturation of the vacuole involves controlled interactions between numerous membranous compartments. Vesicular trafficking is tightly controlled by vesicle tethering and docking machinery that include proteins such as small GTPases and fusion and fission mediators such as SNAREs (Scott *et al.*, 2003). Phagosome maturation can be viewed as a dynamic process that can be monitored by acquisition (or loss) of compartment-specific markers, including those of early endosomes (Rab5, V-ATPase, and syntaxin-13), late endosomes (Rab7 and mannose-6-phosphate receptor), lysosomes (LAMP-1, LAMP-2, and cathepsin D), and the ER (calnexin, calreticulin, and the Bip chaperone) (Scott *et al.*, 2003). Phagosomes that follow the endocytic pathway become acidic and oxidizing, and fuse with lysosomes containing hydrolytic enzymes (Scott *et al.*, 2003).

Some intracellular pathogens have evolved mechanisms to escape destruction by the endocytic pathway by remodelling their phagosome to generate a replication-permissive niche. For example, lipoarabinomannans (LAMs) on the surface of *M. tuberculosis* halt phagosomal maturation by excluding the Rab5 effector EEA-1, inhibit $Ca^{2+}$/calmodulin and PI3K-dependent signaling cascade, and promote p38 mitogen-activated protein (MAP) kinase-dependent activation of the Rab5 GDP-dissociation

inhibitor (Chua *et al.*, 2004). Similarily, immunochemistry and compartment-specific markers has provided a valuable roadmap for the cell biology involved during biogenesis and trafficking of the LCV (Lu and Clarke, 2005; Swanson and Hammer, 2000). To establish a replication-permissive niche, *L. pneumophila* is thought to manipulate host vesicular transport to quickly reprogram the LCV away from the endocytic pathway (Berger and Isberg, 1993; Marra *et al.*, 1992; Roy *et al.*, 1998; Roy and Tilney, 2002; Wiater *et al.*, 1998). The V-ATPase found on early endosomes is not recruited to the LCV, thereby preventing acidification (Lu and Clarke, 2005). The LCV also lacks MHC class I and class II molecules, alkaline phosphatase, CD44, CD63, LAMP-1, LAMP-2, cathepsin D, transferrin receptors, and Rab5 (Clemens *et al.*, 2000; Swanson & Hammer, 2000). Mature LCVs acquire calnexin, calreticulin, Sec22b, several small GTPases (Arf1, Sar1, and Rab1), and several Dot/Icm-secreted factors (RalF, LidA, SidC, and SidM), and contain PI(4)P (Conover *et al.*, 2003; Derré and Isberg, 2004; Fajardo *et al.*, 2004; Kagan *et al.*, 2004; Kagan and Roy, 2002; Lu and Clarke, 2005; Luo and Isberg, 2004; Machner and Isberg, 2006; Nagai *et al.*, 2002; Weber *et al.*, 2006).

As early as 30 minutes post-infection, the phagosome membrane associates with mitochondria and many small ER-derived early secretory vesicles that transform the LCV into a vacuole morphologically similar to the ER (Kagan and Roy, 2002; Lu and Clarke, 2005; Tilney *et al.*, 2001). The phagosome becomes completely enclosed by rough ER membrane within 4-6 hours (Swanson and Isberg, 1995) and the LCV of protozoa and macrophages eventually becomes studded with ribosomes to generate a niche that supports intracellular replication (Katz and Hashemi, 1982; Horwitz, 1983; Swanson and

Isberg, 1995). The exact mechanism by which *L. pneumophila* creates an ER-derived vacuole is unclear, though some hypotheses have been proposed.

#### 2.4.5.1. Role of Autophagy

Autophagy is a catabolic-trafficking mechanism responsible for delivery of double-membraned autophagosomes containing cellular contaminants and defective organelles to lysosomes (Dorn *et al.*, 2002). Two Gram-negative intracellular pathogens with T4SSs are thought to exploit autophagic pathways to generate ER-derived replication vacuoles: *Brucella abortus* and *C. burnetii*. Unlike the LCV, the *Brucella*- and *Coxiella*-containing phagosomes become acidic, acquire some late endosomal markers, and stain positive for autophagic markers (Dorn *et al.*, 2002; Roy, 2002). Furthermore, the induction of autophagy by amino acid depletion or overexpression of autophagy markers increases the number and size of *Coxiella* replicative vacuoles (Gutierrez and Columbo, 2005). It was proposed that *L. pneumophila* may also exploit autophagy, based on similarities between the LCV and autophagosomes such as their generation from ER-derived vesicles (Swanson and Isberg, 1995), acquisition of the autophagic markers Atg7 and Atg8 (Amer and Swanson, 2005), and the eventual fusion with lysosomes (Sauer *et al.*, 2005; Sturgill-Koszycki and Swanson, 2000). It should be noted that all events attributed to autophagy were solely found in macrophages derived from the *L. pneumophila*-permissive A/J murine strain. Amer and Swanson (2005) illustrated that autophagy is an immediate innate response to *L. pneumophila* in restrictive mouse strains like C57BL/6J, and defective in the the permissive A/J strain. Several lines of evidence refute the role of autophagy in other cells during *L. pneumophila* infection. First, formation of the autophagic *C. burnetii*-containing vacuole

is inhibited by wortmanin (inhibitor of the PI3K signalling), but this inhibitor had no effect on LCV biogenesis (Khelef *et al.*, 2001; Weber *et al.*, 2006). Second, early autophagosomes mature after acquisition of V-ATPase and LAMP-1 to form an autolysosome (Dorn *et al.*, 2002), whereas V-ATPase is absent from the LCV in all stages of infection (Lu and Clarke, 2005). Finally, using deletion mutants of *D. discoideum* Otto *et al.* (2004) demonstrated that autophagy was dispensable by showing that LCV biogenesis does not require signalling (Apg1, Apg6) or structural (Apg5, Apg7, Apg8) autophagic components. Therefore, autophagy does not seem to play an equivalent role in all cells.

#### 2.4.5.2. Association with ER-Derived Vesicles

Inhibitors that interfere with GEF functions for Arf GTPases, such as brefeldin A (BFA) inhibit the ability of *L. pneumophila* to replicate in macrophages and reduce the association with the LCV of the ER-localized proteins (Kagan and Roy, 2002). Such experiments have led to the generally accepted view that Dot-Icm-competent *L. pneumophila* creates an ER-like vacuole by intercepting early secretory vesicles exiting from the ER located in the ERGIC region of the cell (Derré and Isberg, 2005; Horwitz *et al.*, 1983; Kagan & Roy, 2002; Roy & Tilney, 2002; Robinson and Roy, 2006; Tilney *et al.*, 2001;). The mechanism used by *L. pneumophila* to create an ER-derived vacuole is likely multifactorial, which would involve recruitment of host proteins to the LCV (Roy *et al.*, 1998; Roy and Tilney, 2002; Wiater *et al.*, 1998). In fact, small GTPases (Sar1, Arf1, and Rab1) and SNARE proteins (Sec22b) that regulate traffic in the early secretory pathway are quickly recruited to the LCV following uptake and play a crucial role in its LCV biogenesis (Kagan and Roy, 2002; Derre and Isberg, 2004; Kagan *et al.*, 2004).

Generation of coatamer protein (COP)-coated vesicles requires the small GTPase Sar1 and Arf1, thereby forming complexes involved in budding events from ER exit sites (Sar1/COPII) and ER-Golgi transport (Arf1/COPI), respectively (Kagan and Roy, 2002; Roy, 2002; Tilney *et al.*, 2001). Interfering with or inhibiting GTPase activity of Sar1, Arf1 or Rab1 abolishes LCV fromation (Derré and Isberg, 2004; Kagan and Roy, 2002; Kagan *et al.*, 2004). Interestingly, recruitment of these GTPases to the LCV requires the Dot/Icm effectors (Derre and Isberg, 2004; Kagan *et al.*, 2004 and 2005; Machner and Isberg, 2006; Nagai *et al.*, 2002). In addition, the SNARE protein Sec22b, which normally controls fusion events between ER-derived vesicles (homotypic fusion) or with Golgi membranes (heterotypic fusion), is also involved in delivery of secretory vesicles to the LCV (Derre and Isberg, 2004; Kagan *et al.*, 2004). Partners that typically interact with Sec22b have not been found on the LCV (Derre and Isberg, 2004; Kagan *et al.*, 2004), yet Dot/Icm-secreted proteins with coiled-coil domains could function as SNAREs. Taken together, observations concerning the Dot/Icm-dependent recruitment of host cell proteins involved in vesicle trafficking to the LCV seem to suggest that *L. pneumophila* secretes molecular mimics of host proteins to facilitate integration into the secretory pathway. The Dot/Icm-dependent process of evasion of the endocytic pathway and fusion with the ER vesicles is crucial for formation of a replication-permissive LCV where ER-derived peptides and nutrients could promote intracellular multiplication (Robinson and Roy, 2006).

Though *L. pneumophila* Dot/Icm plays a central role in the modulation of host cell functions and several putative effectors are injected into host cells, mutants lacking even multiple of these substrates demonstrated no intracellular growth defects, suggesting

functional redundancy. In this respect, Dorer *et al.* (2006) investigated the contribution of several host trafficking pathways that might deliver membrane material and proteins to the LCV. With a few exceptions, the inhibition of only one trafficking pathway by RNA interference was usually not sufficient to cause defects in intracellular replication of *L. pneumophila* in *Drosophila* Kc167 cells. Only a few double-stranded RNA molecules (dsRNAs) targeting ER-Golgi transport could inhibit bacterial replication (Dorer *et al.*, 2006), including those inhibiting production of small GTPases, the SNARE syntaxin 13, and Cdc48/p97. The strongest defects in intracellular replication were observed when dsRNAs were used to target multiple steps in the secretory pathway (Dorer *et al.*, 2006). For example, a dsRNA targetting Sec22b had little effect on intracellular replication unless combined with others targetting components of the transport of protein particles complex (Dorer *et al.*, 2006). The transport of protein particles complex is localized to the ERGIC and has been implicated in vesicle tethering and activation of Rab1, a small GTPase that is crucial for LCV biogenesis (Derre and Isberg, 2004; Machner and Isberg, 2006). Furthermore, a double knockdown of Arf1 and Sec22b, two components implicated in ER retrograde and anterograde transport, respectively, had an additive defect on intracellular growth (Dorer *et al.*, 2006). Thus, the redundancy of Dot/Icm effectors could provide a vaste repertoire of functions that would target multiple pathways to generate the LCV.

Dorer *et al* (2006) also revealed that intracellular replication *L. pneumophila* is enfeebled by inactivation of Cdc48/p97 and its partners, yet the LCV still evaded the endocytic pathway (Dorer *et al.*, 2006). The requirement for replication but not evasion of the endocytic pathway was a particularly interesting finding since Cdc48/p97 is an

AAA-ATPase that forms a complex to act as a chaperone in endoplasmic reticulum-associated degradation (ERAD) (Bar-Nun, 2005). ERAD functions to target misfolded proteins to the surface of the ER where they get ubiquitinated, removed and delivered to proteasomes by Cdc48/p97 for degradation (Bar-Nun, 2005). Inhibition of the proteasome leads to decreased intracellular replication of *L. pneumophila* (Dorer *et al.*, 2006). More importantly, Cdc48/p97 and polyubiquitinated proteins were found to localize to the LCV by a Dot/Icm-dependent process in both macrophages and *Drosophila* cells (Dorer *et al.*, 2006). Since the Cdc48/p97 chaperone complex can remove ubiquitinated and nonubiquitinated proteins from the ER, Dorer *et al.* (2006) demonstrated that Cdc48/p97 could also remove the Dot/Icm substrates LidA and SidC from the LCV. Therefore, *L. pneumophila* seems to exploit the ubiquitination system to temporally modulate the surface composition of the LCV and promote a vacuole suitable for replication.

Though the role and molecular players involved in mitochondrial recruitment and the exact mechanisms behind avoidance of lysosomal fusion remain to be elucidated, remarkable progress has been made in understanding how *L. pneumophila* modulates host responses for LCV biogenesis and trafficking to generate a suitable environment for intracellular replication. After the LCV is generated, *L. pneumophila* is known to differentiate to a replicative form. Since *L. pneumophila* differentiates from an extracellular transmissible form to an intracellular replicating form, current studies have investigated possible germination signals.

### 2.4.6. Possible Germination Signals

Once *L. pneumophila* establishes a replicative vacuole, *L. pneumophila* must acquire essential nutrients such as iron and amino acids. Current views suggest that protection against host-generated ROIs may also play an important role in intracellular survival (discussed below). The following sections describe some factors involved in iron and amino acid transport.

#### 2.4.6.1. Iron Acquisition and Assimilation

Iron, an essential nutrient for bacteria, exists in a dynamic equilibrium between soluble ferrous ($Fe^{2+}$) and the insoluble ferric ($Fe^{3+}$) forms. Robey and Ciaciotto (2002) identified *L. pneumophila* FeoB, a homolog of the ATP-driven iron transporter of *E. coli*, and demonstrated that *feoB* mutants are impaired for intracellular growth in protozoa, human macrophages, and mice. Mutation in *iraAB*, encoding another *L. pneumophila* iron transporter, also inhibits replication in U937 cells and guinea pigs (Viswanathan *et al.*, 2000). Taken together, these results suggest that iron is freely accessible in the LCV, but the source of iron is unclear. Decreased levels of the receptor for transferrin, the key iron transport protein of the host cell, reduced the ability of legionellae to establish intracellular infection (Byrd and Horwitz, 2000). In fact, IFN-γ-activated macrophages restrict iron availability and inhibit intracellular replication of *L. pneumophila* by down regulating transferrin receptors (Byrd and Horwitz, 2000). *L. pneumophila* is known to produce several iron-binding siderophores, and mutation of the Fur-regulated gene *frgA*, encoding a homolog of the siderophore-producing aerobactin synthetase in *E. coli*, significantly impairs replication in U937 cells (Hickey and Cianciotto, 1997). Though *L. pneumophila* likely alters gene expression in response to iron limitation, the regulation by

ferric uptake regulator (Fur) can not be assayed using *fur* mutants, since the *fur* gene is essential in *L. pneumophila* (Hickey and Cianciotto, 1997).

### 2.4.6.2. Amino Acid Uptake

Enclosed in the LCV and unable to synthesize numerous amino acids (Tesh and Miller, 1981), *L. pneumophila* must acquire these nutrients from host cells. The human macrophage amino acid transporter SLC1A5n was shown to be required for infection since interfering with *slc1a5* activity blocked intracellular replication of *L. pneumophila* (Wieland *et al.*, 2005). SLC1A5 was up-regulated during *L. pneumophila* infection and preliminary data suggest that it is localized to the surface of the LCV (Wieland *et al.*, 2005). In this case, delivery of essential amino acids would be assured by SLC1A5 since it is known to exhibit affinities for the amino acids Cys, Ile, Leu, Met, Ser, and Val (Wieland *et al.*, 2005). To use these nutrients, bacteria must first transport these nutrients into their cytoplasm. The major facilitator superfamily (MFS) family of transporters generally mediates transport of compounds such as sugars, amino acids, and Krebs cycle metabolites (Pao *et al.*, 1998). *L. pneumophila* possesses numerous MFS transporters (Chien *et al.*, 2004; Cazalet *et al.*, 2004), one of which, termed phagosomal transporter A (PhtA), has been implicated in transport of the essential amino acid threonine (Sauer *et al.*, 2005). Intracellular *phtA* mutants exhibit severe differentiation and growth defects, yet PhtA function could be bypassed by exogenous threonine-containing peptides or threonine, indicating that *L. pneumophila* may also be equipped with less efficient redundant transport mechanisms (Sauer *et al.*, 2005). Nonetheless, Sauer *et al.* (2005) proposed that *L. pneumophila* uses its Pht transporter family to determine whether

growth-promoting amino acids are available before entering into the replicative phase of the cell cycle.

Ewann and Hoffman (2006) have demonstrated that, unlike what is seen for many other organisms the requirement of *L. pneumophila* for L-cysteine could not be provided by uptake of the oxidized dipeptide cystine. Interestingly, L-cysteine (but not L-cystine) was taken up by an energy-dependent transporter. Cysteine import was shown to have biphasic kinetics (high- and low-affinity transport), a phenomenon usually observed when organisms use either periplasmic binding protein or direct uptake of amino acid, depending on its concentration (Ewann and Hoffman, 2006). Transport was also shown to be pH-dependent, and pH values estimated for the LCV (slightly acidic) increased uptake of cysteine (Wieland *et al.*, 2004). The unique uptake of cyteine by *L. pneumophila* may represent a germination signal that would ensure replication in an intracellular mileu such as protozoa (and macrophages) but not in aerobic biofilms (Ewann and Hoffman, 2006).

### 2.4.7. Egress of *L. pneumophila* from the Spent Host

After exhausting nutrients in its host cell, *L. pneumophila* has evolved two mechanisms that enable the organisms to escape the spent host, necrosis and apoptosis (Bitar *et al.*, 2004; Goa and Abu Kwaik, 1999a and b). First, *L. pneumophila* becomes highly cytotoxic and causes pore formation in the host cell membrane upon termination of intracellular replication in macrophages, ultimately leading to osmotic lysis and egress of the bacteria (Alli *et al.*, 2000; Molmeret *et al.*, 2002; Kirby *et al.*, 1998). *L. pneumophila* mutants termed *rib* (release of intracellular bacteria) are able to evade the endocytic pathway and replicate intracellularly, but are trapped in host protozoa and

macrophages due to a lack of pore-forming ability (Alli *et al.*, 2000; Gao and Abu Kwaik, 2000; Zink *et al.*, 2002). Eventually, *rib* mutants will cause apoptotic death of host macrophages, but due to the lack of caspases, fail to do so in protozoan hosts. The phenotype of *rib* mutants was eventually linked to a truncated IcmT protein where complementation by a functional *icmT* gene restored pore-mediated exit (Bitar *et al.*, 2005; Molmeret *et al.*, 2002a and b). Current models suggest that after intracellular replication an initial step of pore formation disrupts LCV and promotes egress to the cytoplasm where bacteria can still multiply, and subsequently a second step of pore formation disrupts organelles and the host plasma membrane resulting in host cell lysis and bacterial egress (Bitar *et al.*, 2005; Molmoret *et al.*, 2004).

Secondly, *L. pneumophila* is known to induce apoptosis (programmed cell death), a process mediated by cysteine proteases called caspases. The extrinsic pathway is activated by surface-receptor signaling which activates caspase-8, whereas the intrinsic pathway is activated by stress or toxins through cytoplasmic release of cytochrome *c* from mitochondria and activation of caspase-9 (Santic and Abu Kwaik, 2006). Downstream events of either cascade result in caspase-3 activation, the executioner caspase triggering DNA fragmentation (Santic and Abu Kwaik, 2006). Surprisingly, a functional Dot/Icm system is required for caspase-3 activation by extrinsic- and intrinsic-independent events during the early stages of *L. pneumophila* infection in a number of cells, yet DNA fragmentation only occured in the late stages of infection (Abu-Zant *et al.*, 2005; Gao and Abu Kwaik, 1999a and b; Molmeret *et al.*, 2004; Zink *et al.*, 2002). It should be noted that exogenous activation of caspase-3 by the extrinsic and intrinsic

pathways at any point during *L. pneumophila* infection results in abrogation of intracellular replication (Abu-Zant *et al.*, 2005).

The early activation of caspase-3 results in cleavage of rabaptin-5, a major Rab-5 effector that controls fusion events with early endosomes, suggests that this process may allow evasion of the endocytic pathway (Molmeret *et al.*, 2004). In fact, inhibition of the caspase-3-mediated cleavage of rabaptin-5 results in maturation of the LCV through the endosomal-lysosomal pathway (Molmeret *et al.*, 2004). However, this mechanism is controvertial since rabaptin-5 may not even be recruited because Rab5 is absent from the LCV (Coers *et al.*, 1999; Clemens *et al.*, 2000; Roy *et al.*, 1998). On the other hand, lack of Rab5 on the LCV may be attributed to the rapid dissociated of Rab5 from the LCV as a result of the rabaptin-5 cleavage (Molmeret *et al.*, 2004). An unforseen possibility would be that rabaptin-5 could be recruited by other isoforms of Rab5 (Rab5c), which have been implicated in LCV biogenesis (Dorer *et al.*, 2006).

Apoptosis is known to inhibit trafficking of secretory vesicles such as the ER-derived vesicles needed to generate the LCV. Therefore, anti-apoptotic functions might delay host cell death long enough to permit *L. pneumophila* replication (Abu-Zant *et al.*, 2005). Human neuronal apoptosis-inhibitory protein (Naip) has been shown to be a direct inhibitor of caspase-3 (Maier *et al.*, 2002; Sanna *et al.*, 2002). A homolog of Naip termed Naip5 (or BIR-containing 1e, Birc1e), encoded at the *Lgn1* locus of chromosome 13, confers a unique susceptibility to *L. pneumophila* infection in A/J mice (Diez *et al.*, 2003; Fortier *et al.*, 2005; Wright *et al.*, 2003). It has been proposed that either a change in Naip5 expression or loss of function might play a role in the process, whereas other mice encoding a functional Naip5 would be able to restrict *L. pneumophila* growth (Diez

*et al.*, 2003, Fortier *et al.*, 2005; Wright *et al.*, 2003). Naip5 has recently been shown to function as one of the nucleotide-binding oligomerization (NOD) leucine-rich repeat (LRR) proteins, known as NLRs (Ren *et al.*, 2006; Molofsky *et al.*, 2006). By analogy to membrane-bound TLRs, cytoplasmic NLRs could sense pathogen-associated molecular patterns such as peptidoglycan, LPS, and flagellin to promote cellular responses like the inhibition of apoptosis. Interestingly, macrophage killing requires both the Dot/Icm system and flagellin (Molofsky *et al.*, 2006; Ren *et al.*, 2006). It has recently been proposed that flagellin contamination of the host cytoplasm may be mediated by Dot/Icm-dependent pore formation during phagocytosis (Molofsky *et al.*, 2006; Ren and al., 2006). Cytosolic flagellin not only induces caspase 1-dependent pro-inflammatory death in macrophage (called "pyroptosis") resulting in membrane permeability and DNA fragmentation, but also makes *L. pneumophila* vulnerable to the immune system that encodes Naip5 (Molofsky *et al.*, 2006; Ren and al., 2006). Secretion of IL-1β and IL-18 has been attributed to activation of an inflammasome, a large multiprotein complex composed of NLRs, caspases-1, and the adaptor molecules (Molofsky *et al.*, 2006; Ren and al., 2006). As stated above, this model of innate immune defenses applies only to A/J mice. *L. pneumophila* is able to infect a wide variety of cells, suggesting that different mechanisms come into play.

Due to the persistence of *L. pneumophila* and other disease-causing legionellae and the lack of cost-effective prevention strategies to eliminate the organism from aquatic man-made environments, Legionnaires' disease is still a relevant disease. Therefore, investigations into the molecular mechanisms involved in generation of the replication-permissive vacuole in numerous cell types may provide insight into more effective means

for detection, surveillance, and prevention of this respiratory disease. Since *L. pneumophila* has been shown to be especially susceptible to oxidative stresses *in vitro* (Hoffman *et al.*, 1983; Kim *et al.*, 2002; Pine *et al.*, 1986) and is likely faced with an oxidative burst in human phagocytes, antioxidant mechanisms used by *L. pneumophila* to detoxify reactive oxygen species (ROIs) might play a crucial role in pathogenesis.

## 2.5. ROIs, Oxidative Stress, and Antioxidant Defenses

The reduction of molecular oxygen is known to yield toxic byproducts such as superoxide ($\bullet O_2^-$), hydrogen peroxide ($H_2O_2$), and the hydroxyl radical ($\bullet OH$), collectively known as ROIs. Oxidative stress is a term used when organisms are faced with ROIs. In the following sections, possible sources and biological targets of ROIs are presented, as well as some bacterial mechanisms used to resist oxidative stress.

### 2.5.1. Source and Targets of ROIs

*L. pneumophila* is undoubtedly faced with endogenously generated ROIs as byproducts of aerobic metabolism. In fact, aerobic organisms are known to generate $\bullet O_2^-$ in their cytoplasm when molecular oxygen accidentally captures an electron from dehydrogenases of the respiratory chain (Storz and Imlay, 1999; Imlay, 2003). The best known biological target of $\bullet O_2^-$ and $H_2O_2$ is its ability to inactivate the 4[Fe-S] iron-sulfur clusters found in *E. coli* dehydratases such as dihydroxyacid dehydratase, aconitase and fumarase (Fridovich, 1997). The inactivation of these enzymes during oxidative stress results in failure of the pathways leading to synthesis of branched chain amino acids and of the tricarboxylic acid (TCA) cycle (Fridovich, 1997). This would be particularly important for *L. pneumophila*, since this organism obtains its energy from oxidation of

amino acids by means of the TCA cycle. Finally, •OH is formed in the presence of $Fe^{2+}$ and $H_2O_2$ (Fenton reaction) (Liochev and Fridovich, 2002). Superoxide is known to release $Fe^{2+}$ during the destruction of the cytoplasmic iron-sulfur clusters of dehydratases (Imlay, 2006; Keyer *et al.*, 1995; Keyer and Imlay, 1996). The hydroxyl radical has been linked to DNA damage and protein oxidation (Storz and Imlay, 1999; Imlay, 2003).

As a crucial part of innate immune responses, macrophages and neutrophils generate ROIs during phagocytosis by a process known as the respiratory (or oxidative) burst. ROI production is mediated through activation of the reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Sheppard *et al.*, 2005). Signaling cascades triggered during bacterial contact with host cells activate PKC (or other kinases), leading to phosphorylation of cytosolic components ($p47^{phox}$, $p67^{phox}$, $p40^{phox}$), and small GTP-binding proteins Rac1/2 and Rap1a are required for NADPH oxidase activity. Phosphorylated cytosolic components are then translocated to the membrane-bound flavocytochrome $b_{558}$ (composed of $gp91^{phox}$ and $p22^{phox}$) and Rap1a. The assembled NADPH oxidase transfers electrons from cytosolic NADPH to molecular oxygen, resulting in the production of $\bullet O_2^-$ and $H_2O_2$ in the phagosomal lumen (Figure 4). In neutrophils, the most potent bactericidal product, hypochlorous acid (HOCl), is formed by myeloperoxidase (MPO) using $H_2O_2$ and $Cl^-$ as substrates. Furthermore, nitric oxide (NO) produced by the inducible nitric oxide synthase (iNOS) can be combined with $\bullet O_2^-$ to form a toxic reactive nitrogen intermediates (RNIs) like peroxynitrite ($ONOO^-$). Altogether, macrophages and neutrophils produce an arsenal of ROIs and RNIs as an antimicrobial response to phagocytosed bacteria.

### 2.5.2. Resistance to Oxidative Stress and *L. pneumophila* Virulence

The ability to resist the bactericidal action of the NADPH oxidase has been crucial for pathogenesis of numerous intracellular pathogens. Two different strategies might be used by *L. pneumophila* to survive and multiply in host macrophages: i) inhibition of cellular signals leading to activation of the NADPH oxidase; and/or ii) detoxification of the phagosomal milieu by antioxidant enzymes. The following sections provide a brief overview of the oxidative stress defense mechanisms described in *L. pneumophila*, including a discussion of partially purified proteins that might inhibit the NADPH oxidase and the known ROI-scavenging enzymes.

### 2.5.3. *L. pneumophila* and the NADPH Oxidase

Some evidence suggests the NADPH oxidase may play an early but inadequate role in phagocyte defences during infection with *L. pneumophila* and other pathogens (Jacobs *et al.*, 1984; Master *et al.*, 2001; Ng *et al.*, 2004; Piddington *et al.*, 2001). Losses in recoverable bacterial counts in the early stages of *L. pneumophila* were attributed to activation of the NADPH oxidase (Jacobs *et al.*, 1984; Saito *et al.*, 2001). Chronic granulomatous disease (CGD) is a rare disorder of the NADPH oxidase where phagocytes are unable to produce ROIs (Segal *et al.*, 2000). The initial killing of *L. pneumophila* following uptake in a murine model was not observed in a CGD strain with a defective $gp91^{phox}$ (Saito *et al.*, 2001). After an initial decrease in viability following uptake, the remaining *L. pneumophila* cells resisted and were able to replicate (Saito *et al.*, 2001). Similar observations were also made in guinea pigs (Miyamoto *et al.*, 1995). Jacobs *et al.* (1984) used primate alveolar macrophages and the reduction of nitroblue

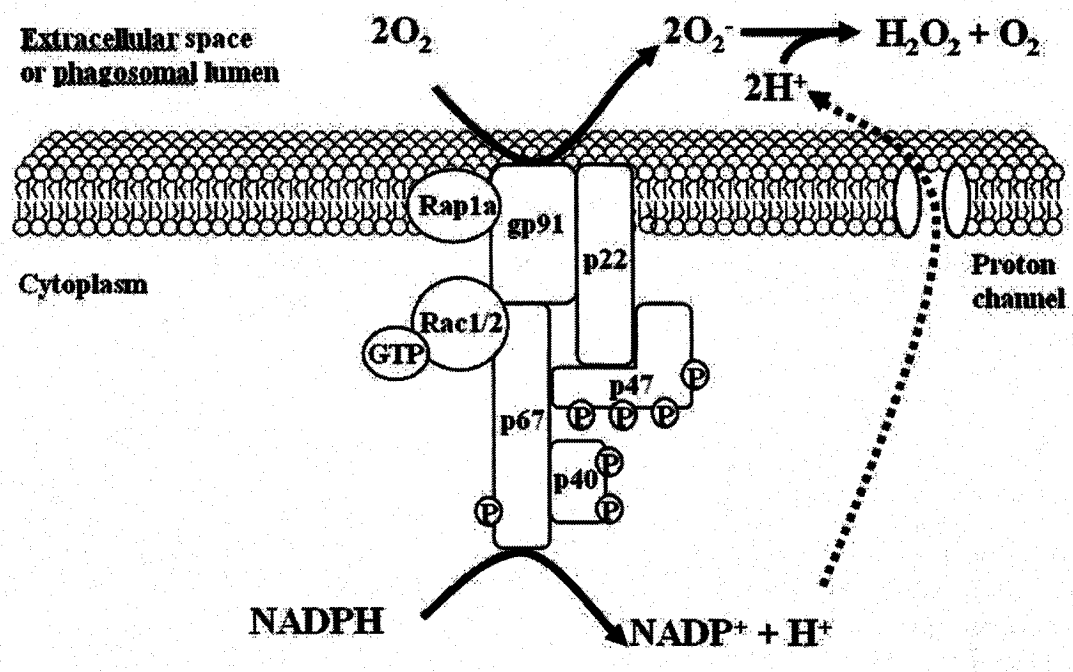



**Figure 4** Generation of an oxidative burst by activated NADPH oxidase. Phosphorylation of the cytosolic components of NADPH oxidase leads to translocation to the membrane-bound cytochrome $b_{558}$ and promotes assembly and generation of superoxide ($\bullet O_2^-$) in the phagosomal milieu or extracellular environment. To prevent membrane depolarization during the NADPH-mediated movement of electrons through the host cell membrane, voltage-gated proton channels compensate to equilibrate the charge distribution (Murphy and Decoursey, 2006). This proton flux also provides essential substrate protons for the conversion of $\bullet O_2^-$ to $H_2O_2$ and then to HOCl by the myeloperoxidase of neutrophils.

tetrazolium (NBT) to show that phagocytosis of *L. pneumophila* is accompanied by a respiratory burst. Though the blue-black formazan arising from NBT reduction by $\bullet O_2^-$ was observed around *L. pneumophila* in the phagosome, although *L. pneumophila* stimulated the reduction of NBT to a lower extent than observed with *E. coli* (Jacobs *et al.*, 1984). These data are consistent with in vitro data where *L. pneumophila* was more susceptible than *E. coli* to ROIs (Locksley *et al.*, 1982). Furthermore, Jacobs *et al.* (1984) demonstrated that the early killing of *L. pneumophila* ingested by primate alveolar macrophages is inhibited by •OH-scavenger mannitol or a combination of superoxide dismutase and catalase. These findings suggest that initially *L. pneumophila* is faced with the oxidative burst generated by the NADPH oxidase; however, the organism must be able to at least partially resist the toxic assault to replicate in its host. The partial resistance to phagocyte-generated ROIs is curious, since *L. pneumophila* is particularly susceptible to $H_2O_2$ and other ROIs (Hoffman *et al.*, 1983; Locksley *et al.*, 1982; Pine *et al.*, 1984). This situation could be attributed to an increased expression of antioxidant enzymes.

On the other hand, another member of the *Legionella* family, *L. micdadei*, has been shown to stimulate an oxidative burst in neutrophils and monocytes to the same extent as observed for *E. coli*, yet 30 minutes following ingestion the organism had an inhibitory effect on phagocyte activation (Donowitz *et al.*, 1990). The attenuation of $\bullet O_2^-$ production varied with the period of time elapsed and with the number of viable *L. micdadei* ingested (Donowitz *et al.*, 1990). As for *L. pneumophila*, the organism is also able to subvert macrophage antimicrobial defences by interfering with the host oxidative metabolism (Jacob *et al.*, 1994). In phorbol myristate acetate (PMA)-stimulated human

macrophages, *L. pneumophila* was shown to down-modulate PKC, which was correlated with decreased phosphorylation of host proteins and as well as decreased $\bullet O_2^-$ production (Jacob *et al.*, 1994). However, *L. pneumophila* only partially inactivates the NADPH oxidase, since PMA-stimulated macrophages retain some residual $\bullet O_2^-$ production (Jacob *et al.*, 1994).

In an attempt to define legionellae proteins involved in the inhibition of NADPH oxidase activity, partially purified lysates of legionellae that contain toxins, phosphatases, phospholipases, and proteases were assayed for $\bullet O_2^-$ production (Friedman *et al.*, 1982; Lochner *et al.*, 1985; Saha *et al.*, 1985; Sahney *et al.*, 1990; Szeto and Shuman, 1990). A crudely purified small toxin from *L. pneumophila* and *L. micdadei* was shown to inhibit the oxidative burst in neutrophils (Friedman *et al.*, 1982; Hedlund, 1981; Lochner *et al.*, 1985). Pre-treatment of neutrophils with the toxin had no effect on $\bullet O_2^-$ production in response to PMA, a PKC agonist widely used for NADPH oxidase activation, but abolished the response to N-formyl-methionine-leucyl-phenylalanine (fMLP), an agonist that signals through PLC (Hedlund, 1981). These data suggested that the toxin might act downstream of PLC but upstream of PKC activation. An acid phosphatase has also been shown to block $\bullet O_2^-$ production (Saha *et al.*, 1985). *L. micdadei* possesses two acid phosphatases ($ACP_1$ and $ACP_2$); however, only $ACP_2$ inhibited the $\bullet O_2^-$ production in neutrophils in response to fMLP but not PMA (Saha *et al.*, 1985 and 1988). The phosphatase might inactivate NADPH oxidase by interfering with PI metabolism involved in its activation. $PIP_2$ [PI(4,5)P] has been shown to be a good substrate for the phosphatase, thereby limiting both the substrate for PLC activity and levels of the second messengers DAG and $IP_3$ (Saha *et al.*, 1988). Interestingly, the remaining phosphoryl

moiety inexplicably does not get removed from PI(4)P (Saha *et al.*, 1988). Similarily, at the expense of ATP a legionellae protein kinase has been shown phosphorylate PI to PI(4)P but not further to $PIP_2$ (Saha *et al.*, 1989). The stability of PI(4)P to further phosphatase or kinase activity is particularly interesting since the Dot/Icm T4SS effector molecule SidC was shown to bind specifically to PI(4)P (Weber *et al.*, 2006). Finally, a partially purified cell-free supernatant of *L. pneumophila* culture containing the major secreted protease (MspA, also known as the Zn-metalloprotease ProA), was shown to inhibit neutrophil $\bullet O_2^-$ production in response to fMLP and PMA without affecting host cell viability (Sahney *et al.*, 1990). However, *mspA L. pneumophila* mutants behaved as wild-type in a guinea pig infection model suggesting that inhibition of the NADPH oxidase does not reqire MspA (Blander *et al.*, 1990).

Though knowledge of the signalling cascades induced during *L. pneumophila* uptake and other lines of evidence seem to suggest that legionellae might be faced with ROIs early in the infection process, numerous partial purified factors have been proposed to inhibit NADPH oxidase activation. Though all known factors that inhibit $\bullet O_2^-$ production in macrophages or neutrophils are known to be T2SS-secreted proteins (Flieger *et al.*, 2000 and 2001; Liles *et al.*, 1999; Hales and Shuman, 1999) and the T2SS was shown to be crucial for *L. pneumophila* intracellular growth in human macrophages and protozoa (Rossier and Cianciotto, 2001), only hydrolytic functions that play minor roles in tissue destruction have been attributed to these factors (Aragon *et al.*, 2000). Some exoprotein mutants have been created in an attempt to evaluate the role of these proteins in pathogenesis, yet none impair the intracellular growth of *L. pneumophila*, suggesting that additional factors are secreted by the T2SS (Blander *et al.*, 1990; Flieger

*et al.*, 2000 and 2001). Further study of T2SS exoproteins should be directed to their possible involvement in the inhibition of the oxidative burst and resistance to the initial killing of *L. pneumophila* in neutrophils and macrophages (Jacob *et al.*, 1994; Jacobs *et al.*, 1984). Interestingly, the *L. pneumophila pilD* mutant, defective in exoprotein secretion, has never been investigated for this ability. It is also curious that there have been no documented reports whether the components of NADPH oxidase are localized to the *L. pneumophila*-containing phagosome. Further investigations are needed to confirm the role of this complex during *L. pneumophila* infection.

### 2.5.4. Bacterial Antioxidant Defenses

Bacterial antioxidant defenses include the concerted actions of both enzymatic and non-enzymatic processes to prevent ROI-mediated damage to proteins, DNA, and membranes (Imlay, 2003; Storz and Imlay, 1999). Some non-enzymatic antioxidants prevent entry of ROIs into the cell, such as the ROI-scavenging LAMs and heat shock protein (Hsp16.3) which have been shown to be required for the growth of *M. tuberculosis* in macrophages (Abulimiti *et al.*, 2003; Chan *et al.*, 1991). Other well-established non-enzymatic mechanisms include the cytoplasmic redox buffer which maintains the cytoplasm as a reducing environment by producing low-molecular-weight thiols such as glutathione and mycothiol (Hand and Honek, 2005). The elimination of oxidized glutathione is performed by NAD(P)H-dependent reduction of disulfide bonds catalyzed by the thioredoxin/thoredoxin reductase or glutaredoxin/glutathione/glutathione reductase systems (Fernandez and Holmgren, 2004; Ritz and Beckwith, 2001).

Bacteria use enzymatic methods to eliminate ROIs, such as the reactions catalyzed by superoxide dismutases (SODs), catalases, alkyl hydroperoxide reductases

(AhpCs), and methionine sulfoxide reductases (Imlay, 2003; Storz and Imlay, 1999). To prevent damage by ROIs, microbial oxidative defenses generally include dismtation of $\bullet O_2^-$ by SOD and the removal of the resulting $H_2O_2$ by catalase or AhpC (Imlay, 2003). Bacterial antioxidant enzymes usually respond to ROIs produced during aerobic respiration (Gonzalez-Flecha and Demple, 1995; Imlay, 2003), but have also been used effectively as survival strategies by intracellular pathogens. Since some evidence suggests that *L. pneumophila* might be faced with an oxidative burst following ingestion by neutrophils, macrophages, and amoebae (Halablab *et al.*, 1990), protection against ROIs could play a crucial role in pathogenesis. Early studies have characterized catalase, peroxidase, and superoxide dismutase (SOD) activities that are not consistent throughout all legionellae (Hoffman *et al.*, 1983; Pine *et al.*, 1984 and 1986). In *L. pneumophila*, the oxidative defense system has been studied in some detail and includes two bifunctional catalase-peroxidases (*katA* and *katB*) (Bandyopadhyay and Steinman, 1998 and 2000), both iron (Fe) and copper-zinc (CuZn) superoxide dismutases (SODs) encoded by *sodB* and *sodC*, respectively (Sadosky *et al.*, 1994; St John and Steinman, 1996), and an alkyl hydroperoxide reductase (*ahpC*) (Rankin *et al.*, 2002).

### 2.5.4.1. Superoxide Dismutases

Superoxide dismutases (SODs) are ubiquitous metalloenzymes that catalyze the reduction of superoxide by the following reaction: $[2\bullet O_2^- + 2H^+ \rightarrow H_2O_2 + O_2]$. Other than their role in detoxifying the $\bullet O_2^-$ generated during aerobic respiration (Gort and Imlay, 1998; Imlay, 2003 and 2006), SODs have also been implicated in the resistance to the oxidative burst in macrophages and neutrophils. Iron or manganese cofactors are generally associated with SODs found in the cytoplasm, whereas copper-zinc SODs are

found in the periplasm. Since $\bullet O_2^-$ can not cross bacterial inner membranes, SODs are thought to act locally in the compartment in which they reside (Fridovich, 1997). Unlike *sod* genes in other organisms (Carlioz and Touati, 1986; Nakayama, 1992), *L. pneumophila sodB* (encoding FeSOD) was found to be an essential gene, suggesting that the periplasmic CuZnSOD is unable to compensate for the lack of FeSOD (Sadosky *et al.*, 1994). Similarily, the cytoplasmic FeSOD does not compensate for the absence of the CuZnSOD (St John and Steinman, 1996). Nonetheless, a strain of *L. pneumophila* expressing multiple copies of *sodB* was shown to increase protection against redox cycling compounds in comparison to the wild-type strain (Sadosky *et al.*, 1994). Using a reporter assay, *L. pneumophila sodB* was found to be constitutively expressed and not induced during oxidative stress or growth in macrophages (Sadosky *et al.*, 1994). Therefore, the cytoplasmic enzyme SodB is thought to provide protection against $\bullet O_2^-$ generated during normal metabolism. However, some cytosolic SODs have been implicated in virulence, such as SodA of *M. tuberculosis*, which is secreted into the host phagosomes to scavenge ROIs (Braunstein *et al.*, 2003).

Other than cytoplasmic SODs, some bacteria also possess periplasmic SODs that have received much attention due their ability to protect against exogenously generated $\bullet O_2^-$ as observed with the oxidative burst generated by phagocytes (Imlay, 2003 and 2006; Fridovich, 1997). In fact, CuZnSODs have been implicated in the intracellular survival of numerous pathogens (De Groote *et al.*, 1997; Gee *et al.*, 2005; Krishnakumar *et al.*, 2004; Piddington *et al.*, 2001). Resistance to the oxidative burst was particularly evident in these organisms due to susceptibility to exogenous $\bullet O_2^-$ generated, the enhanced ROI-dependent microbicidal activity in activated macrophages, and reduced

killing in NADPH oxidase-deficient mice or use of the NADPH oxidase inhibitor aponycin (Piddington *et al.*, 2001; Gee *et al.*, 2005). Furthermore, CuZnSODs may lessen the formation of peroxynitrite, a strong oxidant that is formed in the phagosomes (De Groote *et al.*, 1997). In contrast to the role of periplasmic SOD in resistance to the oxidative burst observed in other intracellular pathogens, the *L. pneumophila sodC* mutant lacking the periplasmic CuZnSOD shows wild-type intracellular growth kinetics in macrophages, even when the respiratory burst is deliberately induced by PMA (St John and Steinman, 1996). In contrast, decreased $\bullet O_2^-$ levels were observed in macrophages infected with *L. pneumophila* incubated with recombinant *L. pneumophila* CuZnSOD (Walti *et al.*, 2002).

Though the *L. pneumophila sodC* mutant is not enfeebled for intracellular growth, it does display a marked decrease in viability during stationary phase (St John and Steinman, 1996). Consistently, CuZnSOD activity was increased during this period (St John and Steinman, 1996). CuZn-SODs have been found to be expressed during stationary phase in other bacteria such as *Caulobacter cresentus* (Schnell and Steinman, 1995) and upon the RpoS-mediated up-regulation of *sodC* in *E. coli* and *Salmonella* (Benov and Fridovich, 1994; Imlay and Imlay, 1996; Gort *et al.*, 1999). Like *L. pneumophila*, *C. crescentus* possesses both FeSOD and CuZnSOD (Schnell and Steinman, 1995). Since *C. crescentus* cohabits with plants that might shed ROI-generating molecules (like polyphenols) into pond water, protection against exogenously generated $\bullet O_2^-$ might play an important role in environmental survival (Schnell and Steinman, 1995). On the other hand, Gort *et al.* (1999) showed that *E. coli* and *S. typhimurium sodC* mutants are no more susceptible to exogenously generated $\bullet O_2^-$, but

display an increased susceptibility to $H_2O_2$ during stationary phase. Therefore, CuZnSODs might also play a role in the detoxification of endogenous $\bullet O_2^-$ (Gort *et al.* 1999; Korshunov and Imlay, 2006), yet the exact nature of cytoplasmic protection from a periplasmic CuZnSOD remains unclear.

### 2.5.4.2. Bifunctional Catalase-Peroxidases

Other than the detoxification of $\bullet O_2^-$ by SODs, the elimination of $H_2O_2$ and other peroxides might also play a crucial role in resistance to the oxidative burst in macrophages. Since $H_2O_2$ diffuses through membranes (Seaver and Imlay, 2001a and b), it can potentially lead to protein oxidation and DNA damage through $Fe^{2+}$-catalyzed formation of •OH (Imlay, 2003 and 2006). The decomposition of $H_2O_2$ is generally performed by heme-containing hydroperoxidases. These enzymes can be classified in three groups: monofunctional catalases (like KatE of *E. coli*), monofunctional peroxidases, and bifunctional catalase-peroxidases (like *E. coli* KatG) (Chelikani *et al.*, 2004). Catalase activity, the ability to disproportionate $H_2O_2$ [$2H_2O_2 \rightarrow 2H_2O + O_2$], can be distinguished from peroxidatic activity [$AH_2$ (donor) + $H_2O_2 \rightarrow$ A (oxidized donor) + $2H_2O$] (Chelikani *et al.*, 2004). Obviously, bifunctional catalase-peroxidases display both activities. Two genes, *katA* and *katB*, have been identified in *L. pneumophila* genomes and are annotated as bifunctional catalase-peroxidases (Amemura-Maekawa *et al.*, 1999; Bandyopadhyay and Steinman, 1998 and 2000). *L. pneumophila* does not possess a monofunctional catalase.

Using zymograms, Bandyopadhyay and Steinman (1998 and 2000) demonstrated that KatA and KatB exhibit predominantly peroxidatic activities, whereas catalase activity is weak (Bandyopadhyay and Steinman, 1998 and 2000). In contrast to other

known catalase-peroxidases (Gee *et al.*, 2004; Loewen, 1996; Manca *et al.*, 1999; Steinman *et al.*, 1997), when *katA* or *katB* mutants, or the *katA katB* double mutants, were subjected to a disk diffusion assay, the mutants were modestly sensitive to $H_2O_2$ compared to the wild-type strain (Bandyopadhyay and Steinman, 1998 and 2000). Furthermore, the weak differences observed during the disk diffusion assay brought into question whether these findings were significant. It is possible to acquire mutations that render the catalase activity of catalase-peroxidases non-functional while the peroxidase activity remains (Chelikani *et al.*, 2004). For example, the periplasmic catalase-peroxidase (KatP) of enterohemorhagic *E. coli* O157:H7 demonstrates strong catalase activity (Varnado *et al.*, 2004), whereas KatY of *Yersinia pestis* displays predominantly peroxidase activity (Gracia *et al.*, 1999). Over twenty years ago, work by Pine *et al.* (1984 and 1986) demonstrated that *L. pneumophila* strains had no catalase activity but were peroxidase positive, whereas other *Legionella* species (*L. gormanii*, *L. micdadei*, and others) were catalase positive and peroxidase negative. Enzymatic analysis using purified proteins should be performed to validate presence (or absence) of catalase and peroxidase activities in both KatA and KatB.

Though KatA and KatB have been reported to be functionally similar, differences were observed in both their expression and cellular localization (Bandyopadhyay and Steinman, 1998 and 2000). Expression of *katB* is maximal during exponential phase and then declines in stationary phase, whereas *katA* expression was increased in stationary phase (Bandyopadhyay and Steinman, 1998 and 2000). By analogy to what is found for *E. coli*, expression of catalase-peroxidase (KatG) activity might be increased during exponential phase due to increased $H_2O_2$ during aerobic respiration (Gonzalez-Flecha and

Demple, 1997; Jenkins *et al.*, 1988; Loewen, 1996). Though not observed for either of the single mutants, the *katA katB* double mutant showed slower exponential growth with a two-fold increase in doubling time (Bandyopadhyay and Steinman, 2000). On the other hand, unlike *E. coli katG*, expression of *katA* and *katB* is not inducible by exogenous $H_2O_2$ (Bandyopadhyay and Steinman, 1998 and 2000). This difference be explained by the slower doubling time of *L. pneumophila* (~ 2h) compared to *E. coli* (~ 30 min), where *L. pneumophila* might not require such a rapid induction to cope with $H_2O_2$ derived from cellular respiration (Bandyopadhyay and Steinman, 1998).

Consistent with expression of *katA* in stationary phase, a *katA* mutant is enfeebled for stationary-phase survival (Bandyopadhyay and Steinman, 2000), as observed for the *sodC* mutant (St John and Steinman, 1996). Similarly, the catalase-peroxidase of *C. cresentus* is known to be important for viability during stationary phase (Steinman *et al.*, 1997). The catalase activity of periplasmic catalase-peroxidases might ensure the removal of $H_2O_2$ before it damages inner-membrane or cytoplasmic targets (Amemura-Maekawa *et al.*, 1999; Bandyopadhyay and Steinman, 1998 and 2000; Bandyopadhyay *et al.*, 2003). The peroxidase activity of catalase-peroxidases may also provide other essential functions by reducing reactive peroxides likely produced by the reactive nitrogen intermediate $ONOO^-$ that could result in oxidation of membrane components.

Since catalase activity in other organisms had been shown to play an important role in the resistance to the NADPH oxidase-derived antimicrobial products (Master *et al.*, 2001; Ng *et al.*, 2004), *L. pneumophila* mutants lacking catalase-peroxidase were assayed in a macrophage infection model. *L. pneumophila katA*, *katB*, and *katA katB* mutants were all defective for intracellular growth compared to the wild-type strain

(Bandyopadhyay and Steinman, 1998 and 2000). It was thus proposed that *L. pneumophila* KatA and KatB play crucial roles for intracellular survival by coping with ROIs generated by the oxidative burst of host macrophages. Furthermore, Bandyopadhyay *et al.* (2003) demonstrated that *katA* and *katB* mutants are not only enfeebled for growth in murine macrophages and protozoa, but display a phenotype that resembles that of *dotA* and *dotB* mutants (Joshi *et al.*, 2001; Sturgill-Koszycki and Swanson, 2000). In fact, phagosomes containing *katA* or *katB* mutants quickly acquired LAMP-1, yet were still able to evade fusion with lysosomes (Bandyopadhyay *et al.*, 2003). Unlike wild-type, the *katA* and *katB* mutants are also resistant to NaCl, a phenotype correlated with avirulent strains like *dot/icm* mutants (Vogel *et al.*, 1996). Since evasion of the endocytic pathway is a hallmark of *L. pneumophila* virulence, the data presented by Bandyopadhyay *et al.* (2003) seemed to suggest that KatA and KatB might play crucial roles in pathogenesis. Indeed, Bandyopadhyay *et al.* (2003) hypothesized that the catalase-peroxidases may ensure a redox state that permits disulfide-bond formation in Dot/Icm components, leading to assembly of a functional complex. Alternatively, KatA and KatB might influence the cellular redox potential or protect certain proteins involved in respiration, where mutants would be unable to generate sufficient energy to promote transition to the replicative phase (Bandyopadhyay *et al.*, 2003). These hypotheses have not yet been confirmed.

It has been proposed that the *L. pneumophila* KatA and KatB catalase-peroxidases might provide catalase activity to detoxify the phagosomal milieu to promote intracellular growth. It is curious that during challenges with $H_2O_2$, the *katA*, *katB*, and *katA katB* mutants were as sensitive as the wild-type strain (Bandyopadhyay and Steinman, 1998

and 2000), calling into question whether KatA and KatB are truly required for the decomposition of $H_2O_2$. KatA and KatB might provide some undefined activity during intracellular growth, like the decomposition of lipid peroxides or peroxynitrite by means of their peroxidase activity. Alternatively, upon loss of catalase function by inactivation of one gene, it is possible that compensatory expression of other antioxidant enzymes might mask their roles in $H_2O_2$ detoxification (Sherman *et al.*, 1996). Due to the fact that the *L. pneumophila katA katB* mutant was viable during aerobic growth and showed wild-type sensitivities to $H_2O_2$, it is likely that other enzymes are responsible for peroxide detoxification in the cell.

#### 2.5.4.3. Alkyl Hydroperoxide Reductases

Members of the ubiquitous family of peroxiredoxins (Prxs) such as AhpCs have received much attention over the years due to their ability to exert hydroperoxidase activity ($ROOH + 2e^- \rightarrow H_2O + ROH$), where organic hydroperoxides are reduced to alcohols (Hofmann *et al.*, 2002; Poole, 2005; Poole *et al.*, 2000; Wood *et al.*, 2003). Since the first report of AhpCs in the late 1980s, these enzymes have been recognized as detoxifiers of $H_2O_2$, organic (lipid) peroxides, and peroxynitrite (Antelmann *et al.*, 1996; Baillon *et al.*, 1999; Baker *et al.*, 2001; Bryk *et al.*, 2000; Bsat *et al.*, 1996; Charoenlap *et al.*, 2005; Christman *et al.*, 1985; Fukumori and Kishii, 2001; Jacobson *et al.*, 1989; Johnson *et al.*, 2004; Master *et al.*, 2002; Mongkolsuk *et al.*, 2000; Olczak *et al.*, 2002; Poole and Ellis, 1996; Rocha *et al.*, 2000; Seaver and Imlay, 2001; Springer *et al.*, 2001; Storz *et al.*, 1989; Tartaglia *et al.*, 1990). Though AhpCs display lower catalytic efficiencies than do catalases ($K_m$ values in the μM range versus in the mM range, respectively), they have been shown to play a crucial role in peroxide detoxification

(Seaver and Imlay, 2001). For example, due to its high affinity, *E. coli* AhpC (and not catalase) is the primary scavenger of endogenously generated $H_2O_2$ (Seaver and Imlay, 2001). Besides protecting cells against peroxides generated during aerobic respiration, AhpC may also protect the cell against exogenous ROIs, such as those produced by the oxidative burst of phagocytic cells (Shi and Ehrt, 2006). In this regard, AhpCs are likely candidates for peroxide-scavenging enzymes in *L. pneumophila*. Many peroxide-detoxifying systems involving AhpC have been well characterized (Hofmann *et al.*, 2002; Poole, 2005; Poole *et al.*, 2000; Wood *et al.*, 2003).

Consistent with the role of AhpCs in peroxide detoxification, *ahpC* mutants in a wide range of organisms are sensitive to peroxides, peroxynitrite, and ROI-producing compounds. Loss of AhpC function is also associated with increased lipid peroxidation, suggesting that AhpC may play an important role in protection of membranes against oxidant-induced damage (Master *et al.*, 2002; Moreau *et al.*, 2001; Wang *et al.*, 2004). The protective role of AhpC against ROIs is further emphasized by its contribution to aerotolerance in microaerophiles (Baillon *et al.*, 1999; Olczak *et al.*, 2002; Wang *et al.*, 2004) and anaerobes (Reynolds *et al.*, 2002; Rocha *et al.*, 2000). The catalytic mechanism involves two highly conserved cysteine residues in AhpC which form an intermolecular disulfide bond within the dimer (Figure 5) and can be reduced by various organism-specific systems (Figure 6). It should be noted that, apart from its role in peroxide detoxification, some less conventional tasks have been attributed to AhpC. These include the prevention of catalase inactivation by organic hydroperoxides like tert-butyl hydroperoxide (tBOOH) (Wang *et al.*, 2004) and involvement as a molecular chaperone (Chuang *et al.*, 2006). Chaperone functions were observed during conditions

of oxidative stress in which AhpC forms high-molecular-weight complexes that refold proteins that could have been damaged in conditions of oxidative stress (Chuang *et al.*, 2006). Interestingly, dimeric, decameric and dodecameric crystal structures of AhpC have been reported (Guimaraes *et al.*, 2005; Hofmann *et al.*, 2002; Wood *et al.*, 2002).

Following exposure to oxidative stress, expression of *ahpC* is induced (Antelmann *et al.*, 1996; Bsat *et al.*, 1996; Charoenlap *et al.*, 2005; Dosanjh *et al.*, 2005; Fukumori and Kishii, 2001; Jacobson *et al.*, 1989; Mongkolsuk *et al.*, 1997; Morgan *et al.*, 1986; Ochsner *et al.*, 2000; Rocha *et al.*, 2000; Zheng *et al.*, 2001). It should be noted that genes encoding the disulfide reductases (*ahpD* and *ahpF*) involved in the reduction of oxidized AhpC are generally found immediately adjacent to *ahpC* and the two genes are transcribed as a polycistronic message. A notable exception was observed in *Xanthomonas campestris*, where *ahpC* and *ahpF/oxyR* transcripts are distinct (Mongkolsuk *et al.*, 1997).

AhpC has been implicated in virulence in some organisms. For example, *M. tuberculosis* mounts a defense against the oxidative burst in host phagocytes by producing both KatG and AhpC (Manca *et al.*, 1999; Sherman *et al.*, 1995; Shi and Ehrt, 2006; Springer *et al.*, 2001). The lack of AhpC function in mycobacteria was correlated to decreased intracellular survival due to a failure to resist to ROIs and RNIs generated by host phagocytes (Shi and Ehrt, 2006; Springer *et al.*, 2001). Furthermore, to remain virulent, catalase-peroxidase mutants of *M. tuberculosis* must compensate by overproducing AhpC (Dhandayuthapani *et al.*, 1996; Heym *et al.*, 1997; Sherman *et al.*, 1996). In *L. pneumophila*, Rankin *et al.* (2002) showed that *ahpC* (termed *ahpC1* in this study) levels increase during intracellular growth in macrophages, consistent with a

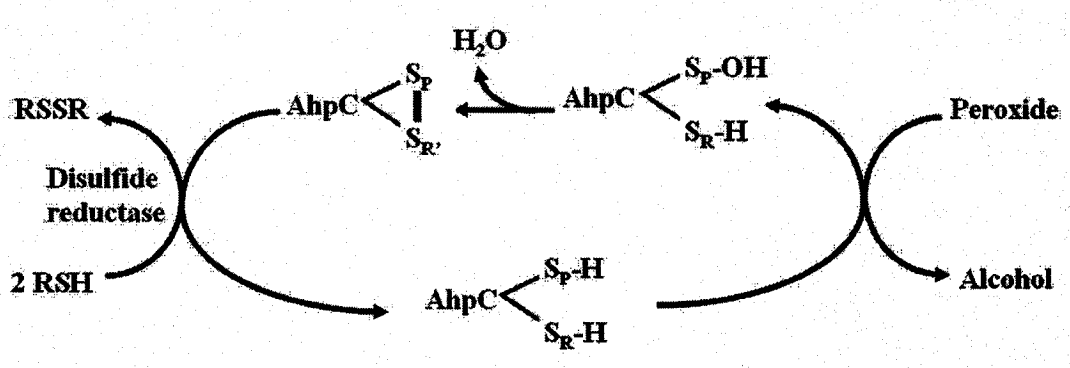



**Figure 5** Peroxide reduction by AhpC. The peroxidase reaction carried out by AhpC involves two conserved cysteine residues: the peroxidatic (Cys-$S_P$H) and resolving (Cys-$S_R$H) cysteines (Ellis and Poole, 1996; Wood *et al.*, 2003). In the first step, the redox-active Cys-$S_P$H attacks the peroxide substrate and is oxidized to a sulfenic acid (Cys-$S_P$OH). Second, the Cys-$S_P$OH from one subunit is attacked by the Cys-$S_R$H of the other subunit. A condensation reaction then results in an intersubunit disulfide bond which must then be reduced by disulfide reductases to regain the peroxidase function of AhpC (Poole *et al.*, 2000; Bryk *et al.*, 2002). The reduction of the intramolecular disulfide bond of oxidized AhpC is mediated by NADPH- or NADH-dependent reactions involving redox partners such as AhpF, AhpD, or thioredoxin, all containing CXXC motifs involved in this process (Jacobson *et al.*, 1989; Hillas *et al.*, 2000; Bryk *et al.*, 2002; Hahn *et al.*, 2002; Koshkin *et al.*, 2003). Reduction of the disulfide reductases completes the catalytic cycle. The peroxidatic cysteine ($S_P$) is depicted as a thiol ($S_P$-H), sulfenic acid ($S_P$-OH) or in a disulfide bond with the resolving cysteine ($S_R$). Disulfide reductases (like AhpF, AhpD, and thioredoxin) are oxidized from the dithiol state (2 RSH) to a disulfide state (RSSR) during AhpC reduction.

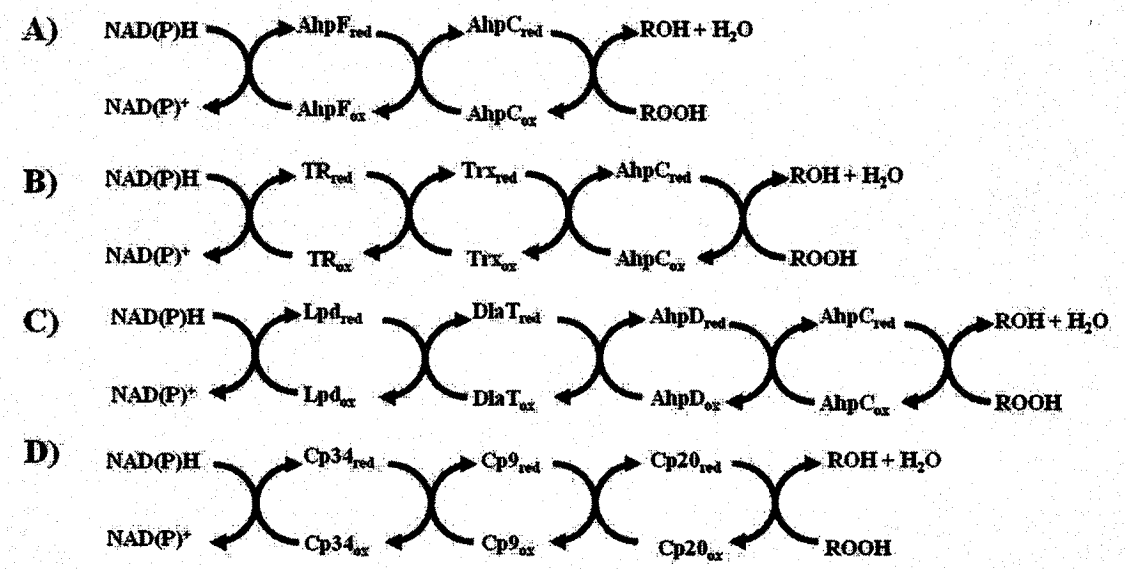



**Figure 6** Disulfide reductase cascades involved in reduction of oxidized AhpC. A) In organisms like *E. coli*, the peroxidase system consists of two components: AhpC and the flavoprotein AhpF, which reduces AhpC in an NAD(P)H-dependent reaction (Jacobson *et al.*, 1989; Poole and Ellis, 1996; Storz *et al.*, 1989; Tartaglia *et al.*, 1990). B) In *H. pylori*, AhpC reduction is catalyzed by thioredoxin (Trx), which is in turn reduced by thioredoxin reductase (TR) (Baker *et al.*, 2001). C) In organisms like *M. tuberculosis*, reduction of AhpC is achieved by a more complex system involving AhpD, dihydrolipoamide acetyltransferase (DlaT, formerly annotated as SucB for dihydrolipoamide succinyltransferase), and dihydrolipoamide dehydrogenase (Lpd) (Hillas *et al.*, 2000; Jaeger *et al.*, 2004; Hahn *et al.*, 2002; Koshkin *et al.*, 2004; Tian *et al.*, 2005). D) In *Clostridium pasterianum* (Reynolds *et al.*, 2002), the NAD(P)H-dependent reduction of peroxides is performed by a three-component system consisting of a peroxiredoxin (Cp20), a glutaredoxin-like protein (Cp9), and a flavoprotein (Cp34). Abbreviations: oxidized (ox); reduced (red); hydroperoxide (ROOH); alcohol (ROH).

putative protective role in detoxification of ROIs in the phagosomal milieu. Without data to support a role in peroxide detoxification, it was concluded that *L. pneumophila* AhpC1 plays a redundant role since it is dispensable for intracellular growth in macrophages (Rankin *et al.*, 2002). It is possible that redundant roles of KatA, KatB, and/or other uncharacterized peroxide-detoxifying enzymes revealed in the *L. pneumophila* genome such as methionine sulfoxide reductases (MsrA), the organic hydroperoxide resistance protein (Ohr) (Stadtman, 2004) or the second uncharacterized AhpC (AhpC2) could be sufficient to protect the cell from ROIs generated in host cells. Alternatively, compensatory expression of antioxidant enzymes could mask the role of AhpC1 during *L. pneumophila* infection. Enhanced *ahpC* expression has been shown to play a compensatory role following inactivation of genes encoding other antioxidant enzymes (Antelmann *et al.*, 1996; Bsat *et al.*, 1996; Charoenlap *et al.*, 2005; Loprassert *et al.*, 2003; Mongkolsuk *et al.*, 2000). Compensatory antioxidant responses are generally mediated by redox-sensitive transcription factors like OxyR.

### 2.5.5. Genetic Responses to Oxidative Stress

Bacteria possess redox-sensitive transcriptional regulators enabling them to cope with oxidative stress (Storz and Imlay, 1999; Mongkolsuk and Helman, 2002). OxyR in enteric bacteria has been viewed as a paradigm model for the protection against peroxide stress (Storz and Imlay, 1999; Mongkolsuk and Helman, 2002). Pretreatment of exponentially growing *E. coli* and *S. typhimurium* cells with sublethal doses of $H_2O_2$ induces an adaptive response that provides resistance to normally lethal doses of $H_2O_2$ and other oxidants (Altuvia *et al.*, 1994; Christman *et al.*, 1985; Morgan *et al.*, 1986). This response is mediated by OxyR-dependent activation of transcription of several genes

including *katG*, *ahpCF*, *dps* (encoding a Fe-chelating DNA-binding protein), *trxC* (thioredoxin), *grxA* (glutaredoxin), *gorA* (glutathione reductase), *fur* (ferric uptake regulator), and the small non-coding *oxyS* RNA which allows the indirect regulation of additional genes including *rpoS* (Altuvia *et al.*, 1994, 1997 and 1998; Christman *et al.*, 1985; Morgan *et al.*, 1986; Ritz *et al.*, 2000; Storz *et al.*, 1989; Tartaglia *et al.*, 1989; Toledano *et al.*, 1994). Many of these gene products have clear roles in antioxidant defences such as the detoxification of peroxide by KatG and AhpCF or the protection by Dps of oxidative damage to DNA. *oxyR* mutants have dramatically increased peroxide sensitivities and rates of spontaneous mutagenesis and lipid peroxidation (Christman *et al.*, 1985; Storz *et al.*, 1990). Furthermore, the peroxide sensitivity is emphasized by the fact that isolated colonies of *oxyR* mutants do not appear on LB medium that is not supplemented with catalase or incubated anaerobically (Hassett *et al.*, 2000).

OxyR is a 34-kDa protein that forms dimers and tetramers in solution (Kullik *et al.*, 1995b; Storz *et al.*, 1990; Zheng *et al.*, 1998). Like other members of the LysR family of bacterial regulators, OxyR possesses an N-terminal helix-turn-helix DNA-binding motif and is known to negatively autoregulate its own expression (Choi *et al.*, 2001; Christman *et al.*, 1989; Zheng and Storz, 2000; Storz *et al.*, 1990). Since exposure to $H_2O_2$ does not influence levels of OxyR, activation of the OxyR regulon was thought to occur by modification of preexisting OxyR (Storz *et al.*, 1990). It is now known that activation of OxyR requires oxidation of two cysteine residues, Cys199 and Cys208 (Kullik *et al.*, 1995; Zheng *et al.*, 1998). Oxidation of Cys199 forms a sulphenic acid (-SOH) intermediate that eventually leads to an intramolecular disulfide bond with Cys208 (Zheng *et al.*, 1998). Conformational change resulting from formation of the disulfide

bond results in refolding of the regulatory domain, and consequently, oxidized OxyR promotes binding of $\sigma^{70}$-containing RNAP to promoters of the OxyR regulon (Choi *et al.*, 2001; Kullik *et al.*, 1995a and b; Storz *et al.*, 1990; Tao *et al.*, 1993; Toledano *et al.*, 1994). Transcriptional activation is thought to occur by interaction of OxyR with the C-terminal domain of the α subunit of RNAP (Tao *et al.*, 1993).

Transcriptional activation by OxyR is halted by reduction of the Cys199-Cys208 disulfide bond (Zheng *et al.*, 1998). In a transcription assay, addition of DTT inhibited OxyR-dependent activation of *ahpC* and *katG* transcription, whereas removal of DTT restored its activity (Christman *et al.*, 1989; Storz *et al.*, 1990; Tao *et al.*, 1991). OxyR has been shown to be preferentially reduced by glutathione (GSH) and by the disulfide reductase glutaredoxin (GrxA) (Zheng *et al.*, 1998). Interestingly, both *grxA* and glutathione reductase (GorA) are are part of the OxyR regulon, suggesting the process is autoregulated (Zheng *et al.*, 1998). It should also be noted that reduction of OxyR is slower than oxidation, enabling the regulator to ensure adequate protection against oxidative stress (Aslund *et al.*, 1999; Tao, 1999). Toledano *et al.* (1994) demonstrated that both oxidized and reduced OxyR are able to bind to target promoters, but only oxidized OxyR promotes transcriptional activation. OxyR binds DNA as a dimer of dimers to four major grooves in DNA (Figure 7). In its reduced form, OxyR aligned with a stretch of DNA with five helical turns and a bend in the third turn, whereas oxidized OxyR aligned with a stretch of four helical turns (Figure 7) (Choi *et al.*, 2001; Toledano *et al.*, 1994). The switch from reduced to oxidized OxyR seems to be accomplished by a rotation in the OxyR monomers, resulting in occupation of different DNA sites and presentation of RNA polymerase contact sites (Choi *et al.*, 2001).

Current models suggest that OxyR activation is sensitive and transient (Aslund *et al.*, 1999; Gonzalez-Flecha and Demple, 1997; Mongkolsuk and Helmann, 2002). Though OxyR responds to intracellular peroxides (Gonzalez-Flecha and Demple, 1997; Kim *et al.*, 2002; Seaver and Imlay, 2001), other stimuli have been proposed. Superoxide generators and S-nitrosothiols have also been shown to induce expression of OxyR-regulated genes, but it is not clear whether these compounds act on OxyR directly (Hausladen *et al.*, 1996; Mongkolsuk *et al.*, 1997; Zheng *et al.*, 2001b). Since the redox potential of OxyR (-185 mV) is higher than that of the *E. coli* cytoplasm (-280 mV), OxyR is found in its dithiol-reduced form in absence of oxidative stress. However, mutants lacking components of the glutaredoxin or thioredoxin systems exhibit activation of OxyR, even in absence of oxidative stress, suggesting that OxyR is activated by disulfide stress as a result of changes in the cellular redox status (Aslund *et al.*, 1999).

At the *oxyR* promoter of enteric bacteria, OxyR binds as a repressor both in oxidized and reduced states (Toledano *et al.*, 1994). In fact, $H_2O_2$ treatment has no influence on the levels of OxyR, suggesting that *oxyR* expression is not peroxide-inducible (Storz *et al.*, 1990). However, OxyR levels increase during exponential growth under the control of the cAMP-activated catabolite repressor protein (Crp), and decrease with transition into stationary phase through RpoS-dependent processes (Gonzalez-Flecha and Demple, 1997a). Increased levels of *oxyR* transcripts during exponential growth are also correlated with enhanced in KatG and AhpCF activities (Gonzalez-Flecha and Demple, 1997b). In *Streptomyces coelicolor*, growth-phase-dependent expression of *oxyR* (and the divergently expressed *ahpC2D* operon) is also observed, where levels increased during early exponential phase (Hahn *et al.*, 2002). Surprisingly,

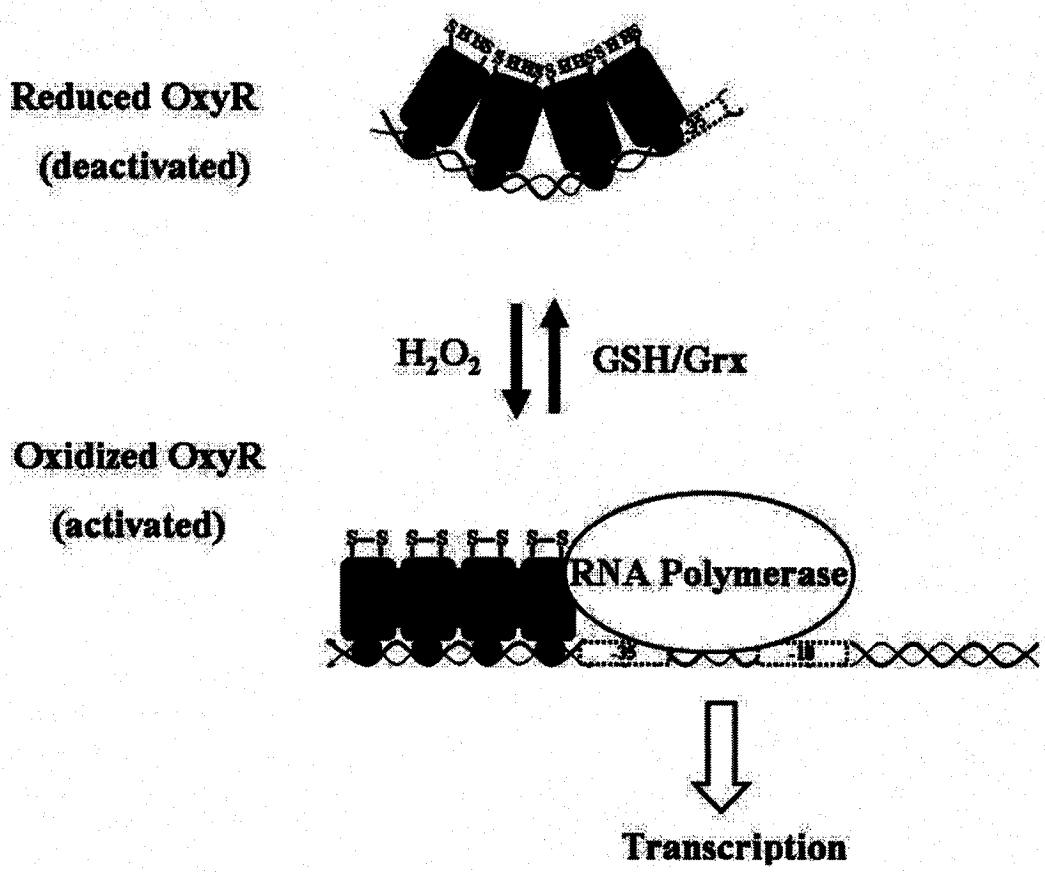



**Figure 7** Transcriptional activation of the OxyR regulon. Oxidation of OxyR by $H_2O_2$ or other oxidants, or changes in the redox status of the cell, results in an intramolecular disulfide bond in each of the four monomers. The resulting conformational change promotes cooperative binding of RNA polymerase and transcription of OxyR-regulated genes. OxyR is inactivated by reduction of the disulfide bond by glutathione (GSH) and glutaredoxin (Grx). OxyR binds DNA in both oxidized and reduced states, yet only oxidized OxyR leads to transcription. In fact, oxidized OxyR was shown to expose the -35 sequences of target promoters, while at its own promoter, the hidden -35 sequence ensured repression (Toledano *et al.*, 1994).

*S. coelicolor* OxyR was shown to act as a transcriptional activator of its own gene in response to peroxide, unlike all other previously identified OxyR homologs; therefore, the regulation of *oxyR* may differ among organisms.

Besides OxyR, numerous regulators have been implicated in the regulation of antioxidant defenses. For example, *E. coli* SoxRS is a well-established system that responds to superoxide stress by increasing the expression of genes for antioxidant enzymes (*sodA*), repair mechanisms (*nfo*), and metabolic products that are more resistant to oxidative stress (*fumC* and *acnA*) (Storz & Imlay, 1999). In *B. subtilis*, a Fur homolog called PerR is responsible for induction of *katA* and *ahpCF* in response to $H_2O_2$ or under conditions of iron deficiency (Bsat *et al.*, 1998; Chen *et al.*, 1995). In *X. campestris* and others, OhrR regulates the organic hydroperoxide resistance (*ohr*) gene in response to lipid peroxides (Fuangthong *et al.*, 2001; Panmanee *et al.*, 2006). To further complicate the pathways of regulation, there can be cross-talk among various regulons (Storz and Imlay, 1999). For example, *E. coli* RpoS is not only important for stationary-phase physiology, but has also been shown to regulate the expression of several antioxidant genes including *katE*, *katG*, *sodC*, *gorA*, and *dps* during stressful conditions (Storz & Imlay, 1999). A second example is the regulation of *fur* by both SoxRS and OxyR (Zheng *et al.*, 1999). During oxidative stress, Fur levels increase to promote repression of iron uptake, which could enhance damage by peroxide *via* Fenton chemistry (Zheng *et al.*, 1999).

In conclusion, numerous antioxidant enzymes provide both periplasmic (SodC and KatA) and cytoplasmic (SodB, KatB, and AhpC) protection against ROIs in *L. pneumophila*. Though much has been learned about its catalase-peroxidases and SODs,

little effort has been undertaken to characterize its AhpCs. AhpCs are well-established detoxifiers of $H_2O_2$ and lipid peroxides in other organisms, and thus could play essential roles in aerobic metabolism and intracellular survival. In fact, *L. pneumophila* is likely faced with ROIs generated during uptake by phagocytes, suggesting that mechanisms used to resist ROIs might promote intracellular survival. In addition, characterization of key players in the peroxide-stress response might be of significance for prevention of *L. pneumophila* infections, since $H_2O_2$ is used to treat problematic bacterial biofilms (Stewart *et al.*, 2000). The *L. pneumophila* genome reveals that this organism contains two *ahpC* genes (*ahpC1* and *ahpC2*), yet only AhpC1 had previously been identified (Rankin *et al.*, 2002). This study was initiated to characterize both AhpC1 and AhpC2 and evaluate whether the previously uncharacterized *L. pneumophila* OxyR homolog could regulate *ahpC* expression in this organism.

# Chapter 3: Materials and Methods

## 3.1. Phylogenetic Analysis

AhpC or OxyR sequences used in phylogenetic analysis were obtained from GenBank by BLASTP search using AhpC1 or OxyR of *L. pneumophila* Philadelphia-1. Pairwise and multiple sequence alignments of unedited protein sequences were performed using ClustalX and consensus sequences generated using BioEdit version 7.0.0. Phylogenetic trees were viewed using TreeView version 1.6.6.

## 3.2. Bacterial Strains and Growth Conditions

All bacterial strains and plasmids used in this study are listed in Table 1.

### 3.2.1. *Escherichia coli*

*E. coli* cells were grown at 37°C in Luria-Bertani (LB) broth or Mueller-Hinton (MH) broth. Sixteen or thirteen grams of agar per litre were included for LB or MH medium. *E. coli* strains J1377 and GS077 were generously provided by Dr. James A. Imlay (University of Illinois, Urbana, IL) and Dr. Gisela Storz (National Institutes of Health, Bethesda, Maryland), respectively. Cells of theses strains were grown at 37°C under anaerobic conditions using the BD GasPak™ EZ (Becton Dickinson, Oakville, ON) and AnaeroGen™ anaerobic atmosphere generation system (Oxoid, Ltd., Nepean, ON). *E. coli* GS077 or derivatives harboring plasmids were the only strains grown in MH broth and on MH solid medium. Proper antibiotic selection for *E. coli* strains was maintained as follows: 100 μg/ml ampicillin; 40 μg/ml kanamycin; 10 μg/ml gentamicin; 20 μg/ml chloramphenicol; 20 μg/ml metronidazole. For transformations of pBlueScript-derived vectors, 40 μl of a 20 mg/ml solution of 5-bromo-4-chloro-3-indolyl-β-D-

galactopyranoside (X-gal) in N, N-dimethylformamide (DMF) was spread on each plate. Where appropriate, isopropyl-β-D-galactoside (IPTG) was added to a final concentration of 1 mM.

### 3.2.2. *Legionella pneumophila*

*Legionella pneumophila* Philadelphia 1 strain was obtained from the Center of Infectious Diseases, Centers for Disease Control (CDC), Atlanta GA. *L. pneumophila* Lp02 is a spontaneous streptomycin-resistant, restriction modification-null, thymidine auxotrophic derivative of *L. pneumophila* Philadelphia 1, which was obtained from Dr. Michele Swanson (University of Michigan Medical School, Ann Arbor, MI). Lp02 *dotB* deletion mutant JV918 was kindly provided by Dr. Joseph Vogel (Washington University, St. Louis, MO). *L. pneumophila* strains were grown at 37°C on buffered charcoal yeast extract (BCYE) or in buffered yeast extract (BYE) broth: 10 g yeast extract, 1 g [2-(2-amino-2-oxoethyl)-amino] ethanesulfonic acid (ACES), and 1 g α-ketoglutaric acid, with pH adjusted to 6.6-6.7 with 6 N KOH. BCYE solid medium included all components of BYE broth as well as 16g/l agar and 1.5 g/l of activated charcoal. After autoclaving, all media received 0.4 g/l L-cysteine pH 6.6-6.7, 0.25 g/l ferric pyrophosphate and 100 μg/ml thymidine. Where appropriate, media were supplemented as follows: 100 μg/ml streptomycin sulphate; 40 μg/ml kanamycin; 10 μg/ml gentamicin; 4 μg/ml chloramphenicol; 20 μg/ml metronidazole. Both *E. coli* and *L. pneumophila* frozen stocks were stored in nutrient broth supplemented with 10% dimethylsulfoxide (DMSO).

**Table 1** Bacterial strains and plasmids used in this study

| Bacterial strain or plasmid | Relevant properties | Reference or source |
|---|---|---|
| ***E. coli*** | | |
| DH5α | F⁻ Φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 endA1 recA1 hsdR17 deoR thi-1 supE44 gyrA96 relA1* | Clontech |
| J1377 | MG1655 F⁻ Δ(*katG17::Tn10*) Δ(*katE12::Tn10*) Δ*ahpCF' kan::'ahpF* ($Km^R$) | Seaver and Imlay, 2001 |
| J1377 ptrc | J1377 + pTrc99A ($Km^R$, $Amp^R$) | This study |
| J1377 ptrcC1 | J1377 + ptrcC1 ($Km^R$, $Amp^R$) | This study |
| J1377 ptrcC2 | J1377 + ptrcC2 ($Km^R$, $Amp^R$) | This study |
| J1377 ptrcD | J1377 + ptrcD ($Km^R$, $Amp^R$) | This study |
| BL21 Codon Plus | F⁻ *ompT hsdS* ($rB^-$ $mB^-$) $dcm^+$ $Tet^R$ *galλ* (DE3) *endA* Hte [*argU ileY leuW*] ($Cm^R$) | Stratagene |
| BL21 petLpOxyR | BL21 Codon Plus + petLpOxyR ($Amp^R$, $Cm^R$) | Stratagene |
| MG1655 | K-12, F- lambda- | ATCC 47076 |
| MG1655 pbad | MG1655 + pBAD22 ($Amp^R$) | This study |
| GS077 | MG1655-derived *oxyR::km* ($Km^R$) | Zheng *et al.*, 2001 |
| GS077 pbad | GS077 + pBAD22 ($Km^R$, $Amp^R$) | This study |
| GS077 pbadEcOxyR | GS077 + pbadEcOxyR ($Km^R$, $Amp^R$) | This study |
| GS077 pbadLpOxyR | GS077 + pbadLpOxyR ($Km^R$, $Amp^R$) | This study |
| ***L. pneumophila*** | | |
| Lp02 | Philadelphia-1 derivative, *rpsL* | Berger and |

| | | |
|---|---|---|
| | *hsdR thyA* ($Sm^R$) | Isberg, 1993 |
| Lp02 pJB908 | Lp02 + pJB908 ($Sm^R$, $Thy^+$) | This study |
| Lp02 pBH6119 | Lp02 + pBH6119 ($Sm^R$, $Thy^+$) | This study |
| Lp02 pC1gfp | Lp02 + pC1gfp ($Sm^R$, $Thy^+$) | This study |
| Lp02 pC2gfp | Lp02 + pC2gfp ($Sm^R$, $Thy^+$) | This study |
| Lp02 pC2(-179/+27)gfp | Lp02 + pC2(-179/+27)gfp ($Sm^R$, $Thy^+$) | This study |
| Lp02 pC2(-156/+27)gfp | Lp02 + pC2(-156/+27)gfp ($Sm^R$, $Thy^+$) | This study |
| Lp02 pC2(-132/+27)gfp | Lp02 + pC2(-132/+27)gfp ($Sm^R$, $Thy^+$) | This study |
| Lp02 pC2(-109/+27)gfp | Lp02 + pC2(-109/+27)gfp ($Sm^R$, $Thy^+$) | This study |
| Lp02 pC2(-91/+27)gfp | Lp02 + pC2(-91/+27)gfp ($Sm^R$, $Thy^+$) | This study |
| Lp02 pC2(-66/+27)gfp | Lp02 + pC2(-66/+27)gfp ($Sm^R$, $Thy^+$) | This study |
| Lp02 pOxyRgfp | Lp02 + pOxyRgfp ($Sm^R$, $Thy^+$) | This study |
| *ahpC1::gm* | Lp02 *ahpC1*mutant ($Sm^R$, $Gm^r$) | This study |
| *ahpC1::gm* pMMBahpC1 | *ahpC1::gm* + pMMBahpC1 ($Sm^R$, $Gm^R$, $Cm^R$ $Thy^+$) | This study |
| *ahpC1::km* | Lp02 *ahpC1*mutant ($Sm^R$, $Km^r$) | This study |
| *ahpC1::km* pMMBahpC1 | *ahpC1::km* + pMMBahpC1 ($Sm^R$, $Km^R$, $Cm^R$ $Thy^+$) | This study |
| *ahpC1::km* pJB908 | *ahpC1::km* + pJB908 ($Sm^R$, $Km^R$, $Thy^+$) | This study |
| *ahpC1::km* pC1 | *ahpC1::km* + pC1 ($Sm^R$, $Km^R$, $Thy^+$) | This study |
| *ahpC1::km* pBH6119 | *ahpC1::km* + pBH6119 ($Sm^R$, $Km^R$, $Thy^+$) | This study |

| | | |
|---|---|---|
| *ahpC1::km* pC2gfp | *ahpC1::km* + pC2gfp ($Sm^R$, $Km^R$, $Thy^+$) | This study |
| *ahpC1::km* pC2(-179/+27)gfp | *ahpC1::km* + pC2(-179/+27)gfp ($Sm^R$, $Km^R$, $Thy^+$) | This study |
| *ahpC1::km* pC2(-156/+27)gfp | *ahpC1::km* + pC2(-156/+27)gfp ($Sm^R$, $Km^R$, $Thy^+$) | This study |
| *ahpC1::km* pC2(-132/+27)gfp | *ahpC1::km* + pC2(-132/+27)gfp ($Sm^R$, $Km^R$, $Thy^+$) | This study |
| *ahpC1::km* pC2(-109/+27)gfp | *ahpC1::km* + pC2(-109/+27)gfp ($Sm^R$, $Km^R$, $Thy^+$) | This study |
| *ahpC1::km* pC2(-91/+27)gfp | *ahpC1::km* + pC2(-91/+27)gfp ($Sm^R$, $Km^R$, $Thy^+$) | This study |
| *ahpC1::km* pC2(-66/+27)gfp | *ahpC1::km* + pC2(-66/+27)gfp ($Sm^R$, $Km^R$, $Thy^+$) | This study |
| *ahpC1::km* pOxyRgfp | *ahpC1::km* + pOxyRgfp ($Sm^R$, $Km^R$, $Thy^+$) | This study |
| *ahpC2D::gm* | Lp02 *ahpC2D* mutant ($Sm^R$, $Gm^R$) | This study |
| *ahpC2D::gm* pMMBahpC2D | *ahpC1::gm* + pMMBahpC2D ($Sm^R$, $Gm^R$, $Cm^R$ $Thy^+$) | This study |
| *ahpC2D::km* | Lp02 *ahpC2D* mutant ($Sm^r$, $Km^r$) | This study |
| *ahpC2D::km* pMMBahpC2D | *ahpC1::km* + pMMBahpC2D ($Sm^R$, $Km^R$, $Cm^R$ $Thy^+$) | This study |
| *ahpC2D::km* pJB908 | *ahpC2D::km* + pJB908 ($Sm^R$, $Km^R$, $Thy^+$) | This study |
| *ahpC2D::km* pC2D | *ahpC2D::km* + pC2D ($Sm^R$, $Km^R$, $Thy^+$) | This study |
| *ahpC2D::km* pBH6119 | *ahpC2D::km* + pBH6119 ($Sm^R$, $Km^R$, $Thy^+$) | This study |
| *ahpC2D::km* pC1gfp | *ahpC2D::km* + pC1gfp ($Sm^R$, $Km^R$, $Thy^+$) | This study |

| | | |
|---|---|---|
| *ahpC2D::km* pOxyRgfp | *ahpC2D::km* + pOxyRgfp ($Sm^R$, $Km^R$, $Thy^+$) | This study |
| *magA::km* | Lp02 *magA* mutant ($Sm^R$, $Km^R$) | Hiltz *et al.*, 2004 |
| JV918 | Lp02 *dotB* mutant ($Sm^R$) | Sexton *et al.*, 2004 |
| MB 379 | Lp02 *rpoS::km* ($Sm^R$, $Km^R$) | Bachman and Swanson, 2001 |
| MB 379 pBH6119 | MB 379 + pBH6119 ($Sm^R$, $Km^R$, $Thy^+$) | This study |
| MB 379 pOxyRgfp | MB 379 + pOxyRgfp ($Sm^R$, $Km^R$, $Thy^+$) | This study |
| MB 414 | Lp02 *letA-22* ($Sm^R$) | Hammer *et al.*, 2002 |
| MB 414 pBH6119 | MB 414 + pBH6119 ($Sm^R$, $Thy^+$) | This study |
| MB 414 pOxyRgfp | MB 414 + pOxyRgfp ($Sm^R$, $Km^R$, $Thy^+$) | This study |
| *ihfA::gm/ihfB::km* | Lp02 *ihfA::gm/ihfB::km* ($Sm^R$, $Km^R$, $Gm^R$) | Morash, 2006 |
| *ihfA::gm/ihfB::km* pBH6119 | *ihfA::gm/ihfB::km* + pBH6119 ($Sm^R$, $Km^R$, $Gm^R$, $Thy^+$) | This study |
| *ihfA::gm/ihfB::km* pOxyRgfp | *ihfA::gm/ihfB::km* + pOxyRgfp ($Sm^R$, $Km^R$, $Gm^R$, $Thy^+$) | This study |
| **Plasmids** | | |
| pBlueScript II KS (+) | High-copy cloning vector | Stratagene |
| pBS::P1/P4Δ*ahpC1* | pBlueScript-derived vector containing upstream (P1/P2) and downstream (P3/P4) sequences of *ahpC1* | This study |

| | | |
|---|---|---|
| pBSΔ*ahpC1::km* | pBS::P1/P4Δ*ahpC1* with kanamycin-resistance cassette of p34S::*km3* inserted in the *Bam*HI site between up- and downstream regions of *ahpC1* | This study |
| pBSΔ*ahpC1::gm* | pBS::P1/P4Δ*ahpC1* with gentamycin-resistance cassette of pPH1J1 inserted in the *Bam*HI site between up- and downstream regions of *ahpC1* | This study |
| pBS::P1/P4Δ*ahpC2D* | pBlueScript-derived vector containing upstream (P1/P2) and downstream (P3/P4) sequences of *ahpC2D* | This study |
| pBSΔ*ahpC2D::km* | pBS::P1/P4Δ*ahpC2D* with kanamycin-resistance cassette of p34S::*km3* inserted in the *Bam*HI site between up- and downstream regions of *ahpC1* | This study |
| pBSΔ*ahpC2D::gm* | pBS::P1/P4Δ*ahpC2D* with gentamicin-resistance cassette of pPH1J1 inserted in the *Bam*HI site between up- and downstream regions of *ahpC2D* | This study |
| pBS::P1/P4Δ*oxyR* | pBlueScript-derived vector containing upstream (P1/P2) and downstream (P3/P4) sequences of *oxyR* | This study |
| pBSΔ*oxyR::km* | pBS::P1/P4Δ*oxyR* with kanamycin-resistance cassette of | This study |

| | | |
|---|---|---|
| | p34S::*km3* inserted in the *Bam*HI site between up- and downstream regions of *oxyR* | |
| pBSΔ*oxyR::gm* | pBS::P1/P4Δ*oxyR* with gentamicin-resistance cassette of pPH1J1 inserted in the *Bam*HI site between up- and downstream regions of *oxyR* | This study |
| pBSC2PROM | Subcloning of $P_{ahpC2}$ (PCR-generated FC2PROM/RC2PROM amplicon) into *Eco*RI/*Bam*HI sites of pBlueScript | This study |
| p34S::*km3* | Origin of the kanamycin-resistance cassette | Dennis and Zylstra,1998 |
| pPH1J1 | Origin of the gentamicin-resistance cassette | Hirsch and Beringer, 1984 |
| pBRDX | Suicide delivery vector with *B. subtilis sacB* (conferring sucrose sensitivity) and *H. pylori rdxA* (conferring sensitivity to metronidazole), chloramphenicol acetyltransferase | Morash, 2006 |
| pBRDXΔ*ahpC1::km* | *ahpC1::km* suicide delivery vector | This study |
| pBRDXΔ*ahpC1::gm* | *ahpC1::gm* suicide delivery vector | This study |
| pBRDXΔ*ahpC2D::km* | *ahpC2D::km* suicide delivery vector | This study |
| pBRDXΔ*ahpC2D::gm* | *ahpC2D::gm* suicide delivery vector | This study |
| pBRDXΔ*oxyR::km* | *oxyR::km* suicide delivery vector | This study |
| pBRDXΔ*oxyR::gm* | *oxyR::gm* suicide delivery vector | This study |

| | | |
|---|---|---|
| pTrc99A | IPTG-inducible expression vector | Amersham |
| ptrcC1 | pTrc99A-derived vector, *ahpC1* under control of the P*trc* promoter | This study |
| ptrcC2 | pTrc99A-derived vector, *ahpC2* under control of the P*trc* promoter | This study |
| ptrcD | pTrc99A-derived vector, *ahpD* under control of the P*trc* promoter | This study |
| pMMB206 | Derivative of pRSF1010; $P_{tac}$ promoter and IPTG-inducible *lacI*$^q$ system; $Amp^R$, $Cm^R$ | Morales *et al.*, 1991 |
| pMMBahpC1 | pMMB206 containing *ahpC1* under control of $P_{tac}$ | This study |
| pMMBahpC2D | pMMB206 containing *ahpC2D* under control of $P_{tac}$ | This study |
| pMMBoxyR | pMMB206 containing *oxyR* under control of $P_{tac}$ | This study |
| pJB908 | pKB5-derivative, RSF1010 ori *lacI*$^q$ P*tac oriTΔ13*, thymidylate synthase (*tdΔi*) | Sexton *et al.*, 2004 |
| pC1 | pJB908 + *grlAahpC1*, *trans*-complement for *ahpC1::km* | This study |
| pC2D | pJB908 + *ahpC2D*, *trans*-complement for *ahpC2D::km* | This study |
| pBH6119 | RSF1010 ori, promoterless *gfpmut3*, thymidylate synthase (*tdΔi*) | Hammer & Swanson, 1999 |
| pC1gfp | *ahpC1* promoter region upstream of *gfpmut3*of pBH6119 | This study |
| pC2gfp or pC2(-199/+27) | $P_{ahpC2}$ region -199 to +27 cloned upstream of *gfpmut3* of pBH6119 | This study |

| | | |
|---|---|---|
| pC2(-179/+27)gfp | $P_{ahpC2}$ region -179 to +27 cloned upstream of *gfpmut3* of pBH6119 | This study |
| pC2(-156/+27)gfp | $P_{ahpC2}$ region -156 to +27 cloned upstream of *gfpmut3* of pBH6119 | This study |
| pC2(-132/+27)gfp | $P_{ahpC2}$ region -132 to +27 cloned upstream of *gfpmut3* of pBH6119 | This study |
| pC2(-109/+27)gfp | $P_{ahpC2}$ region -109 to +27 cloned upstream of *gfpmut3* of pBH6119 | This study |
| pC2(-91/+27)gfp | $P_{ahpC2}$ region -91 to +27 cloned upstream of *gfpmut3* of pBH6119 | This study |
| pC2(-66/+27)gfp | $P_{ahpC2}$ region -66 to +27 cloned upstream of *gfpmut3* of pBH6119 | This study |
| pOxyRgfp | $P_{oxyR}$ cloned upstream of *gfpmut3* of pBH6119 | This study |
| pET29b | IPTG-inducible T7 expression vector | Novagen |
| petLpOxyR | pET29B-derived overexpression vector for $His_6$-LpOxyR | This study |
| pBAD22 | Arabinose-inducible expression vector | Guzman *et al.*, 1995 |
| pbadEcoxyR | pBAD22 expression vector for *E. coli oxyR* | This study |
| pbadLpoxyR | pBAD22 expression vector for *L. pneumophila oxyR* | This study |

$Amp^R$, $Km^R$, $Gm^R$, $Sm^R$, and $Cm^R$ indicate resistance markers to ampicillin, kanamycin, gentamicin, streptomycin, and chloramphenicol, respectively. $Thy^+$ indicates presence of the thymidylate synthase gene enabling the organism to grow in absence of added thymidine.

## 3.3. Molecular Techniques

### 3.3.1. Restriction Endonuclease Digestion

Restriction digestion was performed in 25-µl reactions according to instructions provided by the manufacturer (New England Biolabs [NEB], Ipswich, MA). Unless otherwise indicated, restriction digests were generally incubated at 37°C for 60 to 90 min in a reaction consisting of approximately 1 µg of DNA, the appropriate 1 x reaction buffer, 1 x bovine serum albumin (BSA), and recommended units (U) of restriction enzyme(s). Blunt ends were created by subsequent treatment with 1 µl of T4 DNA polymerase and 1 µl of 10 mM deoxynucleoside triphosphates (dNTPs) (Life Technologies, Burlington, ON) for 30 min at room temperature. When single digests were performed on plasmids, 5'-phosphoryl groups were removed by directly adding 2 µl of calf intestinal phosphatase (CIP) (NEB) into the reaction mixture following restriction endonuclease digestion and incubation at 37°C for 30 min. All reactions were stopped with loading buffer (200 mM Tris-HCl, pH 7.5, 200 µM ethylenediaminetetraacetic acid [EDTA] pH 8.0, 20% glycerol and 0.25% xylene cyanol) (Sambrook and Russell, 2001) and subjected to gel electrophoresis as described below.

### 3.3.2. Agarose Gel Electrophoresis

Electrophoresis of DNA was performed in 1 x Tris acetate EDTA (TAE) buffer, diluted from a 50 x stock, per liter: 242 g Tris base, 57 ml glacial acetic acid, and 100 ml 0.5M EDTA pH 8.0, using 1% agarose gels containing 1 x TAE buffer and 1 µg/ml ethidium bromide. Loading buffer was added to DNA samples prior to loading into the wells of the gel. Agarose gels were subjected to electrophoresis at 125 V/cm for desired

amounts of time. For visualization of DNA, a UV transilluminator (Fotodyne, WI) was used and photographs were captured using Polaroid Polapan 667 black and white film or using a BioRad Gel Doc 2000 System equipped with a CCD camera and the Quantity One software version 4.6 provided by the manufacturer. DNA bands of interest were excised from gels using a clean scalpel blade.

### 3.3.3. Purification of DNA from Agarose Gels

Purification of DNA fragments from agarose gels for subcloning was carried out with a QIAquick gel purification kit (Qiagen Inc., Mississauga, ON) as decribed by the manufacturer following the protocol using a microcentrifuge. For every 100 mg of agarose, 100 µl of QG solubilization and binding buffer was added and agarose was placed at 50°C until the gel slice had completely dissolved. To ensure optimal DNA adsorption and recovery, 10 µl of 3 M sodium acetate and 1 volume of isopropanol were added to the mixture before being loaded into the spin column. After centrifugation (13,000 rpm, 1 min), DNA was washed with 750 µl of buffer PE. The flow-through was discarded and columns were centrifuged for an additional minute. DNA was eluted in 30 µl of elution buffer. To obtain more-concentrated DNA, a MinElute Gel Extraction kit (Qiagen) was used under the same conditions with the following exceptions: 600 µl of buffer QG was added for every 100 µl of sample and final elution was performed in 10 µl. All DNA was stored at -20°C.

### 3.3.4. DNA Ligation

DNA ligations were performed using T4 DNA ligase (NEB). Generally, reactions consisted of equal amounts of DNA, 1 µl of T4 DNA ligase, 5 µl of 4 x T4 DNA ligase

buffer, with final volume was adjusted to a 20 µl with $ddH_2O$. For blunt end ligations, an insert/vector ratio of 10:1 was used. All ligations were conducted at room temperature overnight.

### 3.3.5. Polymerase Chain Reaction

Polymerase chain reaction (PCR) amplifications were performed on a Gene Amp PCR system 2400 (Perkin Elmer, Norwalk, CT) using either *Taq* DNA polymerase (MBI Fermentas, Burlington, ON) or Expand High Fidelity PCR System (Roche Diagnostics, Mississauga, ON). All oligonucleotides (Table 2) synthesized by Integrated DNA Technologies, Inc. (IDT) (Coralville, IA). Typical 25-µl reactions consisted of 0.2 mM dNTPs (GE Heathcare, Baie d'Urfé, QC), 1.5 mM $MgCl_2$, 1x Taq reaction buffer with $(NH_4)_2SO_4$, 1 µM of each primer (Table 2), 0.5 U of *Taq* DNA polymerase (or 1.3 U of enzyme mixture provided in the Expand High Fidelity PCR System), and approximately 50 ng of template DNA. Unless otherwise indicated, the following conditions were used: 94°C (5 min) followed by 35 cycles of 94°C (30 s), 55°C (30 s), and 72°C (1 min per kb of amplicon) and a final extension at 72°C for 7 min. For colony PCR, bacterial cells were harvested using a sterile toothpick, placed in 100 µl of $ddH_2O$, boiled for 10 min, and centrifuged at 13,000 rpm at room temperature to remove cell debris. One microlitre was used as template in PCR reactions. Amplicons were stored at 4°C for use within 24 h, or at -20°C for long-term storage.

**Table 2** Oligonucleotides used in this study

| Name | Sequence (5'→ 3') |
|---|---|
| P1C1 | GCC TCG AGT TCA GTG TAT TGA GGC TTG |

| | |
|---|---|
| | TG (XbaI) |
| P2C1 | CGC GGA TCC AAC TCG GAA GGG CAT ACA AAG (BamHI) |
| P3C1 | CGT GGA TCC GTT GAG GTG GTT GCT GTA TC (BamHI) |
| P4C1 | GCT CTA GAA GTA ACC CAG GAG TTT GAT AG (XhoI) |
| FC1OUT | TCC CAA GAT GCC TCA ATG CG |
| RC1OUT | ATG ATA AAC AAG TGG CGA ATG C |
| FC1INT | TTG CTC TTG ATC ACC GTA TTG |
| RC1INT | CAA TAC GGT GAT CAA GAG CAA |
| P1C2D | CAG CTC GAG GTA CTT GCC ATT TCT CAT TGC (XbaI) |
| P2C2D | CGT GGA TCC TTC AGT AGG GCA GAC AAA GG (BamHI) |
| P3C2D | GCT GGA TCC GCA TTG GCT GTG AGT TAT TCT (BamHI) |
| P4C2D | GAC TCT AGA TTT AGC TGT CAT GAT CCA TGC (XhoI) |
| FC2DOUT | TCT GGC TTT TGG ATT GTA GTT C |
| RC2DOUT | CAC AAG TAA TGG GAA AGC AAT G |
| FC2DINT | GAA GTG GCT AAA GAC ATA C |
| RC2DINT | GTA TGT CTT TAG CCA CTT C |
| P1OXYR | CAT CTC GAG GCT GAG TGC GGA AAT ATG (XbaI) |
| P2OXYR | TCC GGA TCC ACC CCC AAA CGC AAT TCA (BamHI) |
| P3OXYR | GAC GGA TCC ATT TTG GCG AGC AGG AAC (BamHI) |
| P4OXYR | GGA TCT AGA AGT GTA TTA TGT TCG GGA |

| | |
|---|---|
| | C (XhoI) |
| FOXYROUT | See FLPOXYRPROM |
| ROXYRINT | AGT TCG GGC ATT ACC AAG |
| FKM | GAC GGA TCC ACG TTG TGT CTC AAA ATC TCT GA (BamHI) |
| RKM | GAC GGA TCC CGG GTA CCG AGC TCT TAG AA (BamHI) |
| FGM | TTA GGT GGC GGT ACT T |
| RGM | TTG ACA TAA GCC TGT TCG G |
| FC1TRANS | GCC GGA TCC TCA CCC ACC GCT AAT GGA (BamHI) |
| RC1TRANS | CTG AAG CTT ACA AGG ACT CGG AGT GCT (HindIII) |
| FC2DTRANS | TGG GGA TCC GAG GAG AAA ACT TAT GAT TA (BamHI) |
| RC2DTRANS | GGG AGG CTT TAT AGC GTG CGG CGT CAT (HindIII) |
| FC1PTRC | GTC GGA TCC ATG AGT GTT TTA GTT GGG C (BamHI) |
| RC1PTRC | CGT AAG CTT TTA CAA GGA CTC GGA ATG (HindIII) |
| FC2PTRC | GTC GGA TCC ATG ATT ACG GTA GGC AAC (BamHI) |
| RC2PTRC | CGT AAG CTT TTA TTT GAT ATG AAT TGT TTC T (HindIII) |
| FDPTRC | CCG GGA TCC ATG TTA GAG AAT GTT AAA G (BamHI) |
| RC2PTRC | CCG AAG CTT TTA TTT GAT TCG ACC ACT C (HindIII) |
| FC1PROM | GGC GAA TTC GTG CCA TTA CTG AGC GAT |

| | |
|---|---|
| | TC (EcoRI) |
| RC1PROM | GGC GGA TCC GAG TGC CTT TCA TGT AGA GC (BamHI) |
| FC2DPROM | GGC GAA TTC ACC AAT ATT CCT CCA CAG GC (EcoRI) |
| RC2DPROM | GGC GGA TCC CTG GAA ATT TGT TGC CTA CCG (BamHI) |
| RGFP | ACC ATA ACC GAA AGT AGT GAC |
| FGRLRT | GAG CCT ATG TTG CGT GAT G |
| RC1RT | TAA CTC GGA AGG GCA TAC AA |
| FC2RT | TGC TTT ACA GAC CGA TGA AC |
| RDRT | AAC CAT TGA GAG CAG AAA C |
| FRPLJQRT | TGG TGT TTA TCT TCG TGT GGT |
| RRPLJQRT | CAA TGA ATA CTG GAC CTA CAA G |
| FC1QRT | TTG CTC TTG ATC ACC GTA TTG |
| RC1QRT | AGT AAA TCT CAC AGG CCC T |
| FC2QRT | CGG AAG TGG CTA AAG ACA TAC |
| RC2QRT | TGC CTA AAG AAT AAC TCA CAG C |
| FGRLQRT | CCA AGA TGC CTC AAT GCG G |
| RGRLQRT | GTG GTA AAA CTT GAC GAA TAT C |
| FDQRT | CGG AAG TGG CTA AAG ACA TAC |
| RDQRT | TGC CTA AAG AAT AAC TCA CAG C |
| FC1COMP | GGC GGA TCC GTG CCA TTA CTG AGC GAT TC (BamHI) |
| FC2COMP | GGC GGA TCC ACC AAT ATT CCT CCA CAG GC (BamHI) |
| FBLA | TCC GTG TCG CCC TTA TTC C |
| RBLA | AAC TAC GAT ACG GGA GGG C |
| FLPOXYRPET | CAT ATG AAT TTA AGA GAT TTA CAT TAT (NdeI) |

| | |
|---|---|
| RLPOXYRPET | CTC GAG TGC TAA TTT GGA TTG AAC ATT TT (XhoI) |
| FLPOXYRPROM | GCT GAA TTC GAT GGC ACA AAG AGT TGC (EcoRI) |
| RLPOXYRPROM | CTG GGA TCC TGC TTT ACA TCT GCC AGG (BamHI) |
| FC2PROM179 | GGC GAA TTC CCT TCT TTA AAA GTA GTT TAT AAA A (EcoRI) |
| FC2PROM156 | GGC GAA TTC AAA CAT AAA AGC CAA ATA TTT TGC (EcoRI) |
| FC2PROM132 | GGC GAA TTC AAA ATG GAT TAA TAA TTC TTA TAT TG (EcoRI) |
| FC2PROM109 | GGC GAA TTC TTG ATT GAC TTT ATT TAT CGT CTT (EcoRI) |
| FC2PROM91 | GGC GAA TTC CGT CTT TAT AAA AAC AAT TGA TTT T (EcoRI) |
| FC2PROM66 | GGC GAA TTC ATT TAT TAA ATG ATT TCA CCT AAA TT (EcoRI) |
| FECAHPCPROM | GGC ACT GAA GAT ACC AAA GG |
| RECAHPCPROM | TCG ATG AGA TGT AAG GTA ACC |
| FECKATGPROM | CGA AAT GAG GGC GGG AAA AT |
| RECKATGPROM | CGA TAC ACA GCG TTA GAG AG |
| FECOXYRPROM | GGT GCC GCT CCG TTT CTG |
| RECOXYRPROM | GCT GGC TAA CGT GGC AGG |
| Ac-FLPOXYRPROM | Acrydite-CGA TGG CAC AAA GAG TTG CA |
| Ac-RLPOXYRPROM | Acrydite-ACC AAT ATT CCT CCA CAG GC |
| FECOXYRPBAD | GGC GAA TTC ATT ATG AAT CGT GAT CTT GAG (EcoRI) |
| RECOXYRPROM | GGC AAG CTT AAA CCG CCT GTT TTA AAA CTT (HindIII) |

| | |
|---|---|
| FLPOXYRPBAD | GGC GAA TTC ATT ATG AAT TTA AGA GAT TTA CAT TAT (EcoRI) |
| RLPOXYRPBAD | GGC AAG CTT CTA TGA TAA TTT GGA TTG AAC ATT (HindIII) |

Underlined nucleotides represent restriction endonuclease cleavage sites which are indicated in parentheses.

### 3.3.6. Isolation of Genomic DNA

Genomic DNA was isolated from *L. pneumophila* by placing two loopfuls of overnight growth collected from BCYE solid medium into 440 µl of Tris-EDTA buffer (TE) (10 mM Tris-HCl pH 8.0 and 1 mM EDTA pH 8.0). Fifty microlitres of proteinease K (10 mg/ml in 50 mM Tris-HCl pH 8.0 and 1 mM $CaCl_2$) and 10 µl of 10% sodium dodecyl sulfate (SDS) were added. After 1-2 h of incubation at 37°C with gentle rocking, the mixture was sequentially extracted with equal volumes (500 µl) of: i) buffer-saturated phenol; ii) phenol/chloroform 1:1 (v/v); and iii) chloroform/isoamyl alcohol 24:1 (v/v). Between each extraction step, samples were subjected to centrifugation (13,000 rpm, 10 min, 4°C) and supernatants were transferred to the next microtubes. The genomic DNA was then precipitated overnight at -80°C with 0.1 volumes of 0.3 M sodium acetate pH 5.0 and 2 volumes absolute ethanol. Precipitated DNA was spooled on a Pasteur pipette, washed with ice-cold 70% ethanol, and air dried (for ~10 min). Genomic DNA was resuspended in 0.5 ml of 8 mM NaOH, quantitated by spectrophotometry, and stored at 4°C until use.

### 3.3.7. Plasmid Isolation

Plasmid isolations were performed using QIAprep Spin Miniprep kit (Qiagen) following instructions provided by the manufacturer, or using a standard alkaline lysis method as described elsewhere (Sambrook and Russell, 2001). For the miniprep kit, 5-ml *E. coli* cultures were grown overnight, harvested by centrifugation (4000 x *g*, 6 min, room temperature), and resuspended in 250 µl buffer P1. Following addition of 250 µl buffer P2, solutions were mixed by inversion until clarification. Three hundred microlitres of neutralization buffer N3 was added, solutions were mixed by inversion, and solutions were centrifuged at 13,000 rpm for 10 min at room temperature. The resulting supernatants were added to columns and subjected to centrifugation at 13,000 rpm for 1 min. After addition of 750 µl of PE wash buffer, columns were subjected to centrifugation again and the flow-through was discarded. The columns were centrifuged once more under the same conditions to remove residual ethanol. Plasmid DNA was eluted in EB buffer in a final volume of 50 µl.

For larger quantities of plasmid, isolation was performed using a standard alkaline lysis method (Sambrook and Russell, 2001). Briefly, 50-ml *E. coli* cultures were grown overnight at 37°C with agitation (150 rpm). Bacterial cells were harvested by centrifugation (4000 x *g*, 6 min, room temperature), were resuspended in 2 ml of cell resuspension solution (50 mM glucose, 25 mM Tris-HCl pH 8.0, and 10 mM EDTA) and placed on ice for 5 min. Four millilitres of freshly prepared alkaline lysis solution (0.2 N NaOH, 1% SDS) was added and solutions were mixed by inversion until clarification. Following 5 min incubation on ice, 3 ml of neutralization solution (3 M sodium acetate, pH 5) was added and solutions were mixed by inversion until precipitation had occurred.

The solutions were placed at -80°C until frozen, thawed on ice, and centrifuged (4000 x *g*, 15 min, 4°C). The resulting supernatants (approximately 7.5 ml) were transferred to a fresh tube. After addition of 4.5 ml of ice-cold isopropanol, solutions were once again placed at -80°C until frozen. After thawing on ice, the solutions were centrifuged (4000 x *g*, 10 min, 4°C) and washed twice with 70% ethanol (4000 x *g*, 6 min, 4°C). The resulting pellet was resuspended in 1 ml of TE buffer and divided into two microtubes. One microlitre of 10 mg/ml ribonuclease (RNase) was added to each tube and incubated for 30 min at 37°C. The contents of each tube were then phenol-chloroform extracted as described above (see preparation of genomic DNA). After overnight precipitation at -80°C, DNA was pelleted by centrifugation (13,000 rpm, 15 min, 4°C). DNA pellets were washed four times with 70% ethanol (13,000 rpm, 10 min, 4°C) to remove salts. The final pellet was air dried for 10 min and resuspended in 30 µl of sterile dd$H_2O$.

### 3.3.8. Determination of Bacterial Optical Density

Bacterial optical density (OD) was determined at 600 nm ($OD_{600}$) using an Eppendorf xenon flash lamp BioPhotometer (Brinkmann Instruments Ltd., Missisauga, ON) or at 620 nm ($OD_{620}$) using a Unico UV-2100 spectrophotometer. For agar plate grown bacteria, cells were resuspended in 1 ml of phosphate-buffered saline (PBS) [137 mM NaCl, 2.7 mM KCl, 10 mM $Na_2HPO_4$, and 2 mM $KH_2PO_4$] or appropriate culture medium, and vortexed until a homogenous suspension was obtained. Samples were diluted as required, transferred to a cuvette, and $OD_{600}$ or $OD_{620}$ was determined. For broth cultures, 1-ml samples were removed, washed by centrifugation (4,000 × *g*, 6 min) and suspended in fresh medium (or dd$H_2O$) before $OD_{600}$ or $OD_{620}$ determination.

### 3.3.9. Preparation of *E. coli* Rubidium Chloride Competent Cells

Five-millilitre *E. coli* cultures were grown overnight at 37°C with agitation (150 rpm). These cultures were subcultured 1:20 into 50 ml of pre-warmed LB broth and cultures were grown to an $OD_{620}$ of ~0.6. Bacterial cells were harvested by centrifugation (3,000 x *g*, 15 min, 4°C) and incubated for 5 min on ice in 40 ml cold transformation buffer 1 [30 mM potassium acetate, 100 mM rubidium chloride ($RbCl_2$), 10 mM $CaCl_2 \cdot H_2O$, 50 mM $MnCl_2 \cdot 4H_2O$, and 15% glycerol]. After centrifugation under the same conditions, the bacterial pellets were resuspended in 4 ml of transformation buffer 2 [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]. Bacterial cells were aliquoted into 200 µl fractions and stored at -80°C until use.

#### 3.3.9.1. Transformation of *E. coli*

Transformation of $RbCl_2$-competent cells was performed as follows. Two hundred microlitres of competent cells were thawed on ice prior to addition of 10 µl of ligation mixture. Cells were then placed at 37°C for 90 s, on ice for 5 min then added to 1 ml of pre-warmed LB broth for 1 h incubation at 37°C with slow agitation (100 rpm). Cells were then harvested by centrifugation (4000 x *g*, 6 min, 4°C) and resuspended in 111 µl of LB broth. Various volumes (1, 10, 100 µl) of bacterial suspensions were spread onto LB solid medium containing the appropriate selection agent and incubated overnight at 37°C. Colonies were replica-plated onto fresh LB solid medium and once again incubated overnight at 37°C. Transformants were confirmed by PCR analysis and restriction endonuclease digestion of harboured plasmids.

### 3.3.10. Cloning of *L. pneumophila* Genes for Expression in *E. coli*

To determine whether *L. pneumophila ahpC1* and *ahpC2* were functional in *E. coli*, both of these genes were expressed under the control of the IPTG-inducible promoter of pTrc99A in a catalase/peroxidase-deficient *E. coli* strain J1377 (obtained from Dr. James A. Imlay, University of Illinois, Urbana, IL). Similar procedures were taken for complementation *E. coli oxyR::km* strain GS077 (generously provided by Dr. Gisela Storz, National Institutes of Health, Bethesda, MD) with *E. coli* MG1655 *oxyR* (termed EcOxyR) or *L. pneumophila oxyR* (termed LpOxyR) using the arabinose-inducible expression systems of pBAD22 (Guzman *et al.*, 1995).

#### 3.3.10.1. Cloning of *L. pneumophila ahpC1* and *ahpC2* into pTrc99A

To generate IPTG-inducible expression vectors, *L. pneumophila ahpC1* and *ahpC2* genes were amplified using primer pairs FC1PTRC/RC1PTRC (for *ahpC1*) and FC2PTRC/RC2PTRC (for *ahpC2*). Following restriction digestion with BamHI and HindIII, amplicons were ligated into similarly restricted pTrc99A. The resulting constructs (ptrcC1 and ptrcC2) were transformed into *E. coli* DH5α cells and verified by PCR analysis using the primers named above. Plasmids were transformed into electrocompetent *E. coli* J1377 cells. Ampicillin-resistant transformant strains were confirmed by PCR analysis using the primers described above and the FBLA/RBLA primers for the ampicillin-resistance cassette. PCR-positive transformants were named J1377 ptrc (empty-vector control), J1377 ptrcC1, and J1377 ptrcC2.

### 3.3.10.2. Cloning of *E. coli* and *L. pneumophila oxyR* into pBAD22

To generate arabinose-inducible expression vectors, amplicons generated from PCR reactions using primers pairs FECOXYRPBAD/RECOXYRPBAD (for EcOxyR) and FLPOXYRPBAD/RLPOXYRPBAD (for LpOxyR), were digested with EcoRI and HindIII and cloned into EcoRI/HindIII-cleaved pBAD22. The resulting constructs (pbadEcOxyR and pbadLpOxyR) were transformed into *E. coli* DH5α cells and verified by PCR analysis using the primers described above. Plasmids were extracted and electroporated into *E. coli* GS077. Ampicillin-resistant *E. coli* GS077 transformants were confirmed by PCR analysis and termed GS077 pbadEcOxyR and GS077 pbadLpOxyR, respectively. The pBAD22 empty vector was transformed into wild-type *E. coli* MG1655 and GS077 cells, verified by PCR analysis using primer pair FBLA/RBLA, and the resulting strains termed MG1655 pBAD22 and GS077 pBAD22, respectively.

### 3.3.10.3. Disk Diffusion Assay

To determine if *L. pneumophila ahpC1* and *ahpC2* are able to complement the catalase/peroxidase-deficient *E. coli* strain J1377, a standard disk diffusion assay was performed. Cultures of all strains were grown to mid-exponential phase ($OD_{620}$ of ~0.6) and cells corresponding to 0.1 $OD_{620}$ were inoculated into 4 ml of pre-warmed 0.75% LB (or MH) top agar and poured onto LB (or MH) ampicillin-containing solid medium with or without 1 mM IPTG. Once the medium had solidified, 10 µl of 3% or 30% $H_2O_2$ was placed on sterile ¼-inch antibiotic disks that were placed in the center of the plates. The cultures were then incubated at 37°C overnight under anaerobic conditions and the resulting zones of inhibition were measured and reported as the mean ± standard deviation (SD) for three independent experiments. Disk diffusion assays for

complementation of *E. coli oxyR::km* (GS077) were performed following the same procedure with the following exceptions: i) expression of *E. coli oxyR* or *L. pneumophila oxyR* from respective pBAD22-derived plasmids was induced with various concentrations of arabinose (0, 0.0002, 0.002, 0.02, 0.2%) or repressed using 0.2% glucose for 60 min in broth prior to being inoculated into LB top agar and poured onto LB (or MH) solid medium containing arabinose or glucose; ii) expression of EcOxyR and LpOxyR was confirmed by SDS-PAGE.

### 3.3.11. Preparation of *E. coli* and *L. pneumophila* Electrocompetent Cells

From frozen stocks, overnight 5-ml *E. coli* cultures were subcultured 1:50 into 250 ml of LB and allowed to grow to an $OD_{620}$ of ~0.5. From frozen stocks, cells of *L. pneumophila* strains were grown at 37°C for 48 h on BCYE medium with appropriate selection conditions and transferred to fresh media for an additional 24 h incubation before use. The resulting bacterial lawns were removed from medium using sterile disposable loops and placed in 20 ml of sterile $ddH_2O$. *E. coli* and *L. pneumophila* cells were harvested by centrifugation (3,000 x *g*, 10 min, 4°C) and resuspended as follows: i) in 20 ml of cold 15% glycerol; ii) two washes in 10 ml 15% glycerol; and iii) a final resuspension in 200 µl of cold 15% glycerol. Forty-microlitre portions were stored at -80°C.

#### 3.3.11.1. Electroporation of Plasmids into *E. coli* or *L. pneumophila*

Electrocompetent *E. coli* or *L. pneumophila* cells were thawed on ice and approximately 10 µg of plasmid DNA was added. Cells/DNA mixtures were loaded into 2-mm gapped electroporation cuvettes and placed on ice for 5 min. Electroporation was

performed at 2.1 kV, 200 Ω, 25 μFD. Contents of the cuvette were immediately transferred to 1 ml of pre-warmed media (for *E. coli*: pre-warmed LB without antibiotics; for *L. pneumophila*: BYE without antibiotics, ferric pyrophosphate or L-cysteine) and incubated at 37°C for 1 h with gentle agitation (100 rpm). One hundred microlitres of the transformation was then spread on medium (LB or BCYE, respectively) with appropriate selection and incubated at 37°C for 24 h (for *E. coli*) or 4-6 days (for *L. pneumophila*). The resulting colonies were replica plated onto fresh medium and incubated overnight at 37°C. The resulting transformants were confirmed by PCR analysis.

### 3.3.12. Construction of *L. pneumophila* Mutants

Chromosomal deletion mutants were created as described in Morash (2006). Briefly, upstream and downstream sequences of the target genes were amplified by PCR. Between the two fragments, a kanamycin- or gentamicin-resistance marker was inserted. The resulting construct was inserted into a suicide delivery plasmid for allelic recombination in *L. pneumophila*.

#### 3.3.12.1. Construction of *ahpC1*, *ahpC2D*, and *oxyR* Mutants

Approximately 500 bp of upstream (P1/P2) and downstream (P3/P4) flanking DNA sequences of the respective gene were amplified by PCR (Figure 8). Primers pairs P1C1/P2C1 and P3C1/P4C1 were used for *ahpC1*, P1C2D/P2C2D and P3C2D/P4C2D for *ahpC2D*, and P1OXYR/P2OXYR and P3OXYR/P4OXYR for *oxyR*. The P1/P2 amplicons encoded XbaI and BamHI restriction sites and P3/P4 amplicons encoded BamHI and XhoI restriction sites, respectively, thereby permitting sequential ligation into pBlueScript II KS (Stratagene, La Jolla, CA). The resulting plasmids were termed

pBSΔ*ahpC1*, pBSΔ*ahpC2D*, and pBSΔ*oxyR*, respectively. Between the upstream and downstream regions of *ahpC1*, *ahpC2D*, and *oxyR*, BamHI restriction and subsequent ligation of the kanamycin-resistance determinants of p34s-*km3* (Dennis, and Zylstra, 1998) [or gentamicin-resistance cassette of pPH1J1 (Hirsch and Beringer, 1984)] resulted in plasmids pBSΔ*ahpC1::km* (or pBSΔ*ahpC1::gm*), pBSΔ*ahpC2D::km* (or pBSΔ*ahpC2D::gm*), and pBSΔ*oxyR::km* (or pBSΔ*oxyR::gm*). The constructs were subcloned into the NotI and XhoI sites of pBRDX, a suicide vector containing *sacB* and *rdxA* (Goodwin *et al.*, 1998) as counter-selection markers. After transformation into *E. coli* DH5α cells and confirmation by PCR analysis, the resulting plasmids [pBRDXΔ*ahpC1::km* (or pBRDXΔ*ahpC1::gm*), pBRDXΔ*ahpC2D::km* (or pBRDXΔ*ahpC2D::gm*), and pBRDXΔ*oxyR::km* (or pBRDXΔ*oxyR::gm*)], were purified by alkaline lysis and introduced into *L. pneumophila* by electroporation. Allelic recombinants of *L. pneumophila* were selected for loss of plasmid from a population of kanamycin (or gentamicin) resistant colonies by replica-plating onto medium supplemented with 5% (w/v) sucrose, 20 μg/ml metronidazole or 4 μg/ml chloramphenicol. Kanamycin-resistant ($Km^R$) [or gentamicin-resistant ($Gm^R$)], streptomycin-resistant ($Sm^R$) metronidazole-resistant ($Mtz^R$), sucrose-resistant ($Sac^R$) and chloramphenicol-sensitive ($Cm^S$) strains were screened by PCR using primers that bind outside of the cloned upstream (FC1OUT for *ahpC1*, FC2DOUT for *ahpC2D*, and FOXYROUT for *oxyR*) and downstream regions (RC1OUT for *ahpC1* and RC2DOUT for *ahpC2D*), internal to the deleted coding region (FC1INT or RC1INT for *ahpC1*, FC2DINT or RC2DINT for *ahpC2D*, and ROXYRINT for *oxyR*), or combinations with primers designed for the kanamycin- (FKM or RKM) or gentamicin- (FGM1 and RGM)

resistance cassette. Reverse transcriptase PCR (RT-PCR) was also used to confirm the absence of *ahpC1 or ahpC2D* mRNA. Mutants were designated *ahpC1::km* (or *ahpC1::gm*) and *ahpC2D::km* (or *ahpC2D::gm*), respectively.

To construct *ahpC1ahpC2D* double mutants, pBRDXΔ*ahpC1::gm* or pBRDXΔ*ahpC2D::gm* constructs were introduces into the *ahpC2D::km* and *ahpC1::km* mutants, respectively by electroporation. These constructs were also introduced by electroporation into wild-type Lp02 to generate *ahpC1::gm* and *ahpC2D::gm*. As a second attempt to obtain double mutants using reciprocal markers, pBRDXΔ*ahpC2D::km* and pBRDXΔ*ahpC1::km* constructs were introduced by electroporation into the *ahpC1* and *ahpC2D* gentamicin-resistant mutants, respectively. To validate the simultaneous use of kanamycin- and gentamicin-resistance markers, the pBRDXΔ*ahpC::gm* suicide vectors were electroporated into a *magA::km* mutant (Hiltz *et al.*, 2004). Other strategies included addition of an excess of catalase to BCYE medium or electroporation of pBRDX-derived constructs into *ahpC1* or *ahpC2D* mutants strains that had been complemented with pMMBahpC2D or pMMBahpC1 (which contain *ahpC2D* or *ahpC1* genes under the control of the IPTG-inducible promoter of pMMB206) (Morales *et al.*, 1991). For these attempts, 1 mM IPTG and 4 μg/ml chloramphenicol were added to BCYE medium.

### 3.3.12.2. Complementation of the *ahpC* Mutants

Promoter and coding sequences of *grlAahpC1* and *ahpC2ahpD* were amplified using primer pairs FC1COMP/P4C1 and FC2COMP/P4C2D, respectively, then cloned into the BamHI and XbaI sites of pJB908 (Sexton *et al.*, 2004). The resulting plasmids, pC1 and pC2D (Figure 9), were introduced by electroporation into Lp02 *ahpC1::km* or

*ahpC2D::km*, generating strains *ahpC1::km* pC1 or *ahpC2D::km* pC2D, respectively. Mutants capable of growth on BCYE without thymidine were verified by PCR, and expression of the target genes was confirmed by qPCR. Strains containing empty-vectors were confirmed by PCR using the primer pair FBLA/RBLA.

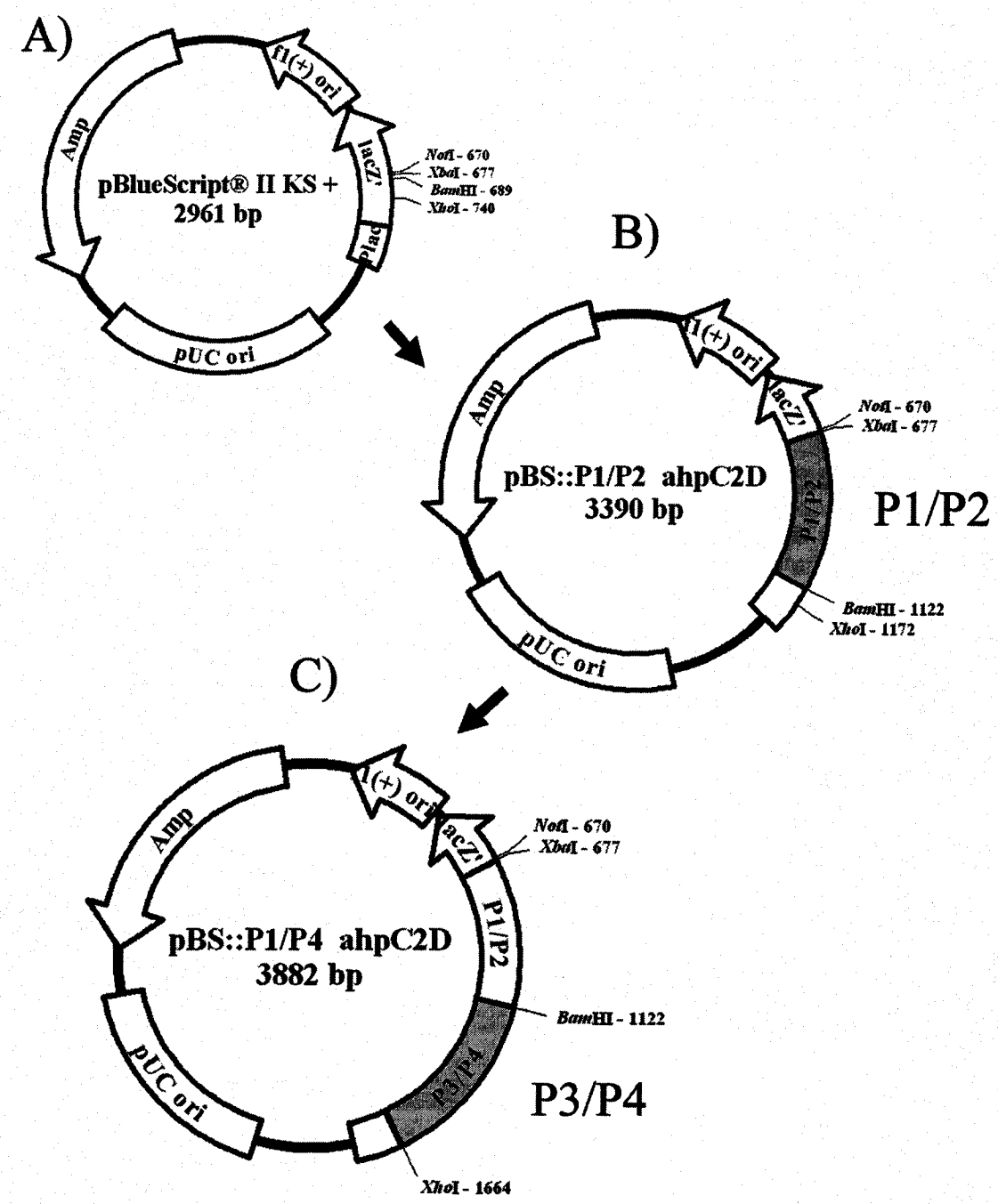
### 3.4. Characterization of the *L. pneumophila ahpC1* and *ahpC2D* Mutant

#### 3.4.1. Genomic Organization of the *ahpC* Loci

##### 3.4.1.1. RNA Isolation

Total RNA was obtained by the Trizol method as described by instructions provided by the manufacturer (InVitrogen, Burlington ON). *L. pneumophila* was grown on BCYE for 24 h and approximately $10^8$ bacterial cells were harvested (4000 x *g*, 6 min, room temperature), lysed by repetitive pipetting in 1 ml of Trizol reagent and incubated at room temperature for 5 min. After addition of 0.2 ml of chloroform, samples were shaken vigorously by hand for 15 s, followed by 3 min incubation at room temperature. The samples were centrifuged (12,000 x *g*, 15 min, 4°C) and the aqueous layers were transferred to fresh microtubes. After addition of 0.5 ml of ice-cold isopropanol, samples were incubated at room temperature for 10 min, followed by centrifugation (12,000 x *g*, 15 min, 4°C). The resulting pellets were washed with ice-cold 70% ethanol and centrifuged (7500 x *g*, 5 minutes at 4°C). The ethanol was removed and the pellets were air dried for ~10 min, resuspended in 30 µl of $ddH_2O$ containing 0.1% diethylpyrocarbonate (DEPC), and were incubated at 55°C for 30 min to facilitate suspension.

**Figure 8** Construction of *L. pneumophila ahpC::km* suicide delivery plasmids. PCR with primer pairs P1C2D/P2C2D and P3C2D/P4C2D was used to amplify upstream (P1/P2) and downstream (P3/P4) regions of *ahpC2D*. A)-C): to generate pBS::P1/P4Δ*ahpC2D*, the P1/P2 and P3/P4 fragments were sequentially cloned into pBlueScript II KS (+) using XbaI/BamHI and BamHI/XhoI, respectively. D) The kanamycin (*km*)-resistance marker from p34S*::km3* was cloned into the *Bam*HI site generated with primers P2 and P3. The resulting construct was termed pBSΔ*ahpC2D::km*. E) The XbaI site from primer P4 and the NotI site of pBlueScript were used to subclone the *ahpC2D::km* constructs into the pBRDX suicide delivery plasmid, generating pBRDXΔ*ahpC2D::km* (Morash, 2006). Similar procedures were used to generate the pBRDXΔ*ahpC1::km* and pBRDXΔ*oxyR::km* contructs and the pBRDXΔ*ahpC1::gm*, pBRDXΔ*ahpC2D::gm* and pBRDXΔ*oxyR::gm* constructs, containing the gentamicin (*gm*)-resistance marker from pPH1J1. All plamids were drawn using pDRAW32 version 1.0 (Acaclone Software). All inserts are indicated in grey.


A)
pBlueScript® II KS +
2961 bp
Amp
f1(+) ori
lacZ'
Plac
pUC ori
NotI - 670
XbaI - 677
BamHI - 689
XhoI - 740
B)
pBS::P1/P2 ahpC2D
3390 bp
P1/P2
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C)
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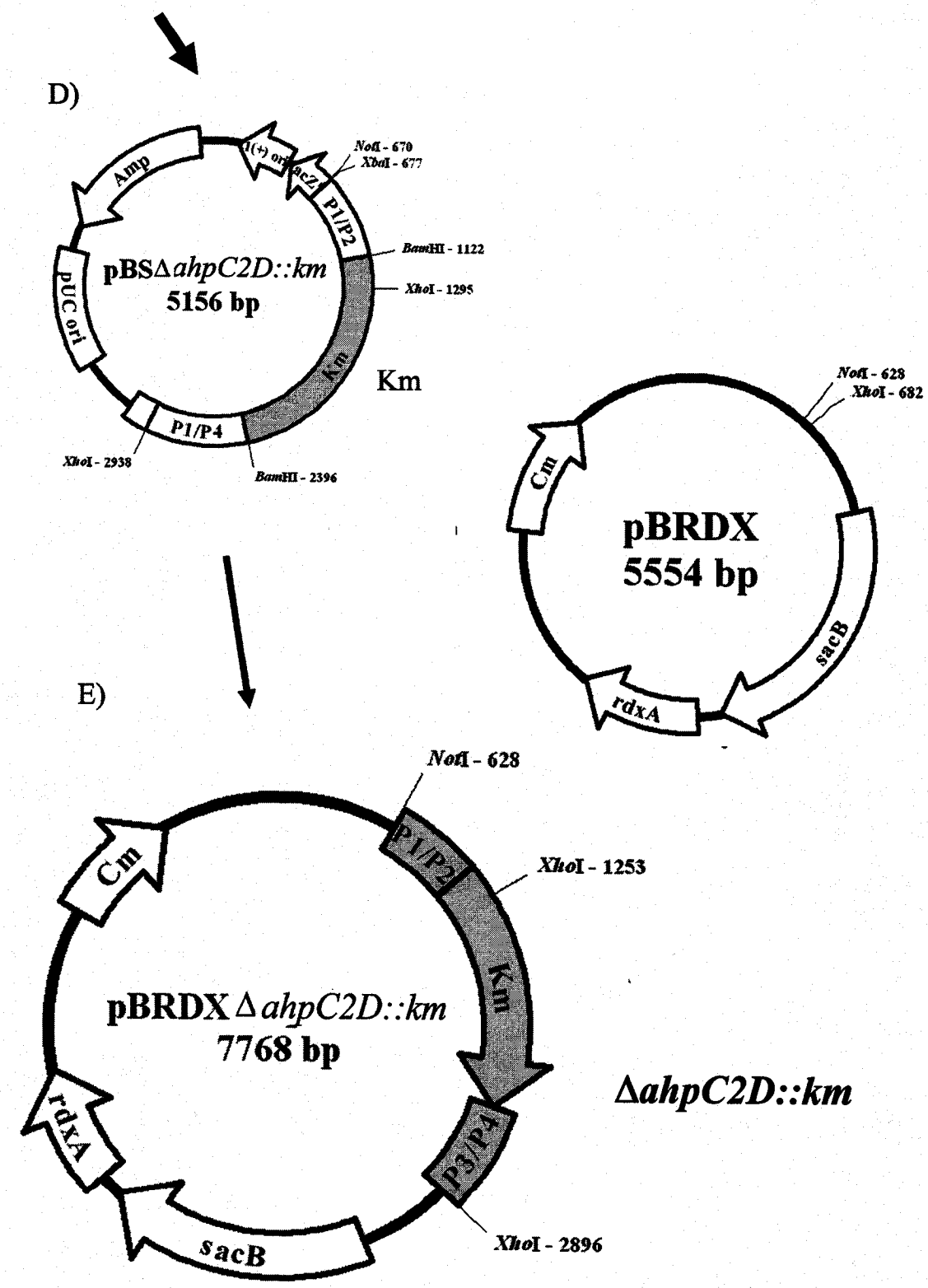
P3/P4
XhoI - 1664

Continued...


D)
Amp
f1(+) ori
lacZ
P1/P2
pUC ori
pBSΔahpC2D::km
5156 bp
Km
P1/P4
NotI - 670
XbaI - 677
BamHI - 1122
XhoI - 1295
XhoI - 2938
BamHI - 2396
Km
NotI - 628
XhoI - 682
Cm
pBRDX
5554 bp
sacB
rdxA
E)
NotI - 628
Cm
P1/P2
XhoI - 1253
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7768 bp
Km
rdxA
P3/P4
sacB
XhoI - 2896
ΔahpC2D::km

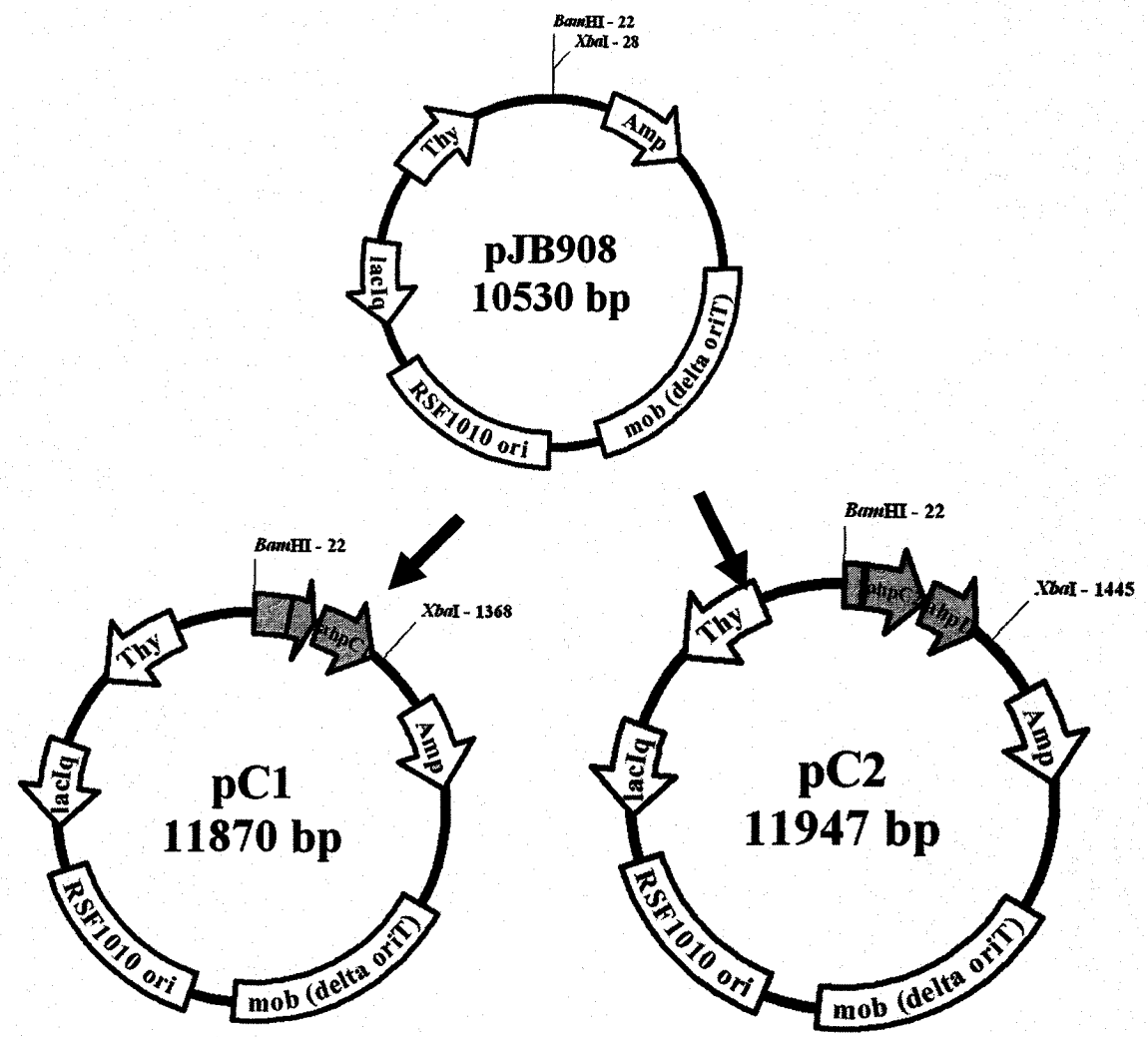



**Figure 9** Complementation of *L. pneumophila ahpC* mutants. Complementation of the *ahpC1* and *ahpC2D* mutants used pJB908-derived plasmids containing coding sequences and promoters of *grlAahpC1* or *ahpC2ahpD*, respectively. The pKB5-derived (Berger and Isberg, 1993) pJB908 plasmids contain: an ampicillin-resistance cassette (Amp) for selection in *E. coli*, the thymidylate synthase (*thyA*) gene known to complement the thymidine auxotrophy of *L. pneumophila* Lp02, a broad-range RSF1010 origin to permit replication in *L. pneumophila*, and a mutated origin of conjugative transfer (*oriTΔ13*) to prevent intracellular growth defects in macrophage (Sexton *et al.*, 2004).

### 3.4.1.2. Deoxyribonuclease I (DNaseI) Treatment of RNA

DNaseI was prepared as a 5 U/µl solution in storage DNaseI storage buffer [50% glycerol, 20 mM sodium acetate pH 6.5, 5 mM $CaCl_2$ and 0.1 mM phenylmethylsulphonylfluoride (PMSF)]. Four microlitres of crude RNA preparation was added to 4 µl of $ddH_2O$ (DEPC), 1 µl of 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 min, and the enzyme was inactivated at 70°C for 10 min. To confirm the absence of large fragments of DNA, 2 µl of this preparation was subjected to PCR analysis under the same conditions as described for cDNA in RT-PCR reactions described below. This reaction was termed the no-RT control.

### 3.4.1.3. Reverse Transcriptase PCR (RT-PCR)

For cDNA synthesis, 2 µl of DNaseI-treated RNA was added to a reaction mixture containing 1 µl of a 1 µg/µl solution of random hexamers, 1 µl of 10 mM dNTPs, and 9 µl of $ddH_2O$. The reaction mixture was heated to 65°C for 5 min and quickly chilled on ice. After brief centrifugation, 4 µl of 5 × first-strand bufer, 2 µl of 0.1 M dithiothreitol (DTT) and 1 µl RNaseOUT™ Recombinant Ribonuclease Inhibitor (InVitrogen) were added to each tube. After 2 min incubation at 37°C, 200 U of Moloney Murine Leukemia Virus (M-MuLV) reverse transcriptase (InVitrogen) were added to the mixture. The cDNA synthesis reaction was carried out for 50 min at 37°C followed by enzyme inactivation at 70°C for 15 min. Aliquots of cDNA were stored at -80°C.

Amplification of cDNA was performed by standard PCR techniques. To confirm the absence of DNA contamination in RNA samples, a no-RT control was performed

under the same PCR conditions. Amplicon size was verified by electrophoresis through 2% agarose gels stained with ethidium bromide. To determine if *grlAahpC1* and *ahpC2D* mRNAs were expressed as polycistronic messages, standard PCR amplification was performed using 2 µl of cDNA template and FGRLRT/RC1RT and FC2RT/RDRT primers, respectively. To confirm absence of *ahpC1* and *ahpC2D* mRNA in *ahpC1::km* (or *ahpC1::gm*) and *ahpC2D::km* (or *ahpC2D::gm*) deletion mutants or to confirm the expression of *ahpC1* and *ahpC2D* in respective complements (pC1 and pC2), RT-PCR reactions were performed with using primer pairs FC1RT/RC1INT (for *ahpC1*) or FC2RT/RC2INT for (*ahpC2D*).

### 3.4.2. Bacterial Growth

For all experiments, cells of *L. pneumophila* strains were grown from frozen stocks for 48 h on BCYE solid medium before being spread onto fresh medium and incubated for an additional 24 h. These cells were used to inoculate starter cultures consisting of 50 ml BYE containing appropriate selective agents, which were grown for 18-20 h at 37°C with gentle agitation (125 rpm). These starter cultures were sub-cultured into 50 ml pre-warmed BYE to an $OD_{620}$ of 0.2 and were incubated for desired times. For growth-curve determination, samples were taken every 3 h over a two-day period. For stationary-phase survival experiments, *L. pneumophila* cells were grown as above and samples were taken at 24, 36, 48, 60, and 72 h to determine $OD_{620}$ and viability (colony-forming units per ml; cfu/ml). To determine culture viability, samples were washed twice with BYE, serially diluted in PBS, and spread onto BCYE medium under proper selection conditions. All experiments were performed using triplicate cultures and values are reported as the mean ± SD from three independent experiments.

### 3.4.3. Sensitivity to Oxidative Stress

To determine sensitivity to oxidative stress of wild-type *L. pneumophila* and *ahpC* mutants, a microdilution assay and a peroxide challenge of BYE-grown bacteria was performed.

#### 3.4.3.1. Microdilution Susceptibility Assay

*L. pneumophila* strains were harvested from BYE broth after 18-20 h of growth, washed twice with PBS, and diluted in 2 × BYE to an $OD_{620}$ of 0.2. In triplicate wells of a 96-well microtiter plate, 50 µl of bacterial suspension (giving 0.01 $OD_{620}$ final) was added to an equal volume of various concentrations (final concentration per well of ranging from 0 to 1 mM) of the following peroxides: $H_2O_2$, *tert*-butyl hydroperoxide (tBOOH), cumene hydroperoxide (CHP) or methyl viologen (PQ). All peroxides were purchased from Sigma-Aldrich, Oakville, ON. Following incubation at 37°C for 24 h with gentle agitation, the lowest concentration resulting in no visible growth was deemed to be the minimal inhibitory concentration (MIC). From wells where no growth was evident, quadruplicate 10-µl samples were spotted onto BCYE solid medium to assess minimal bactericidal concentration (MBC). Since both MIC and MBC were identical, results are reported as the mean ± SD for three independent experiments.

#### 3.4.3.2. Peroxide Challenge

To eliminate the effect of the culture medium on peroxide sensitivity, *L. pneumophila* cells were washed with PBS, diluted to 0.1 $OD_{620}$ and then challenged for 30 min with various concentrations (0, 250, 500, 1000, and 2000 µM) of tBOOH or with 500 µM tBOOH for varying periods of time (0, 30, 60, 90, and 120 min). Cells were

washed twice with PBS before being serially diluted and spread on BCYE to determine viability. Results represent the mean ± SD deviation of triplicate values obtained from three independent experiments.

### 3.4.4. Cell Culture and Infection Models

To dertermine if *L. pneumophila ahpC* mutants are enfeebled for intracellular growth, *L. pneumophila* infections of HeLa and U937 cells were performed, as well as a plaque assay using L929 cells.

#### 3.4.4.1 HeLa Cell Culture

HeLa cells were grown in 25-$cm^2$ Falcon tissue culture flasks (Becton-Dickinson, Oakville, ON) as described (Garduno *et al.*, 1998 and 2002). HeLa cells were cultured in 1 × minimal essential medium (MEM) supplemented with 4 mM L-glutamine (Sigma), 1 × antibiotic-antimycotic solution (100 U/ml penicillin G, 100 µg/ml streptomycin and 250 ng/ml amphotericin B) (Sigma) and 10% fetal bovine serum (FBS) (Sigma). Monolayers were grown until confluent and removed from the flask using trypsin-EDTA (0.25% trypsin, 0.1% EDTA). Cells were counted using a Neubauer hemocytometer and seeded at $10^6$ cells/well into 12-well Falcon tissue-culture plates. Cells were grown overnight and monolayer formation was verified using an inverted microscope. Monolayers were washed three times with 1 ml sterile PBS [137 mM $NaCl_2$, 10 mM $Na_2HPO_4$] and 900 µl fresh 1× MEM (supplemented with 10% FBS, 4 mM L-glutamine, 100 µg/ml streptomycin, 100 µg/ml thymidine, and without antibiotic-antimycotic solution) was added to each well.

### 3.4.4.2. U937 Cell Culture

U937 cells (ATCC CRL-1593.2) were routinely grown in suspension in 175-$cm^2$ tissue-culture flasks in RPMI 1640 supplemented (Sigma) with 4 mM L-glutamine, 1 × antibiotic-antimycotic solution, and 10% FBS (Pearlman *et al.*, 1988). To differentiate U937 cells into non-replicating adherent macrophage-like forms, cells were incubated in for 48 h in fresh medium containing 50 ng/ml of phorbol 12-myristate 13-acetate (PMA). To transfer PMA-differentiated U937 cells to tissue-culture plates for bacterial infection experiments, cells were detached from the bottom of flasks by methods described by Berger and Isberg (1993b). Briefly, 10 ml of PBS, pH 7.2, containing 2 mM EDTA was transferred into 50-ml flasks and incubated for approximately 5 min until cells had detached from plastic. EDTA-detached cells were transferred to 50-ml Falcon tubes and $MgCl_2$ was added to obtain a final concentration of 5 mM. Cells were pelleted by centrifugation (500 × g, 5 min, room temperature) and resuspended in fresh medium (supplemented with 10% FBS, 4 mM L-glutamine, 100 µg/ml streptomycin, 100 µg/ml thymidine, and without antibiotic-antimycotic solution or PMA). Cells were counted using a Neubauer hemocytometer and transferred to 12-well plates to approximately 1 × $10^6$ U937 cells/well.

### 3.4.4.3. *L. pneumophila* Infection of Cultured Cells

For infection, *L. pneumophila* cells from 18-20 h broth cultures were harvested (4000 x *g*, 6 min, room temperature) and washed twice using sterile PBS. Bacteria were resuspended in supplemented medium (supplemented with 10% FBS, 4 mM L-glutamine, and 100 µg/ml of thymidine) and added to triplicate wells of quadruplicate plates. For HeLa cell infection, bacteria corresponding to an $OD_{620}$ of 1.0 in 100 µl of supplemented

MEM were added to each well (giving 0.1 $OD_{620}$ bacteria per well). For U937 cell infection, bacteria corresponding to an $OD_{620}$ 0.1 (approximately $10^8$ cells) were resuspended in 10 ml of supplemented RPMI 1640, and 100 µl of this bacterial suspension was added to each well, representing approximately $10^6$ bacteria/well or a multiplicity of infection (MOI) of 1. All inocula were serially diluted to determine cfu/ml. Following addition of bacteria, all plates were centrifuged (500 x *g*, 10 min, room temperature) and incubated for 2 h at 37°C, 5% $CO_2$, to facilitate bacteria/cell interaction. To remove extracellular bacteria, wells were washed three times with fresh medium, and incubated for 90 min at 37°C, 5% $CO_2$ in 1 ml of medium containing 50 µg/ml gentamicin. Following three additional washes, fresh MEM or RPMI 1640 medium (supplemented with 10% FBS, 4 mM L-glutamine, 100 µg/ml streptomycin, and 100 µg/ml thymidine) was added to each well. At several time points (immediately for zero time or 24, 48, and 72 h post-infection), medium was harvested from HeLa and U937 monolayers and cells were lysed with cold deionised water. After centrifugation (4000 x *g*, 6 min, room temperature), bacteria recovered from culture media or following host cell lysis were pooled, serially diluted, and spread on BCYE to determine cfu/ml. Since *L. pneumophila* does not replicate in MEM (Pearlman *et al.*, 1988) or RPMI 1640 (Sexton *et al.*, 2004), daily determination of cfu/ml is an accurate measure of intracellular growth.

#### 3.4.4.4. L929 Plaque Assay

Murine L929 cells (ATCC clone CCLI) were grown and maintained in 1 × MEM supplemented with 10% FBS. For plaque assay, approximately $5 \times 10^5$ cells were added to each well of a 24-well tissue-culture plate. After allowing the cells to adhere for 2-3 h,

the confluent monolayers were washed once with PBS, and received 200 µl of 1 × MEM (with 10% FBS). Overnight *L. pneumophila* cells from BCYE solid medium were harvested and standardized to an $OD_{620}$ of 0.5 in 1 ml of 1 × MEM (with 10% FBS). Cells were then serially diluted 10-fold in 900 µl of 1 × MEM (with 10% FBS). Immediately after removing the culture medium from the L929 cells, 400 µl of each dilution (six per strain) was added to duplicate wells. Plates were then subjected to centrifugation (1,000 × g, 10 minutes, room temperature) and incubated for 3 h at 37°C, 5% $CO_2$. Monolayers were washed six times with PBS before addition of 1 ml of 0.6% agarose in 1 × MEM (with 10% FBS) supplemented with 4 mM glutamine, 100 µg/ml thymidine and appropriate antibiotic for selection. Cells were then incubated for four days at 37°C, 5% $CO_2$ in a Tupperware container containing damp paper towels. Each inoculum dilution was then serially diluted and spread on BCYE with appropriate selection to determine viable counts (cfu/ml). After four days, each monolayer then received 1 ml of PBS containing 10% formalin and was incubated for an additional 24 h at room temperature. The PBS/formalin solution was washed with $ddH_2O$, the agarose plugs were carefully removed, and the monolayers were stained for 1 h with 1% crystal violet. Excess stain was then removed with $ddH_2O$, and the plates were left to air dry. Plaques in the monolayer were counted and plaquing efficiency was calculated as follows: (number of plaques formed/viable count of the inoculum) x 100.

### 3.4.5. Green Fluorescence Protein (GFP) Reporter Assay

GFP-transcriptional fusions of *ahpC1*, *ahpC2D*, and *oxyR* promoter regions ($P_{ahpC1}$, $P_{ahpC2}$, and $P_{oxyR}$, respectively) were constructed using primer pairs FC1PROM/RC1PROM, FC2PROM/RC2PROM, and

FLPOXYRPROM/RLPOXYRPROM, cleaved using BamHI and EcoRI, and cloned into the *Eco*RI and *Bam*HI sites of pBH6119 (gift from Michele Swanson, University of Michigan Medical School, Ann Arbor, MI) (Hammer and Swanson, 1999), generating constructs pC1gfp, pC2gfp, and pOxyRgfp, respectively. For mapping of potential OxyR binding sites within $P_{ahpC2}$, PCR-generated 5'-deletion fragments of $P_{ahpC2}$ (using primers FC2PROM179, FC2PROM156, FC2PROM132, FC2PROM109, FC2PROM91, and FC2PROM66 in combination with RC2PROM) were amplified, cleaved, and cloned into pBH6119 as described above, generating plasmids pC2(-179/+27)gfp, pC2(-156/+27)gfp, pC2(-132/+27)gfp, pC2(-109/+27)gfp, pC2(-99/+27)gfp, and pC2(-66/+27)gfp, respectively. All reporter constructs were first transformed into *E. coli* DH5α cells and correct insert orientation was determined by PCR using the respective combination of forward primer and the reverse RGFP primer. The empty-vector control and selected pBH6119 derivatives were introduced by electroporation into wild-type *L. pneumophila* Lp02 or (*ahpC1*, *ahpC2D*, *rpoS*, *letA*, or *himAB*) mutant strains, as indicated. Transformant cells selected on medium without thymidine were verified to contain respective plasmids by PCR analysis using primers described above or using the FBLA/RBLA primers for the ampicillin-resistance marker.

For fluorometric determination of GFP levels, bacterial samples were taken from BYE broth cultures at 3 h intervals and following determination of $OD_{620}$, bacteria were harvested by centrifugation (4000 x *g*, 6 min, room temperature) and suspended in PBS. Fluorescence was measured on a TD-700 fluorometer (Turner Designs Inc., Sunnyvale, CA) using 310-390 nm excitation and a 486 nm emission filter. Values are expressed as

relative fluorescence units (rfu) per $OD_{620}$ and represent triplicate values obtained from three independent experiments.

### 3.4.6. Evaluation of Gene Expression by Quantitative Real-Time PCR (qPCR)

qPCR was performed on a 36-well rotor of the RotorGene 3000 system (Corbett Research, Kirkland, QC). PCR amplification was performed on 2.5 µl of cDNA template in a 25-µl reaction containing 0.5 U *Taq* polymerase, 10,000-fold dilution of SYBR Green I nucleic acid stain (Invitrogen Canada Inc., Burlington, ON), 500 nM dNTPs, 1 × reaction buffer (Karsai *et al.*, 2002), and 200 nM of the primer sets FRPLJQRT/RRPLJQRT, FC1QRT/RC1QRTR, and FC2QRT/RC2QRT for *rplJ*, *ahpC1*, and *ahpC2*, respectively. PCR conditions are as follows: initial denature (94°C, 30 s), 45 cycle (denature 94°C, 30 s; anneal 55°C, 30 s; extend 72°C, 30 s), and melt (72-95°C). After emission at 470 nm, fluorescence acquisition was performed at 80°C and detected at 510 nm. All qPCR samples were accompanied by a respective no-template control (NTC) consisting of all PCR reagents except template, and a no-RT control to ensure absence of DNA in the DNaseI-treated RNA. Samples were analyzed by melt-curve analysis and by electrophoresis through 2% agarose gels. Sample concentrations were determined by standard curves generated under the same conditions using 10-fold dilutions of genomic DNA as template. All samples were normalized to *rplJ* levels and values are expressed as the fold increase/decrease relative to *ahpC2* (calibrator sample = 1 ×) levels in the wild-type strain. Values represent the mean ± SD of quadruplicate samples obtained from three independent experiments.

### 3.5. Overproduction and Purification of LpOxyR

To obtain purified LpOxyR, *L. pneumophila oxyR* was cloned into the ITPG-inducible pET29b plasmid, expressed in *E. coli* BL21, and purified using affinity chromatography.

#### 3.5.1. Cloning of *L. pneumophila oxyR* into pET29b

The pET29b expression system was used to overproduce LpOxyR. Briefly, PCR using the primer pair FLPOXYRPET/RLPOXYRPET was used to generate an amplicon containing NdeI and XhoI restriction sites, respectively. Following digestion, the NdeI-XhoI fragment was cloned into the corresponding sites of pET29b, generating petLpOxyR. Presence of the coding sequence of *L. pneumophila oxyR* behind the T7 promoter and in frame with the C-terminal hexameric histidine ($His_6$) tag was confirmed by DNA sequencing (DalGen Microbial Genetics Center, Halifax, NS).

#### 3.5.2. Overexpression of *L. pneumophila oxyR* in *E. coli* BL21

For overexpression, an overnight culture of *E. coli* BL21(DE3) Codon Plus (Novagen) harboring the petLpOxyR overexpression construct was inoculated 1% (v/v) into 200 ml of fresh LB broth and grown to an $OD_{620}$ of 0.4. IPTG was added to a final concentration of 1 mM and incubation was continued for an additional 60 min at 37°C. Bacteria were then harvested by centrifugation (4000 × g for 6 min) and resuspended (5 ml/g [wet weight]) in binding buffer (20 mM Tris-HCl pH 7.9, 500 mM NaCl, 5 mM imidazole, and 1 mM PMSF).

### 3.5.3. Purification of LpOxyR

To isolate $His_6$-LpOxyR, *E. coli* BL21 cells were first lysed on ice by sonication (6 bursts of 10 s at 200 W with 60 s of cooling between each burst). The lysate was clarified by centrifugation (10,000 × g for 15 min, 4°C) and the resulting supernatant was added to 5 ml nickel ($Ni^{2+}$)-nitriloacetic acid (NTA) resin (EMD Biosciences). After gentle agitation for 60 min on a rocking platform, the slurry was packed into a 10-ml PD-10 column (GE Healthcare). The column was washed three times with 10 ml of wash buffer (20 mM Tris-HCl pH 7.9, 500 mM NaCl, 10 mM imidazole, and 1 mM PMSF). $His_6$-LpOxyR bound to the $Ni^{2+}$-NTA resin was eluted with elution buffer (20 mM Tris-HCl pH 7.9, 500 mM NaCl, 100 mM imidazole, and 1 mM PMSF). Protein fractions were pooled and dialyzed in 6,000 to 8,000 molecular-weight cut-off dialysis tubing (Spectrapor, Spectrum Medical Industries, Los Angeles, CA) against dialysis buffer 1 (20 mM Tris-HCl pH 7.9, 400 mM KCl, 200 µM EDTA and 10% glycerol) for 2 h then dialysis buffer 2 (20 mM Tris-HCl pH 7.9, 200 mM KCl, and 20% glycerol) for 12 h, and finally against storage buffer (20 mM Tris-HCl pH 7.9, 100 mM KCl, and 50% glycerol). Purified protein was quantified by standard Brandford assay (Bradford, 1976) and portions were stored at -70°C. Samples taken from each step of the purification were analyzed by SDS-PAGE with silver staining as described below.

#### 3.5.3.1. Quantification of Proteins by Bradford Assay

Protein lysates or purified $His_6$-LpOxyR were serially diluted in $ddH_2O$, and 80 µl of diluted lysate received 20 µl of Bradford reagent (Bio Rad Laboratories, Ltd., Mississauga, ON) (Bradford, 1976). Spectrophotometric determination was measured at 595 nm. As a blank, 20 µl of Bradford reagent was added to 80 µl of $ddH_2O$. Protein

concentration was estimated from a standard curve ($y = 0.024x + 0.0515$ in μg/ml; $R^2 = 0.9931$) generated with various dilutions of BSA.

### 3.5.3.2. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

SDS-PAGE analysis of proteins was performed by standard techniques described in Sambrook and Russell (2001). Briefly, polyacrylamide gels were cast using a 5% stacking gel [per 6 ml: 4.2 ml $ddH_2O$, 1 ml 30% acrylamide:bisacrylamide (29:1), 760 μl of 1.5 M Tris-HCl pH 6.8, 60 μl of 10% SDS, 60 μl of 10% ammonium persulfate (APS), and 6 μl of N, N, N', N'-tetramethylethylenediamine (TEMED)] and a 12% resolving gel [per 10 ml: 3.2 ml $ddH_2O$, 4 ml 30% acrylamide:bisacrylamide (29:1), 2.6 ml of 1.5 M Tris-HCl pH 8.8, 100 μl of 10% SDS, 100 μl of 10% APS, and 4 μl of TEMED]. Proteins samples were obtained from 0.75 $OD_{620}$ of cells were harvested from bacterial cultures. Pellets were resuspended in SDS sample buffer (250 mM Tris-HCl pH 6.8, 20% glycerol, 2% SDS, 5% β-mercaptoethanol, 0.001% bromophenol blue) and were boiled for 10 min. After centrifugation to pellet cell debris, 15 μl of protein lysate were loaded into the wells of the polyacrylamide gel and electrophoresis was performed at 125 V/cm in SDS electrophoresis buffer (15 g/l Tris base, 72 g/l glycine, and 5 g/l SDS). Gels were then transferred to Coomassie blue stain, or to 50% methanol 10% acetic acid solution for silver staining. Coomassie staining of SDS-PAGE gels were performed as follows: i) overnight staining in Coomassie stain (0.25% Coomassie Brilliant Blue R-250 in 50% (v/v) methanol and 10% (v/v) acetic acid; ii) a rapid destain for 1-2 h in 50% (v/v) methanol and 10% (v/v) acetic acid, and iii) a slow destain in 5% (v/v) methanol and 7% (v/v) acetic acid. Gels were preserved for short periods in 20% (v/v) methanol and 3% (v/v) glycerol; for long-term preservation, a Tut's Tomb drying

frame was used to seal gels in cellophane (Matsudaira and Burgess, 1978). Silver staining of gels was carried out as follows: i) overnight fixation in a 50% methanol 10% acetic acid solution; ii) 15-min fixation in 50% (v/v) methanol; iii) five consecutive 1-min washes with Ultra Pure distilled water (Invitrogen); iv) sensitization for 1 min with 0.02% sodium thiosulfate; v) two washes with distilled water; and vi) treatment with 0.2% silver nitrate for 25 min. Gels were developed using 3% sodium carbonate containing 0.025% formalin until desired intensity was achieved. The reaction was stopped by incubation with 1.4% EDTA for 10 min and gels were washed twice in distilled water. Proteins were excised in a laminar-flow hood using a sterile scalpel blade and submitted for mass spectrometry analysis (DalGen Microbial Genetics Center, Halifax, NS).

### 3.6. Electrophoretic Mobility Shift Assay (EMSA)

DNA fragments were generated by PCR amplification using primer pairs FC1PROM/RC1PROM, FC2PROM/RC2PROM, and FOXYRPROM/ROXYRPROM, respectively. Fifty nanograms of the DNA fragments were mixed with varying amounts of $His_6$-LpOxyR (ranging from 0, 3.9, 7.8, 15.6, 31.3, 62.5, 125, 250, and 500 ng per lane) in a 20-µl reaction mixture containing 20 mM Tris-HCl pH 8.0, 5 mM $MgCl_2$, 20 mM KCl, 50 µg BSA, 20% glycerol, 1 mM PMSF, and either 1 or 200 mM DTT (Promega) for oxidizing or reducing conditions, respectively. After an incubation of 30 min at room temperature, protein-DNA complexes were resolved by electrophoresis using a Mini-Protein II apparatus (Bio-Rad) in 0.5× Tris borate EDTA (TBE) buffer (for 5 × TBE: 54 g/l Tris Base, 27.5 g/l boric acid, and 1 mM EDTA). Electrophoresis was performed for approximately 1 h at 125 V/cm on a non-denaturing 0.5 × TBE 5%

polyacrylamide gel (for 10 ml: 1.66 ml 30% acrylamide:bisacrylamide (29:1), 7.26 ml $ddH_2O$, 1 ml 5 × TBE, 80 µl of 10% APS, and 8 µl TEMED) which had been pre-run for 20 min under the same conditions. Gels were then soaked in 10,000-fold diluted SYBR Green I in TE buffer pH 7.5 and washed twice in distilled water, and DNA was visualized using a Typhoon 9410 system (GE Heathcare) under blue light excitation at 488 nm and using a 520 BP 40 emission filter. Densitometry was performed using Molecular Dynamics ImageQuant version 5.2. Mapping of the $P_{ahpC2}$ was performed as above using 500 ng of $His_6$-LpOxyR and 50 ng of various sized 5'-deletion DNA fragments generated by PCR using a common reverse primer (RC2PROM) with different forward primers (FC2PROM, FC2PROM179, FC2PROM156, FC2PROM132, FC2PROM109, FC2PROM91, and FC2PROM66). All promoter fragments were also subcloned into pBH6119, introduced into into *L. pneumophila* wild-type or *ahpC1::km* by lectroporation, and subjected to the GFP-reporter assay described above. For binding assays to *E. coli ahpCF*, *katG*, and *oxyR* promoters, primer pairs FECAHPCPROM/RECAHPCPROM, FECKATGPROM/RECKATGPROM, and FECOXYRPROM/RECOXYRPROM, respectively were used to generate amplicons and EMSA analysis was performed under oxidizing or reducing conditions as described above.

### 3.7. DNaseI Footprinting

DNaseI protection experiments were performed using a 226-bp DNA fragment of $P_{ahpC2}$ generated from PCR reactions using primer pair FC2PROM/RC2PROM (encoding EcoRI and BamHI restriction sites, respectively). After restriction endonuclease digestion (EcoRI for top strand; BamHI for bottom strand), 5'-phosphoryl groups were

removed using CIP and reactions were purified using a QIAquick gel extraction kit. End-labelled DNA was prepared by exchange reaction from the γ-phosphoryl group from [γ-$^{32}$P]-dATP by using Ready-To-Go T4 polynucleotide kinase (GE Heathcare) as described by the manufacturer. End-labelled DNA was purified with a QIAquick nucleotide removal kit (Qiagen). Binding reactions were performed by adding approximately 20,000 counts per minute (cpm) of labeled probe and various concentrations of $His_6$-LpOxyR protein (0, 90, 250, 430 pmol) to an assay buffer consisting of 20 mM Tris-HCl pH 8.0, 10 mM $MgCl_2$, 100 mM KCl, 1 mM $CaCl_2$, 10 % glycerol, 0.05 mg/ml BSA, 4 μg/ml poly dI:dC and either 1 or 200 mM DTT for oxidizind or reducing conditions, respectively. After 20 min incubation, 0.5-1 U of DNase I was added, incubated for 1 min, then 100 μl of a stop solution (200 mM EDTA pH 8.0, 5 M ammonium acetate, and 100 μg of salmon sperm DNA) was added. The mixture was extracted with phenol-chloroform and ethanol precipitated. Dry pellets were mixed with a formamide loading buffer (80% (w/v) deionized formamide, 10 mM EDTA pH 8.0, 1 mg/ml xylene cyanol, 1 mg/ml bromophenol blue), heated to 80°C and loaded onto an 6% polyacrylamide-urea sequencing gel consisting of 42 g urea, 20 ml 30% acrylamidebisacrylamide (29:1), 20 ml 5 × TBE, adjusted to 100 ml with $ddH_2O$ and polymerized using 450 μl APS and 45 μl TEMED. The DNA sequence ladder was constructed using SequiTherm EXCEL II DNA Sequencing Kit (Epicenter Technologies). Briefly, 4 μl of a premix solution [1.5 μl of 100 ng/μl of FC2PROM (or RC2PROM) primer, 1 μl [α-$^{35}$S]-dATP (≥ 1000 μCi), 7.2 μl of sequencing buffer, 2 μl of pBSC2PROM template (50-200 fmoles), 4.3 μl $ddH_2O$, and 1 μl SequiTherm EXCEL II DNA polymerase (5 U/μl)] was added to each tube containing 2 μl of one of the dideoxynucleotide-deoxynucleotide termination mixes (G,

A, T, C). Reactions were overlayed with mineral oil before being transferred to a pre-heated (95°C) thermocycler. The reactions were heated for 5 min at 95°C and cycled 30 times (30 s at 95°C, 1 min at 70°C). Reactions were terminated using 3 µl of formamide loading buffer and loaded onto the same 6% polyacrylamide-urea gel as the DNaseI footprinting samples. Electrophoresis of samples was performed for approximately 2 h at 1500 V/cm. Gels were transferred onto a 3-mm Whatman paper, covered with cellophane, and dried for 1 h using a vacuum dryer. Gels were then exposed to a phosphor screen and processed using a Typhoon 9410 system.

### 3.8. Acrylamide Capture of DNA-Bound Complexes

To identify transcription factors that bind to $P_{oxyR}$, an acrylamide capture assay was performed following a modified version of described methods in Nelson *et al.* (2002). To validate the technique, parallel experiments were performed with $P_{ahpC2}$. Briefly, PCR reactions were performed using forward primers containing a 5'Acrydite™ moiety (Ac) and unmodified reverse primer (Ac-FLPOXYRPROM/ROXYRPROM and Ac-FC2PROM/RC2PROM for *oxyR* and *ahpC2*, respectively). Amplicons were purified using MinElute Gel Extraction kit (Qiagen). Protein lysates were obtained as follows: bacteria were harvested (4000 x *g*, 6 min, 4°C) from 500-ml 24 h cultures of *L. pneumophila*. Pellets were washed in cold 50 mM Tris HCl pH 7.5, and resuspended in sonication buffer (50 mM Tris-HCl pH 7.5, 1 mM EDTA pH 8.0, 50 mM $NaCl_2$, 10% glycerol with 0.1 mM PMSF and 1 mM DTT). Cells were lysed using using a French press and subjected to ultracentrifugation at 100,000 × g. Protein lysates were aliquoted and stored at -80°C. Samples protein was quantitated using a standard Bradford assay (Bradford, 1976). Before use, protein lysates were diluted in a buffer consisting of 100

mM Tris-HCl pH 7.5 and 10 mM $MgCl_2$ and treated for 15 min at room temperature with 100 mM of acetyl phosphate lithium potassium salt (Sigma) to capture possible phosphorylated response regulators. Binding reactions were performed in a 25 µl volume and consisted of 0 (no DNA for control), 2, 4 or 8 µg of Ac-DNA, 6 µg of protein lysate, 7.5 µl buffer D (20 mM 4-2-hydroxyethyl-1-piperazineethanesulfonic acid (HEPES) pH 7.8, 100 mM KCl, 10% glycerol, 1 mM DTT), 1 µg poly dI:dC, and 3.5 µl of 30% acrylamide:bisacrylamide (29:1). Reactions were incubated for 15 min before addition of 1.25 µl of 5 % APS and 1.25 µl of TEMED (diluted 1:30 in buffer D). The reactions were immediately loaded into the wells of a pre-cast 5% polyacrylamide 0.5 × TBE gel (Biorad). After polymerization (approximately 5 min), electrophoresis was performed in 0.5 × TBE for 1 h at 125 V/cm to remove unbound proteins. Proein-bound DNA was excised from each well of the gel, transferred to a microtube containing 100 µl of SDS sample buffer (NEB) with 5% β-mercaptoethanol and heated for 10 min at 94°C to remove captured proteins from the acrylamide. After a brief centrifugation, 50-µl portions were loaded on a pre-cast 4-15% Tris-HCl polyacrylamide gel (Biorad). SDS-PAGE and silver staining was performed as described above. Bands distinct were excised and submitted for mass spectrometry analysis.

## Chapter 4: Results

Early after the discovery of *L. pneumophila* in the late 1970s, it was shown that, unlike other members of the *Legionellaceae* family, this organism lacked catalase activity and was particularly susceptible to ROIs. These findings were further supported by the necessity of ROI-scavenging components in its microbiological medium (BCYE), including α-ketoglutaric acid and charcoal. More recently, since mutants of the two *L. pneumophila* catalase-peroxidases (KatA and KatB) were shown to display altered vesicular trafficking of the LCV and loss of viability in stationary phase, it was postulated that decomposition of $H_2O_2$ may be crucial for intracellular survival. Although KatA and KatB may provide some functions necessary for intracellular growth, the proposed model was unfounded since no catalase activity was attributed to these enzymes. In other organisms, AhpC has been shown to detoxify $H_2O_2$, organic hydroperoxides and peroxynitrite, and thus could provide this invaluable function. Although no data validated the peroxide-detoxifying properties of AhpC in *L. pneumophila*, previous work (Rankin *et al.*, 2002) reported that *ahpC1* is up-regulated during intracellular growth in macrophages and showed that the *ahpC1::km* mutant was not enfeebled for growth in macrophage. The recent access to the sequences of three *L. pneumophila* genomes has revealed the presence of a second *ahpC* gene in this organism, which is designated here as *ahpC2*. This study was initiated to characterize both AhpC1 and AhpC2. Further efforts were also taken to identify a homolog of the peroxide-inducible transcriptional regulator OxyR as a possible regulator of *ahpC2* expression.

## 4.1. Phlogenetic Analysis and Structural Organization of the Two *ahpC* Loci

Comparative genomics revealed that *L. pneumophila* and the closely related organism *C. burnetii* contain two *ahpC* loci in their respective chromosomes. Our results also indicate that *L. pneumophila*, *C. burnetii*, and other organisms such as *S. typhimurium, P. putida*, and *V. vulnificus* also possess two AhpCs with identity and similarity values (between both AhpCs) ranging between 35-40% and 58-65%, respectively. First, *L. pneumophila* AhpC1 which had been previously identified by Rankin *et al.* (2002), shares 70% identity and 86% similarity with its *C. burnetii* homolog. It also shares approximately 68-76% similarity and 84-88% identity with other AhpC/Tsa family members found in γ-proteobacteria, including the thiol-specific antioxidant (Tsa) of *S. typhimurium* (Farr & Kogoma, 1991) and the AhpCs of the genera *Vibrio*, *Francisella*, and *Pseudomonas* (Figure 10 clade I). The AhpC1 class is often partnered with thioredoxin reductases (Baker *et al.*, 2001; Farr & Kogoma, 1991). Upstream of *L. pneumophila ahpC1* is *grlA*, which encodes a glutaredoxin-related protein (GrlA) which might be involved in AhpC reduction (Figure 12A) (Rankin *et al.*, 2002). Upstream of this locus and encoded in the opposite direction is the essential FeSOD gene *sodB* (Sadosky *et al.*, 1994) (Figure 12A). Second, *L. pneumophila* AhpC2 shares 72% identity and 85% similarity with its *C. burnetii* homolog and approximately 60-72% similarity and 70-85% identity with other AhpCs found in γ-proteobacteria (*Rickettsiella*), α-proteobacteria (*Brucella* and *Bradyrhizobium*), β-proteobacteria (*Bordetella* and *Burholderia*), and various actinobacteria including *Streptomyces*, *Mycobacterium*, and *Corynebacterium* (Figure 10 clade III). The second *ahpC* gene (designated as *ahpC2*) is adjacent to *ahpD*, also an AhpC partner that has been well

characterized in members of the genera *Mycobacterium* and *Streptomyces* (Hahn *et al.*, 2002), and can be found in similar positions in the genomes of all the members of clade III in Figure 10. Downstream of *ahpD* and in the opposite orientation is *sodC*, encoding the periplasmic Cu,Zn-SOD (St John and Steinman, 1996) (Figure 12A). Since no AhpF homolog could be found in the genomes of *L. pneumophila* and *C. burnetii*, it was not surprising to see that neither AhpC1 nor AhpC2 shows strong similarity to proteins in organisms containing *ahpCF* operons (*E. coli*, *S. typhymurium*, *B. anthracis*, and *S. aureus*) (Figure 10 clade II). It should also be noted that both AhpC1 and AhpC2 possess, among other highly conserved residues, the peroxidatic and resolving cysteines (Figure 11).

To evaluate the putative operon structures at the *L. pneumophila ahpC* loci, RT-PCR reactions were performed with primers (small arrows in Figure 12A) designed to span the intergenic sequences between *grlA* and *ahpC1*, or between *ahpC2* and *ahpD*. As seen in Figure 12B, generation of amplicons that span these intergenic regions indicated that both gene sets can be transcribed as polycistronic messages. This indicates that *ahpC1* and *ahpC2* are found in operons with *grlA* and *ahpD*, respectively.

### 4.2. Complementation of an *ahpC* and Catalase-Deficient *E. coli* Mutant

Since both *ahpC* genes were expressed in *L. pneumophila*, efforts were made to determine if both AhpC1 and AhpC2 could effectively provide peroxidase activity to protect cells against peroxides. To do so, both *ahpC1* and *ahpC2* of *L. pneumophila* were cloned independently into the IPTG-inducible expression vector pTrc99A and transformed into an *ahpCF*- and *katG, katE*-defective *E. coli* mutant J1377, a strain that

**Figure 10** Phylogenetic analysis of *L. pneumophila* AhpCs. The phylogenetic tree was constructed by multiple sequence alignments of AhpC sequences describe below. Strains, abbreviations and accession numbers are as follows : *Aeromonas hydrophila*, Ahy (AAU9339); *Bacillus anthracis*, Ba (YP_016961); *Bordetella pertussis*, Bp (CAE43811); *Bradyrhizobium japonicum*, Bj (NP_768417); *Brucella suis*, Bs (AAN33893); *Burkholderia fungorum*, Bf (ZP_00280258); *Corynebacterium diphtheriae* (CAE49951); *Coxiella burnetii*, Cb1 (NP_820687) and Cb2 (NP_820460); *E. coli*, Ec (AAC73706); *Francisella tularensis*, Ftu (YP_513702); *Haemophilus influenzae*, Hin (AAS67289); *Helicobacter pylori*, Hp (CAE47416); *L. pneumophila*, Lp1 (YP_125339) and Lp2 (YP_096359); *Mycobacterium tuberculosis*, Mt (NP_216944); *Pasteurella multocida*, Pmu (AAK02879); *Pseudomonas putida*, Pp1 (AAN66709) and Pp2 (NP_744587); *Rickettsiella grylli*, Rgr (ZP_01300617); *Salmonella typhimurium*, St1 (AAL19356) and St2 (AAL19559); *Shewanella amazonensis*, Sam (ZP_00587734); *Staphylococcus aureus*, Sa (YP_039855); *Streptomyces coelicolor*, Sc (CAC05877); *Vibrio vulnificus*, Vv1 (AAO08975) and Vv2 (NP_762462). *L. pneumophila* AhpC1 (Lp1) and AhpC2 (Lp2) are indicated by arrows.

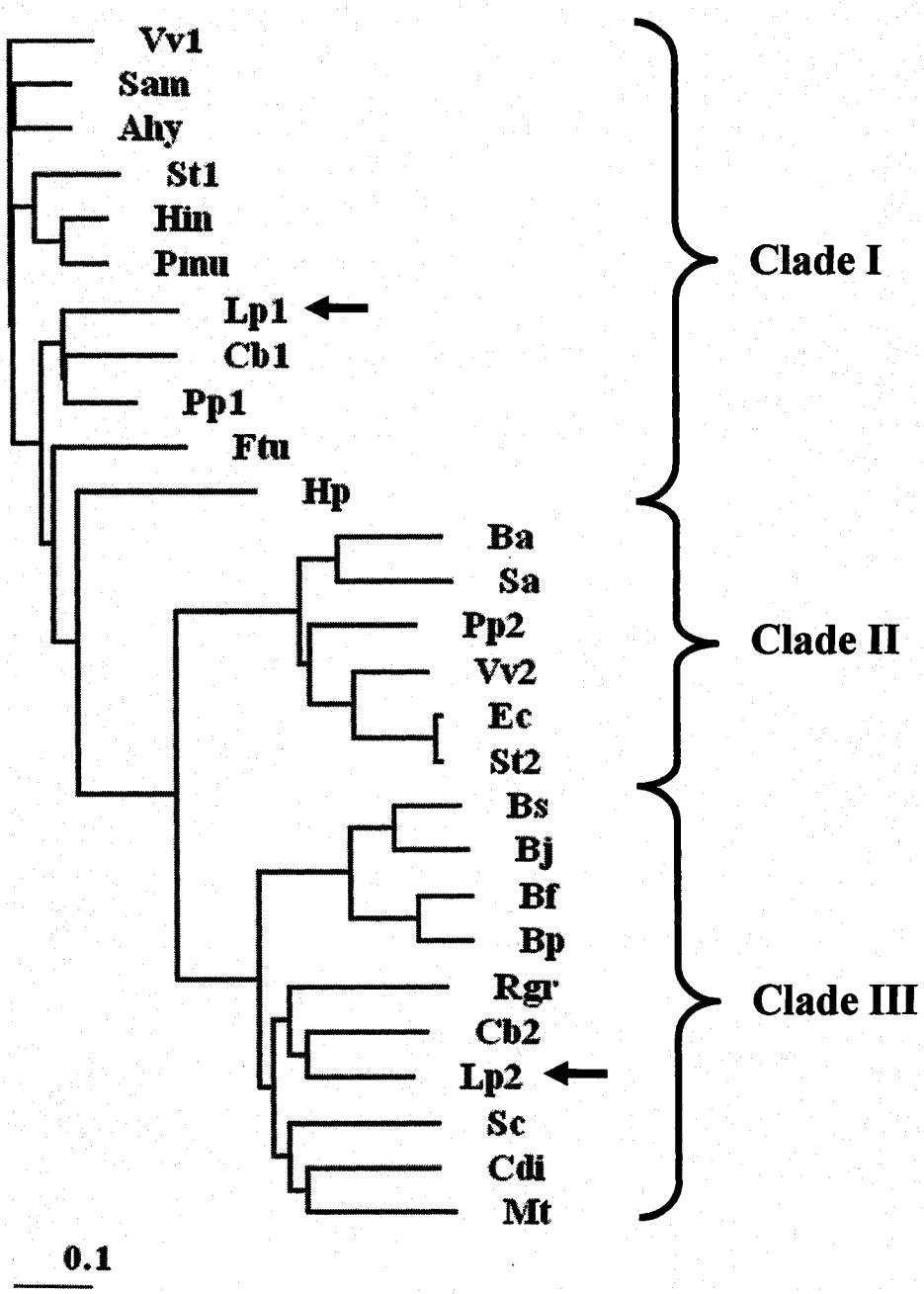
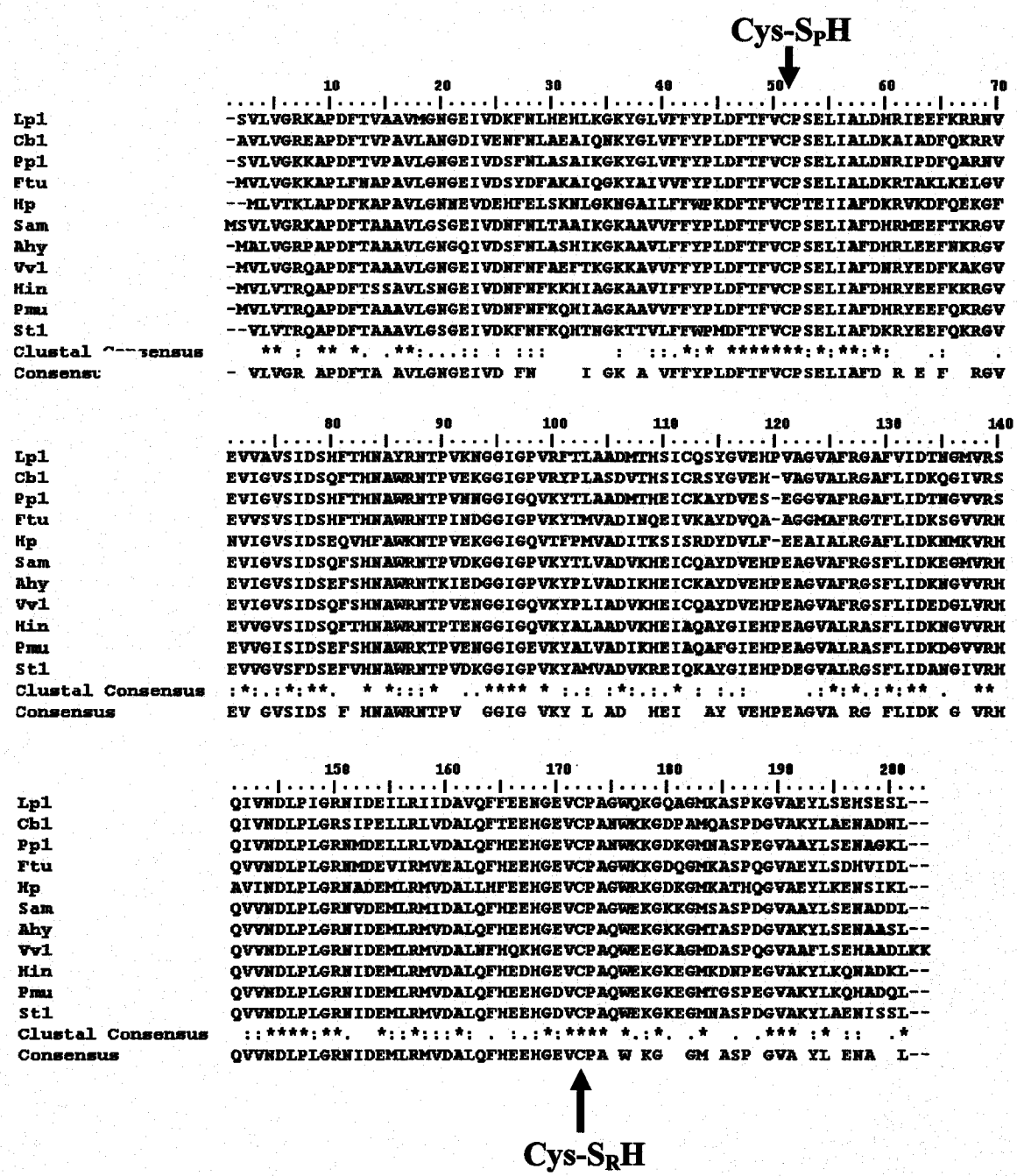

Vv1
Sam
Ahy
St1
Hin
Pmu
Lp1
Cb1
Pp1
Ftu
Hp
Ba
Sa
Pp2
Vv2
Ec
St2
Bs
Bj
Bf
Bp
Rgr
Cb2
Lp2
Sc
Cdi
Mt
Clade I
Clade II
Clade III
0.1

**Figure 11** Multiple sequence alignments of *L. pneumophila* AhpCs. A) Alignment of AhpC1 homolgues: *A. hydrophila* (Ahy), *C. burnetii* (Cb1), *F. tularensis* (Ftu), *H. influenzae* (Hin), *H. pylori* (Hp); *L. pneumophila* (Lp1), *P. multocida* (Pmu), *P. putida* (Pp1), *S. typhimurium* (St1), *S. amazonensis* (Sam), and *V. vulnificus* (Vv1). B) Alignment of AhpC2 homologs: *B. pertussis* (Bp), *B. japonicum* (Bj), *B. suis* (Bs), *B. fungorum* (Bf), *C. diphtheriae* (Cdi), *C. burnetii* (Cb2), *L. pneumophila* (Lp2), *M. tuberculosis* (Mt), *R. grylli* (Rgr), and *S. coelicolor* (Sc). Peroxidatic and resolving cysteines (Cys-$S_P$H and Cys-$S_R$H, respectively) are indicated by arrows.

A)

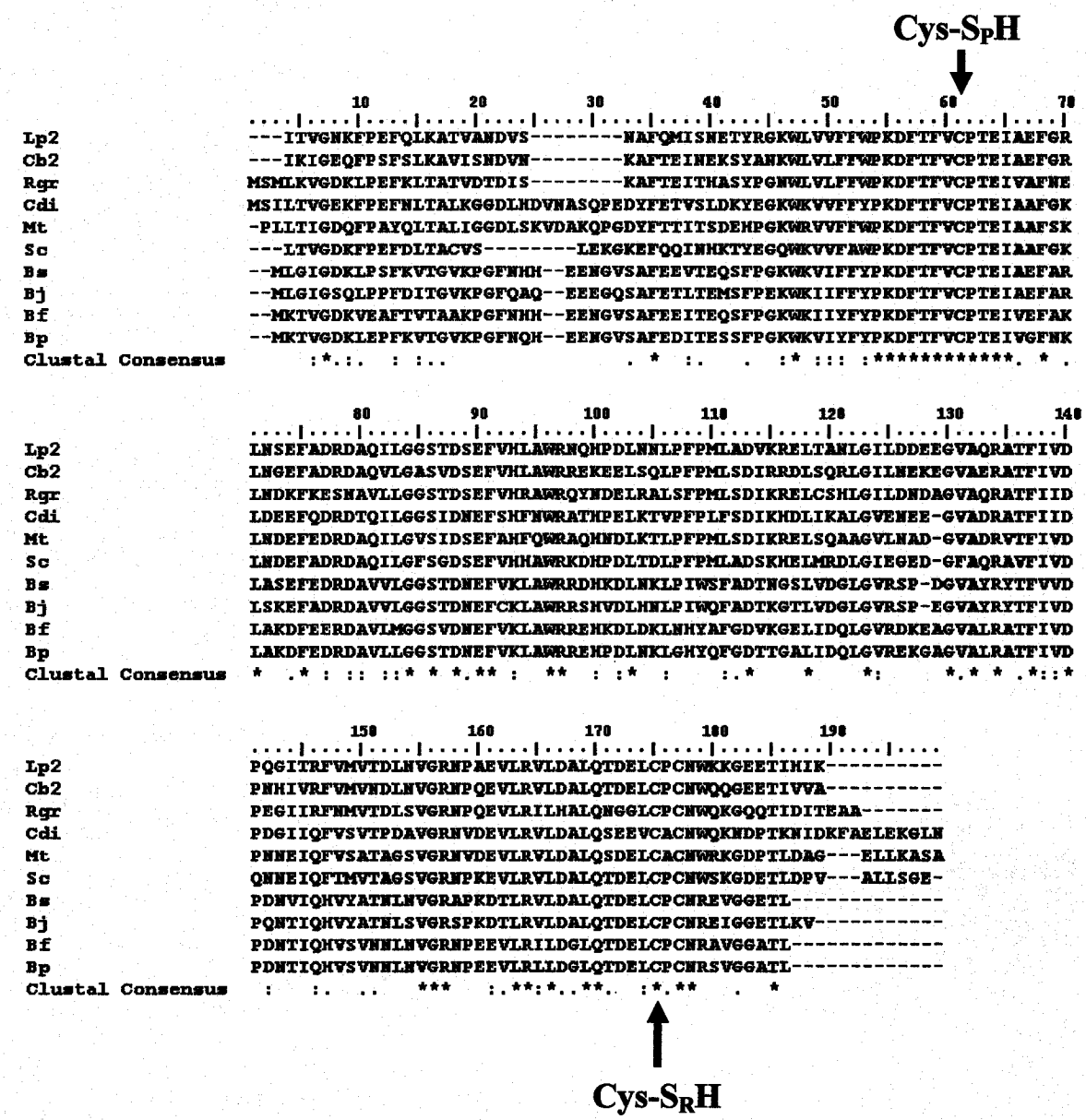



```
                                                                                Cys-SPH
                                                                                   |
                    10        20        30        40        50        60        70
                    |         |         |         |         |         |         |
Lpl                 -SVLVGRKAPDFTVAAVMGNGEIVDKFNLHEHLKGKYGLVFFYPLDFTFVCPSELIALDHRIEEFKRRNV
Cbl                 -AVLVGREAPDFTVPAVLANGDIVENFNLAEAIQNKYGLVFFYPLDFTFVCPSELIALDKAIADFQKRRV
Ppl                 -SVLVGKKAPDFTVPAVLGNGEIVDSFNLASAIKGKYGLVFFYPLDFTFVCPSELIALDNRIPDFQARNV
Ftu                 -MVLVGKKAPLFNAPAVLGNGEIVDSYDFAKAIQGKYAIVVFYPLDFTFVCPSELIALDKRTAKLKELGV
Hp                  --MLVTKLAPDFKAPAVLGNNEVDEHFELSKNLGKNGAILFFWPKDFTFVCPTEIIAFDKRVKDFQEKGF
Sam                 MSVLVGRKAPDFTAAAVLGSGEIVDNFNLTAAIKGKAAVVFFYPLDFTFVCPSELIAFDHRMEEFTKRGV
Ahy                 -MALVGRPAPDFTAAAVLGNGQIVDSFNLASHIKGKAAVLFFYPLDFTFVCPSELIAFDHRLEEFNKRGV
Vvl                 -MVLVGRQAPDFTAAAVLGNGEIVDNFNFAEFTKGKKAVVFFYPLDFTFVCPSELIAFDNRYEDFKAKGV
Hin                 -MVLVTRQAPDFTSSAVLSNGEIVDNFNFKKHIAGKAAVIFFYPLDFTFVCPSELIAFDHRYEEFKKRGV
Pmu                 -MVLVTRQAPDFTAAAVLGNGEIVDNFNFKQHIAGKAAVVFFYPLDFTFVCPSELIAFDHRYEEFQKRGV
Stl                 --VLVTRQAPDFTAAAVLGSGEIVDKFNFKQHTNGKTTVLFFWPMDFTFVCPSELIAFDKRYEEFQKRGV
Clustal Consensus      ** : ** *. .**:...:: : :::       :  ::.*:* *******:*:**:*:   .:    .
Consensus           - VLVGR APDFTA AVLGNGEIVD FN    I GK A VFFYPLDFTFVCPSELIAFD R E F  RGV

                         80        90        100       110       120       130       140
                         |         |         |         |         |         |         |
Lpl                 EVVAVSIDSHFTHNAYRNTPVKNGGIGPVRFTLAADMTHSICQSYGVEHPVAGVAFRGAFVIDTNGMVRS
Cbl                 EVIGVSIDSQFTHNAWRNTPVEKGGIGPVRYPLASDVTHSICRSYGVEH-VAGVALRGAFLIDKQGIVRS
Ppl                 EVIGVSIDSHFTHNAWRNTPVNNGGIGQVKYTLAADMTHEICKAYDVES-EGGVAFRGAFLIDTNGVVRS
Ftu                 EVVSVSIDSHFTHNAWRNTPINDGGIGPVKYTMVADINQEIVKAYDVQA-AGGMAFRGTFLIDKSGVVRH
Hp                  NVIGVSIDSEQVHFAWKNTPVEKGGIGQVTFPMVADITKSISRDYDVLF-EEAIALRGAFLIDKNMKVRH
Sam                 EVIGVSIDSQFSHNAWRNTPVDKGGIGPVKYTLVADVKHEICQAYDVEHPEAGVAFRGSFLIDKEGMVRH
Ahy                 EVIGVSIDSEFSHNAWRNTKIEDGGIGPVKYPLVADIKHEICKAYDVEHPEAGVAFRGSFLIDKNGVVRH
Vvl                 EVIGVSIDSQFSHNAWRNTPVENGGIGQVKYPLIADVKHEICQAYDVEHPEAGVAFRGSFLIDEDGLVRH
Hin                 EVVGVSIDSQFTHNAWRNTPTENGGIGQVKYALAADVKHEIAQAYGIEHPEAGVALRASFLIDKNGVVRH
Pmu                 EVVGISIDSEFSHNAWRKTPVENGGIGEVKYALVADIKHEIAQAFGIEHPEAGVALRASFLIDKDGVVRH
Stl                 EVVGVSFDSEFVHNAWRNTPVDKGGIGPVKYAMVADVKREIQKAYGIEHPDEGVALRGSFLIDANGIVRH
Clustal Consensus   :*:.:*:**.  * *:::*  ..**** * :.: :*:.:.* : :.:     .:*:*.:*:** .  **
Consensus           EV GVSIDS F HNAWRNTPV  GGIG VKY L AD  HEI  AY VEHPEAGVA RG FLIDK G VRH

                              150       160       170       180       190       200
                              |         |         |         |         |         |
Lpl                 QIVNDLPIGRNIDEILRIIDAVQFFEEHGEVCPAGWQKGQAGMKASPKGVAEYLSEHSESL--
Cbl                 QIVNDLPLGRSIPELLRLVDALQFTEEHGEVCPANWKKGDPAMQASPDGVAKYLAENADNL--
Ppl                 QIVNDLPLGRNMDELLRLVDALQFHEEHGEVCPANWKKGDKGMNASPEGVAAYLSENAGKL--
Ftu                 QVVNDLPLGRNMDEVIRMVEALQFHEEHGEVCPAGWKKGDQGMKASPQGVAEYLSDHVIDL--
Hp                  AVINDLPLGRNADEMLRMVDALLHFEEHGEVCPAGWRKGDKGMKATHQGVAEYLKENSIKL--
Sam                 QVVNDLPLGRNVDEMLRMIDALQFHEEHGEVCPAGWEKGKKGMSASPDGVAAYLSENADDL--
Ahy                 QVVNDLPLGRNIDEMLRMVDALQFHEEHGEVCPAQWEKGKKGMTASPDGVAKYLSENAASL--
Vvl                 QVVNDLPLGRNIDEMLRMVDALNFHQKHGEVCPAQWEEGKAGMDASPQGVAAFLSEHAADLKK
Hin                 QVVNDLPLGRNIDEMLRMVDALQFHEDHGEVCPAQWEKGKEGMKDNPEGVAKYLKQNADKL--
Pmu                 QVVNDLPLGRNIDEMLRMVDALQFHEEHGDVCPAQWEKGKEGMTGSPEGVAKYLKQHADQL--
Stl                 QVVNDLPLGRNIDEMLRMVDALQFHEEHGDVCPAQWEKGKEGMNASPDGVAKYLAENISSL--
Clustal Consensus    ::****:**.  *::*:::*: . :.:*:**** *.:*. .*  . .*** :* ::  .*
Consensus           QVVNDLPLGRNIDEMLRMVDALQFHEEHGEVCPA W KG  GM ASP GVA YL ENA  L--
                                                  |
                                                  |
                                                Cys-SRH
```

**B)**


Cys-SpH
Cys-SRH
Lp2
Cb2
Rgr
Cdi
Mt
Sc
Bs
Bj
Bf
Bp
Clustal Consensus

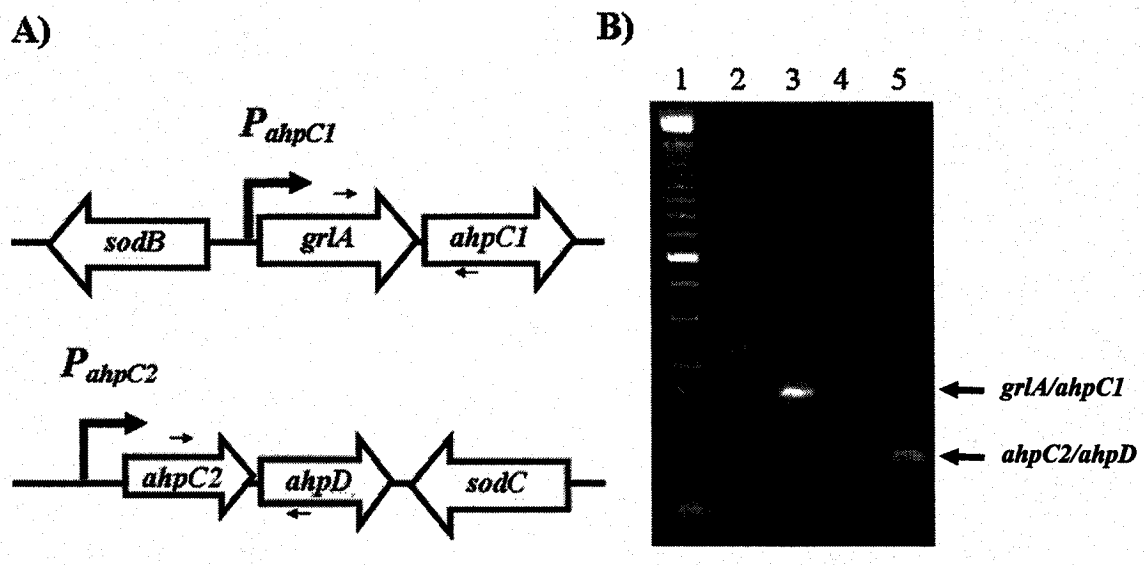



**Figure 12** Genetic organizations of the *ahpC1* and *ahpC2* loci. A) Schematic representation of the two *ahpC* operons. Small arrows represent primers used in RT-PCR reactions. Large arrows indicate putative promoter regions ($P_{ahpC1}$ and $P_{ahpC2}$). B) RT-PCR validation of operon structure. Invitrogen 100-bp DNA ladder was used as a size reference (lane 1). Negative controls (lanes 2 and 4) consisted of a no-RT reaction for DNaseI-treated RNA or positive RT-PCR reactions for the *grlA/ahpC1* (lane 3) or *ahpC2/ahpD* operon (lane 5).

exhibits virtually no hydroperoxides activity. When these cells are exposed to $H_2O_2$ in a standard disk diffusion assay any peroxide resistance conferred by *L. pneumophila ahpC* genes should manifest itself as a decrease in the zone of inhibition (in other words, an increase in $H_2O_2$ resistance) (Figure 13).

Under repressing conditions (absence of IPTG), *E. coli* J1377 cells harboring pTrc99A-derived plasmids containing either *L. pneumophila ahpC1* (ptrcC1) or *ahpC2* (ptrcC2) showed a level of sensitivity to $H_2O_2$ similar to that of J1377 harboring the empty-vector control (ptrc). Indeed, zones of inhibition for theses strains were 49 ± 2, 49 ± 2, and 49 ± 1 mm for J1377 ptrc, J1377 ptrcC1, and J1377 ptrcC2, respectively. In contrast, IPTG-mediated induction resulted in a decrease of the zone of inhibition for strains harboring the *L. pneumophila ahpC* genes (48 ± 1 versus 33 ± 4 and 31 ± 2 mm for J1377 ptrc, J1377 ptrcC1, and J1377 ptrcC2, respectively). No differences were observed between J1377 ptrc and J1377 ptrcD containing *L. pneumophila ahpD*, in the presence (50 ± 2 mm) and absence of IPTG (49 ± 3). These data are not depicted in Figure 13. These data indicate that both *L. pneumophila* AhpC1 and AhpC2 can complement the *E. coli* peroxidase/catalase-deficient mutant, a phenotype consistent with the proposed role for these AhpCs in protecting against toxic levels of peroxides.

### 4.3. Construction and Complementation of *L. pneumophila* Mutants

#### 4.3.1. Construction of *L. pneumophila ahpC1* and *ahpC2D* Mutants

To assess the roles of *L. pneumophila* AhpC1 and AhpC2 in oxidative stress and virulence, chromosomal deletion mutants were created using the pBRDX suicide delivery vector that was developed in our laboratory (Morash, 2006). An example of construction

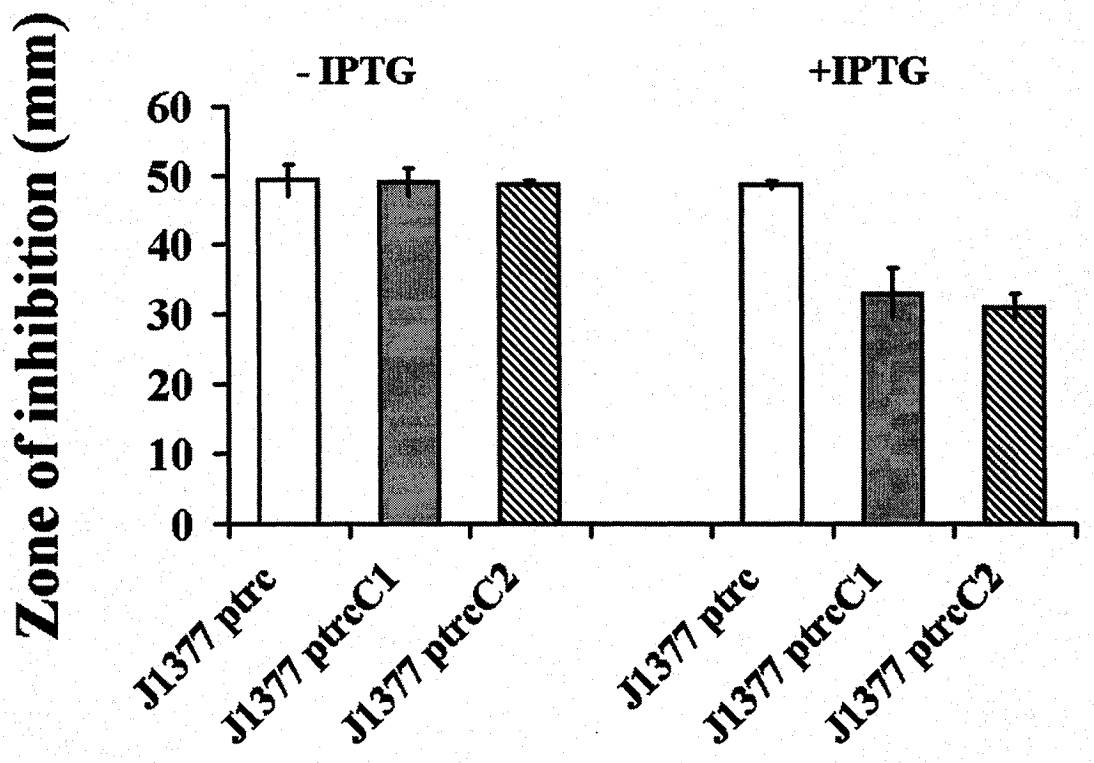


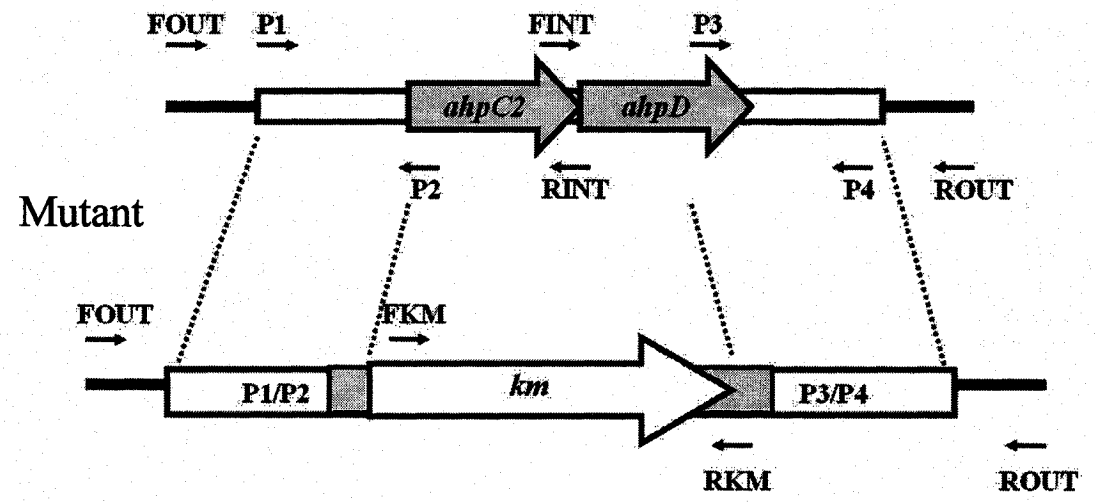

**Figure 13** Expression of *ahpC1* and *ahpC2* in *E. coli* J1377. Disk diffusion assays were performed for peroxide detoxification by *E. coli* J1377 cells harboring the empty-vector control (J1377 ptrc), *L. pneumophila ahpC1* (J1377 ptrcC1) or *ahpC2* (J1377 ptrcC2) in the presence or absence of 1 mM IPTG. Assay was performed in triplicate and results indicate the mean diameter of clearing ± SD.

of the pBRDXΔ*ahpC2D::km* construct is provided in Figure 8. This plasmid was previously created in our laboratory by Dr. Michael G. Morash and Dr. Ann Karen C. Brassinga. It carries *H. pylori rdxA* (Goodwin *et al.*, 1998), encoding a NADPH nitroreductase which converts metronidazole (2-methyl-5-nitroimidazole-1-ethanol) to its DNA-damaging reactive intermediate (Sisson *et al.* 2000). Therefore, bacteria harboring pBRDX-derived plasmids are rendered sensitive to metronidazole. In addition, pBRDX ncodes a chloramphenicol acetyltransferase (*cat*), which confers resistance to chloramphenicol, and *sacB*, a gene encoding levansucrase from *B. subtilis* that confers sensitivity to sucrose (a second counter-selection).

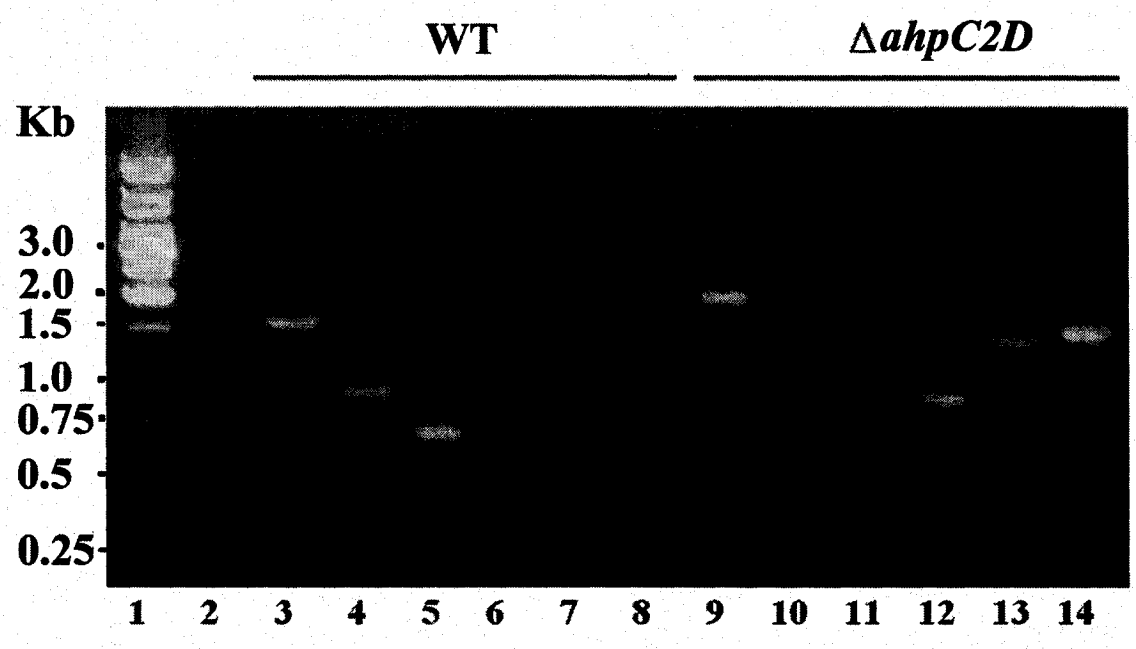
All pBRDX-derived plasmids were subjected to electroporation into electrocompetent Lp02 and transformants were spread on the appropriate antibiotic selection medium. The resulting colonies were transferred on fresh medium with antibiotics (master plate), or media containing appropriate selection. It was essential to differentiate merodiploid clones containing the unrecombined plasmid from the desired clones that have acquired kanamycin or gentamicin resistance by chromosomal insertion of the *ahpC::km* (or *ahpC::gm*) sequences at the *ahpC* locus. To select for the double crossover event (clones that have undergone allelic replacement and loss of the pBRDX-derived plasmids), transformants were replica plated on selective medium. Supplementation with metronidazole, sucrose or chloramphenicol was used to eliminate clones that had not undergone allelic recombination. Screening for mutants (gene replacement) was done by PCR analysis using oligonucleotides sequence specific for the deleted regions of respective genes or for the antibiotic-resistance marker. An example for the validation of the *ahpC2D::km* mutant is provided in Figure 14. For each of the

**Figure 14** Identification of *L. pneumophila* mutants by PCR analysis. A) Schematic representation of the *ahpC* loci in wild-type or the putative *ahpC::km* mutant. B) To validate chromosomal insertion of *ahpC::km* and distinguish a mutant strain from wild-type, the following primer pairs were used: FOUT/ROUT (amplicons are smaller in WT than in *ahpC::km*, compare lanes 3 and 9); FOUT/RINT (positive PCR for WT, negative for *ahpC::km*, compare lanes 4 and 10); FINT/ROUT (positive PCR for WT, negative for *ahpC::km*; compare lanes 5 and 11); FKM/RKM (negative for WT, positive PCR for *ahpC::km*, compare lanes 6 and 12); FKM/ROUT (negative for WT, positive PCR for *ahpC::km*, compare lanes 7 and 13); FOUT/RKM (negative for WT, positive PCR for *ahpC::km*, compare lanes 8 and 14).

A)


Wild-type
FOUT
P1
FINT
P3
ahpC2
ahpD
P2
RINT
P4
ROUT
Mutant
FOUT
FKM
P1/P2
km
P3/P4
RKM
ROUT

B)


WT
ΔahpC2D
Kb
3.0
2.0
1.5
1.0
0.75
0.5
0.25
1 2 3 4 5 6 7 8 9 10 11 12 13 14

transformants, approximately 100 colonies were screened, which yielded approximately 4 mutants for each of the *ahpC* genes. No *oxyR::km* or *oxyR::gm* mutants were obtained using similar techniques.

### 4.3.2. Construction of *ahpC* Double Mutants

Attempts to construct *ahpC* double mutants (*ahpC1::km ahpC2D::gm* or *ahpC1::gm ahpC2D::km*) in *L. pneumophila* were unsuccessful. To eliminate the possibility that the gentamicin-resistance cassette may not be properly expressed in Lp02 *ahpC::km* mutants, chromosomal deletions for each of the *ahpC* genes were created using the gentamicin-resistance cassette. As a second attempt to obtain double mutants using reciprocal markers, the pBRDXΔ*ahpC::km* constructs were introduced into gentamicin-resistant mutants. The pBRDXΔ*ahpC1::gm* and pBRDXΔ*ahpC2D::gm* suicide vectors were transformed into a *magA::km L. pneumophila* mutant (Hiltz *et al.*, 2004). Gentamicin-resistant *ahpC* mutants could be recovered in the *magA*-deficient background, thereby validating simultaneous use of the kanamycin- and gentamicin-resistance markers. However, no *ahpC1 ahpC2D* double mutant could be obtained, suggesting that alkyl hydroperoxide reductase function may be essential for viability. In all experiments, supplementation of BCYE with an excess of bovine catalase during recovery of possible double mutants (or *oxyR* mutants) did not aid in the ability to obtain mutants. Unlike the situation for *E. coli*, it was not possible to isolate these mutants in the absence of molecular oxygen or under microaerobic condition given that *L. pneumophila* is a strict aerobe.

Using IPTG-inducible promoters of pMMB206, we investigated whether complementation of *ahpC* mutants might permit *ahpC* double mutants to survive in the

presence of IPTG (but die in its absence). Electroporation of pBRDXΔ*ahpC1::gm* or pBRDXΔ*ahpC2D::gm* constructs into the *ahpC2D::km* pMMBahpC2D or *ahpC1::km* pMMBahpC1 mutants, respectively, yielded inexplicable recombination events. Parallel experiments using complemented gentamicin-resistant mutants yielded similar results. These results may, in part, be attributed to the design of the pBRDX-derived constructs, since some coding sequence is still present in the P1/P2 or P3/P4 portions. Thus, the coding sequence remaining in the Δ*ahpC::gm* (or Δ*oxyR::km*) may promote recombination with the intact *ahpC* genes found on the pMMB206-derived constructs. No attempts were made to repeat these experiments using pBRDX-derived constructs (and mutants) that lack the respective coding sequences. Since the *oxyR* and *ahpC* double mutants could not be obtained, their functions were thought to be essential for viability and efforts were therefore focus on the characterization of the *ahpC1::km* and *ahpC2D::km* mutants.

### 4.4. Characterization of *L. pneumophila ahpC* Mutants

#### 4.4.1 Sensitivity of *ahpC1* and *ahpC2D* Mutants to Oxidative Stress

To address the apparent redundancy of the two AhpC systems, MIC and MBC values for $H_2O_2$, tBOOH, CHP, and PQ were determined for wild-type cells, *ahpC* mutants, and the respective *trans*-complemented derivatives. PQ (paraquat or methyl viologen) was also used in this study, since it is a redox cycling compound known to generate intracellular superoxide which, in turn, is converted to $H_2O_2$ by SOD. Since minimal inhibitory and minimal bactericidal concentrations values were identical (MIC = MBC), Table 3 reflects the minimal concentrations of peroxide necessary to both inhibit the growth and kill the various *L. pneumophila* strains grown in BYE medium.

MIC/MBC values for wild-type *L. pneumophila* were 1000 μM for both $H_2O_2$ and tBOOH, 500 μM for CHP, and 250 μM for PQ. In contrast, the values for the *ahpC1* and *ahpC2D* mutants were 2- to 4-fold lower, indicating the mutants are more sensitive to peroxides. Moreover, the *ahpC1* mutant was approximately 2-fold more sensitive to peroxides than was the *ahpC2D* mutant, suggesting that AhpC1 may be more abundant or more efficient in removal of peroxide. Presence of the pJB908 empty vector did not influence peroxide sensitivities of wild-type or *ahpC* mutants, validating its suitability for *trans*-complementation of the *ahpC* mutants. Complementation of *ahpC1* and *ahpC2D* mutants with pJB908-derived contructs containing both promoter and coding sequences of *grlAahpC1* or *ahpC2ahpD* (pC1 and pC2, respectively) restored wild-type resistance to $H_2O_2$, tBOOH, CHP and PQ.

**Table 3** Sensitivity of *L. pneumophila* strains to oxidative stress

| Strains | MIC[1] or MBC[1] (μM) | | | |
|---|---|---|---|---|
| | $H_2O_2$ | tBOOH | CHP | PQ |
| Lp02 | 1000 ± 0 | 1000 ± 0 | 500 ± 0 | 250 ± 0 |
| Lp02 pJB908 | 1000 ± 0 | 1000 ± 0 | 500 ± 0 | 250 ± 0 |
| *ahpC1*::*km* | 125 ± 0 | 125 ± 0 | 188 ± 63 | 94 ± 31 |
| *ahpC1*::*km* pJB908 | 125 ± 0 | 125 ± 0 | 250 ± 0 | 125 ± 0 |
| *ahpC1*::*km* pC1 | 750 ± 250 | 750 ± 250 | 500 ± 0 | 250 ± 0 |
| *ahpC2D*::*km* | 250 ± 0 | 250 ± 0 | 250 ± 0 | 125 ± 0 |
| *ahpC2D*::*km* pJB908 | 250 ± 0 | 250 ± 0 | 250 ± 0 | 125 ± 0 |
| *ahpC2D*::*km* pC2D | 750 ± 250 | 750 ± 250 | 500 ± 0 | 250 ± 0 |

[1]Minimal inhibitory and bactericidal concentrations were equivalent in this experiment.

In a related series of experiments, wild-type *L. pneumophila* and *ahpC* mutants were grown to mid-exponential phase in BYE broth, harvested, and resuspended in various concentrations of tBOOH. After 30 min of incubation, both *ahpC1* and *ahpC2D* mutants displayed a marked loss in viability compared to wild-type *L. pneumophila* (Figure 15A). When challenged with 500 μM tBOOH for varying periods of time, wild-type exhibited 2-log decrease in viability by 2 h post-challenge, whereas *ahpC1* and *ahpC2D* mutants exhibited a 7-log decrease in viability (Figure 15B). There was a progressive decrease in viability over longer periods of time. Complementation of *ahpC* mutants restored concentration- and time-dependent kinetics of killing by tBOOH to those observed for wild-type (Figure 15A and B). It should also be noted that in both sets of experiments no differences in peroxide resistance were observed when the pJB908 empty-vector control was present in the wild-type or mutant strains. These results show that deletion of either *ahpC* gene renders cells sensitive to tBOOH, suggesting that both *ahpC1* and *ahpC2D* are required for full resistance to peroxides.

### 4.4.2. In vitro and in vivo Growth Rates

As seen in Figures 16 and 17, *ahpC1* and *ahpC2D* mutants displayed growth kinetics in BYE broth, in HeLa cells, and in U937 cells similar to the wild-type Lp02 cells. Following inoculation of BYE broth with 0.2 $OD_{620}$ of cells, all cultures entered mid-exponential and stationary phases of growth at approximately 12-15 h and 24 h, respectively. Presence of pJB908 (empty vector) in wild-type or mutant cells, or complementation of *ahpC* mutants with pJB908-derived plasmids (pC1 and pC2), had no influence on growth kinetics (data not shown). To eliminate any discrepancies of correlating growth and optical density in the BYE culture study, particularly for

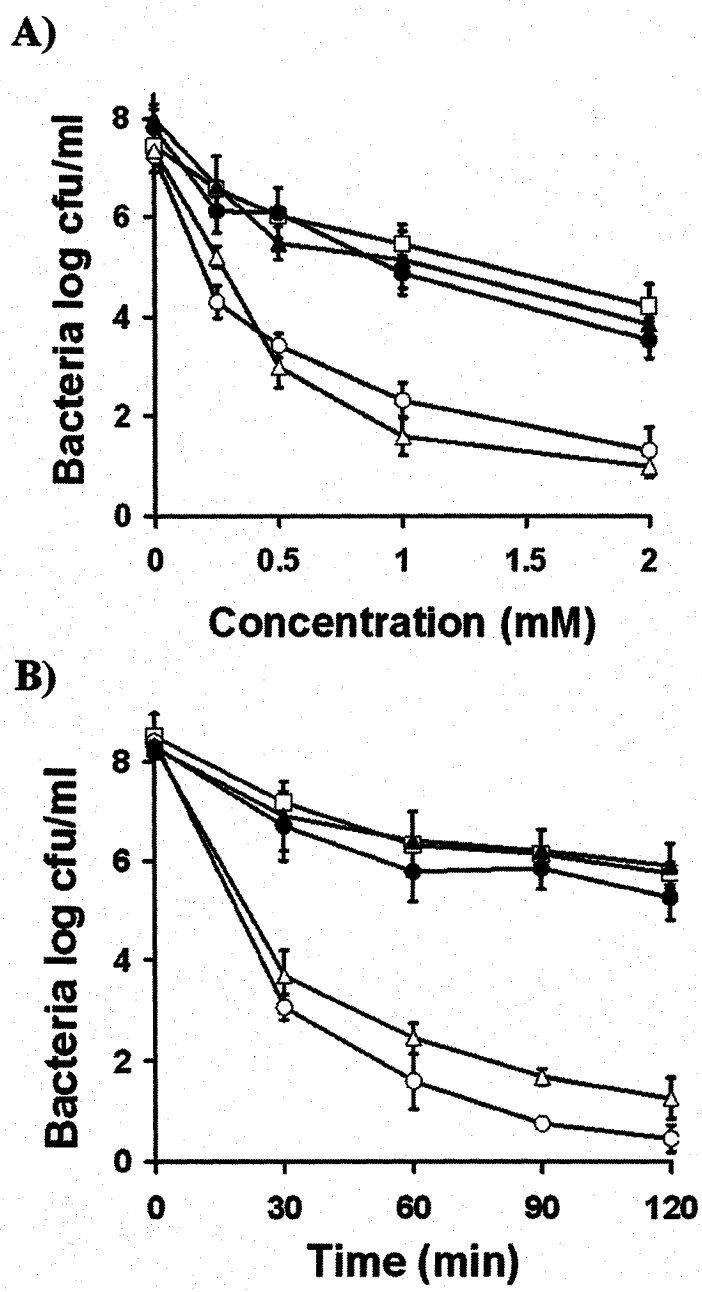
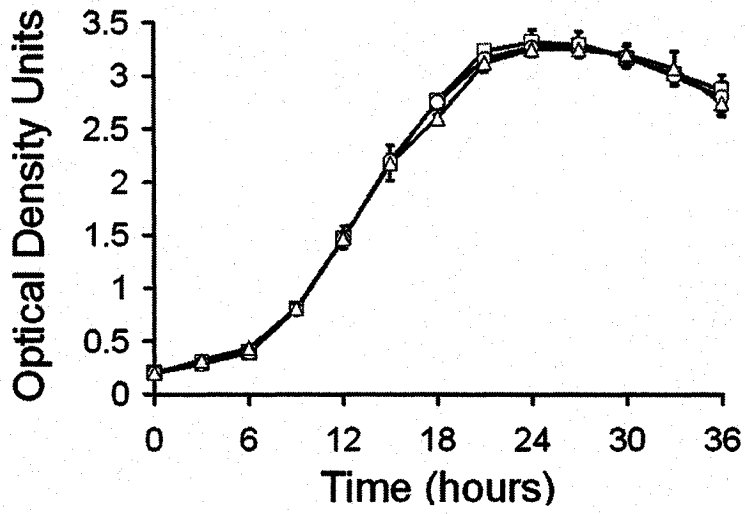



**Figure 15** Peroxide sensitivity of the *L. pneumophila ahpC* mutants. Wild-type *L. pneumophila* (□), *ahpC* mutant strains *ahpC1::km* (○) and *ahpC2D::km* (Δ), and complemented mutants *ahpC1::km* pC1 (●) and *ahpC2D::km* pC2D (▲) were challenged A) for 30 min with varying concentrations of tBOOH ranging from 250 to 2000 μM or B) with 500 μM tBOOH for 0, 30, 60, 90, and 120 min. Results are expressed as $\log_{10}$ of cfu/ml and represent mean values ± SD obtained from three independent experiments.

A)




B)

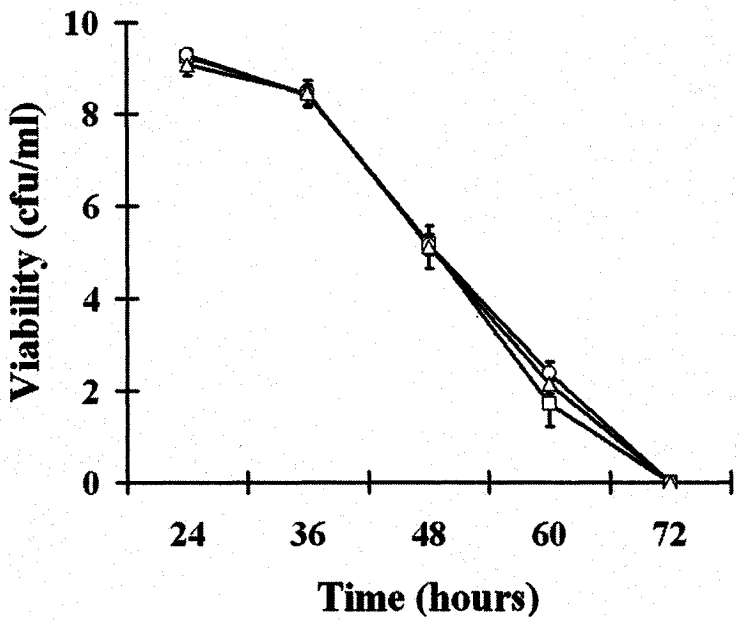
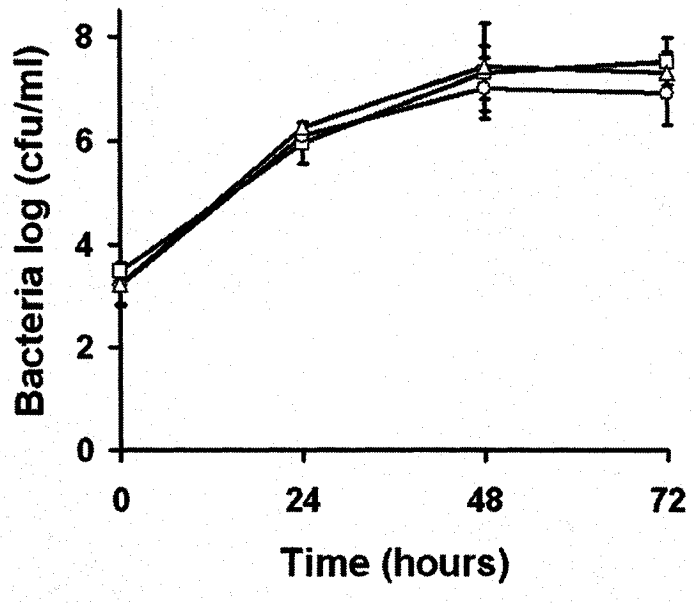



**Figure 16** Growth rates and stationary phase survival of the *ahpC* mutants. A) Growth rates of wild-type *L. pneumophila* (□), *ahpC1::km* (○) and *ahpC2D::km* (Δ) in BYE broth cultures. Optical density reported at 620 nm. B) Stationary phase survival curves of wild-type, *ahpC1::km*, and *ahpC2D::km* cells. Data are reported as the mean $\log_{10}$ cfu/ml ± SD of three independent experiments.

A)




B)

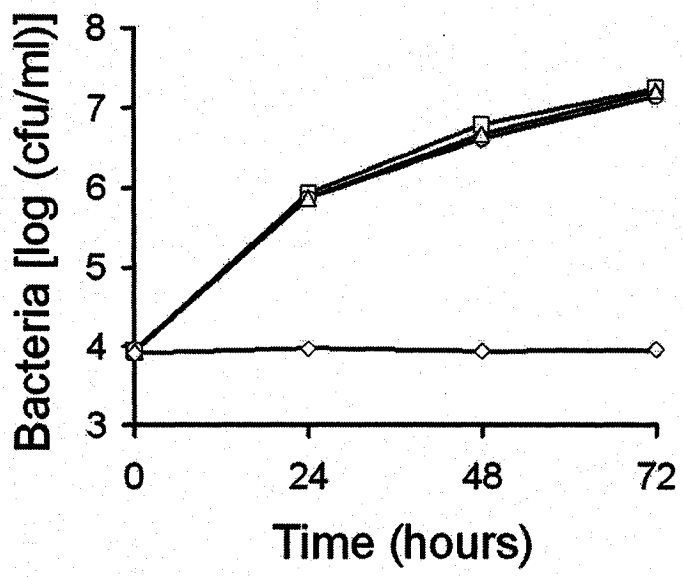



**Figure 17** Intracellular growth rates of the *L. pneumophila ahpC* mutants. A) HeLa and B) U937 cells were infected with *L. pneumophila* wild-type (□), *ahpC1::km* (○) and *ahpC2D::km* (Δ) or *dotB::km* (◊) mutant strains. Data are reported as the mean $\log_{10}$ cfu/ml ± SD of three independent experiments.

stationary-phase survival, viability was assessed following growth up to 96 h in BYE broth. No differences in viability could be observed between wild-type and mutant strains even with extended periods (72 h) of incubation.

For intracellular growth assays the non-phagocytic epithelial-cell derived HeLa cells were used, since they have previously been validated in our laboratory for *L. pneumophila* intracellular growth (Garduno *et al.*, 1998). Intracellular growth in U937 cells was also evaluated, since these cells have long been an established model for *L. pneumophila* intracellular growth used to distinguish virulent from avirulent forms of this organism (Pearlman *et al.*, 1988). PMA-differentiated U937 cells are known to display macrophage-like characteristics, including the ability to induce phagocytosis with generation of an oxidative burst in the phagocytic vacuole (Roberts *et al.*, 1991). Therefore, it was hypothesized that the peroxide-sensitive *ahpC* mutant would likely be exposed to ROIs in the host phagosome of PMA-treated U937 cells. Data in Figure 17 indicate that both wild-type and *ahpC* mutant cells were able to proliferate in HeLa and in U937 cells, which was particularly evident at 24 h post-infection where strains exhibited an approximate 2-log increase in growth. At later time points (48 h and 72 h post-infection), the relative numbers of bacteria recovered were also increased. However, it is generally accepted that *L. pneumophila* infection eventually leads to host cell lysis and would thus not be able to sustain growth kinetics as observed between the 0 and 24 h time points. A *dotB* mutant of Lp02 was included as a negative control in the U937 infection model, since it is known to be defective for intracellular multiplication in the macrophage (Sexton *et al.*, 2004). It should be noted that L929 plaque assays (Fernandez *et al.*, 1994) were also attempted, but yielded no significant differences in plaquing

efficiencies between mutant and wild-type strains (0.15 ± 0.04 for WT, 0.09 ± 0.06 for the *ahpC1* mutant, and 0.11 ± 0.03 for the *ahpC2D* mutant). Since both *ahpC1* and *ahpC2D* mutants showed growth kinetics that were indistinguishable from those of the parent strain Lp02 in HeLa, U937 and L929 cells, no investigations were performed using the complemented *ahpC* strains.

### 4.4.3. Growth-Phase and Compensatory Expression of *ahpC1* and *ahpC2*

To determine if *ahpC1* and *ahpC2* are differentially expressed during growth in BYE broth, a GFP-reporter assay was performed. As seen in Figure 18, *ahpC1* expression decreased during lag and early-exponential phases of growth (0 to 12 h) and increased again in mid-exponential and early-stationary phases (12 to 30 h). In contrast, *ahpC2D* levels increased during lag of growth (0 to 6 h) and decreased during early-exponential, late-exponential and stationary phases (12 h to 36 h). Thus, *ahpC2* is thought to be repressed under these conditions. The GFP-reporter studies also indicated that *ahpC1* is more highly expressed than *ahpC2D*, which is consistent with MIC and MBC findings described earlier (Table 3). Since the growth rates of either of the *ahpC* mutants were similar to that of wild-type, it was proposed that in each mutant the remaining *ahpC* gene might compensate through increased levels of gene expression. To determine if the expression profiles of *ahpC1* and *ahpC2* are altered in the *ahpC* mutants, GFP-reporter constructs were introduced into strains of the opposing mutant background (pC1gfp in the *ahpC2D* mutant and pC2gfp in the *ahpC1* mutant). Although the growth-cycle gene expression profile was not altered, an increased expression (~ 2-fold) over levels detected in the wild-type cells was observed when the pC2gfp construct was present in the *ahpC1* mutant. Furthermore, a high basal level of expression (~ 2- to 3-

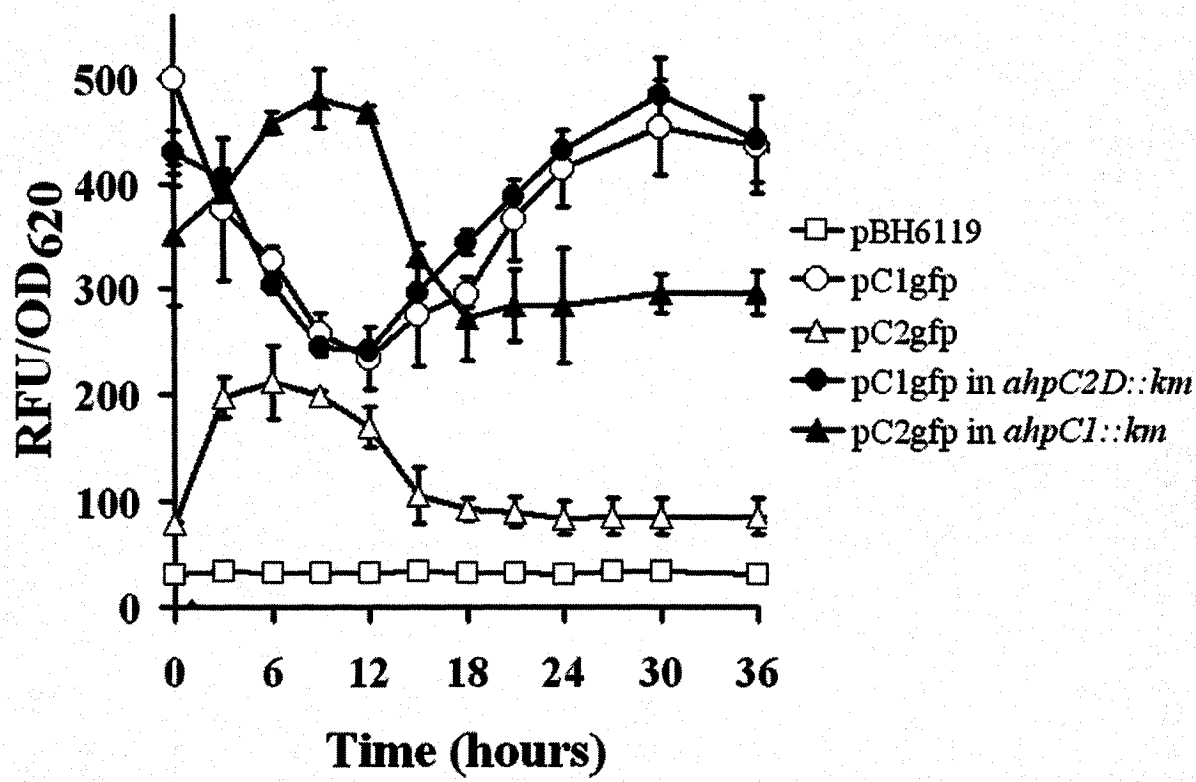



**Figure 18** Growth-phase and compensatory expression of *ahpC1* and *ahpC2*. Samples were taken every three hours of BYE-grown strains Lp02 bearing indicated plsmids. Fluorescence was determined at 488 nm. Relative fluorescence units (rfu) were normalized to 1.0 $OD_{620}$ of cells. Data are reported as the mean ± SD of three independent experiments.

fold higher than wild-type levels) was maintained through late-exponential and stationary phases of growth. In contrast, no significant increase in fluorescence was observed with the pC1gfp construct in the *ahpC2D* mutant (Figure 18).

### 4.4.4. Analysis of Compensatory Gene Expression by qPCR

Since results from the GFP-reporter assay indicate that AhpC2D may compensate for deficiencies in AhpC1 activity, reverse-transcriptase quantitative PCR (RT-qPCR, real-time PCR or qPCR) was used to compare mRNA levels for *ahpC1* and *ahpC2*. Relative quantification was chosen (versus absolute) since it is the analytic method of choice for many real-time PCR studies and is best applied when multiple genes or various conditions are being analyzed. For accurate quantification of cDNA levels in qPCR, an internal control is required to compensate for variations in the amounts of starting material, enzyme efficiencies, and differences in overall transcriptional activity. Since our laboratory had some success with amplification of the structural gene for the L10 ribosomal protein (*rplJ*), it was analyzed for suitability as an internal control. RNA was extracted from exponentially growing wild-type, *ahpC1::km*, *ahpC2D::km*, *ahpC1::km* pC1, and *ahpC2D::km* pC2 cells and normalized to equivalent amounts (100 ng) (by spectrophotometry; 1.0 $OD_{260}$ corresponds to 40 μg/ml of RNA), and after reverse transcription, *rplJ* cDNA was quantified by qPCR. Since *rplJ* cDNA template gave similar threshold-cycle ($C_T$) values (approximately 25) for all strains, the *rplJ* mRNA was deemed an appropriate internal control (Figure 19). However, this control was only validated for RNA extracted from exponentially growing populations of cells, and therefore should not be applied blindly for other time points or for different experimental conditions.

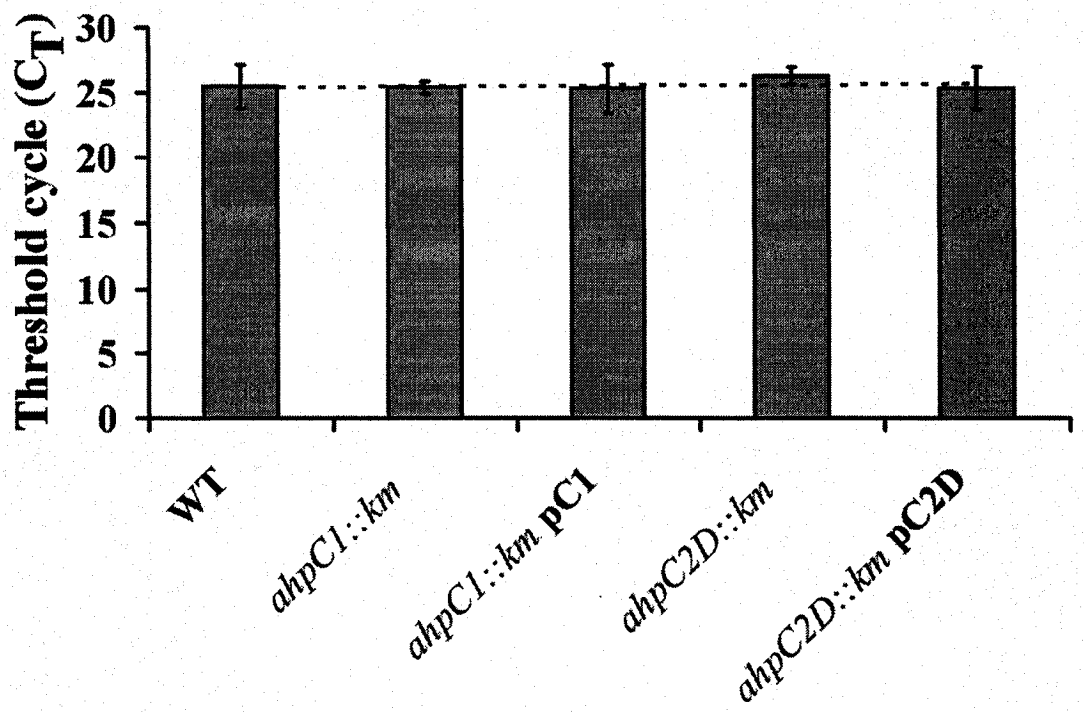



**Figure 19** Validation of *rplJ* for use as an internal control for qPCR analysis. Equivalent amounts (100 ng) of total RNA extracted from exponentially growing cultures of wild-type, *ahpC1::km*, *ahpC2D::km*, *ahpC1::km* pC1, and *ahpC2D::km* pC2 cells was subjected to reverse transcription and levels of *rplJ* cDNA were quantifed by qPCR. Threshold cycle ($C_T$) values (the number of PCR cycles necessary to reach a point where the fluorescent signal is first recorded above the background) are reported as the mean ± SD of triplicate samples obtained from three independently grown cultures. The equation for the trend line (dotted line) is $y = 0.0068 + 25.35$ ($R^2 = 0.0634$).

To accurately quantify cDNA levels, quantitative PCR methods require that the PCR efficiencies of all genes be similar, and preferably ≥90%. PCR efficiency (E) was measured using standard curves generated by 10-fold serial dilutions of amplicons synth using genomic DNA as template and primers specific for *rplJ*, *ahpC1*, and *ahpC2* (Figure 20). PCR efficiencies of each gene were then calculated using the following formula: E = 10 (1/-slope) -1 (a slope of -3.32 is equivalent to 100% PCR efficiency). Since efficiencies ranged from 91-93% for all genes analyzed, the standard curves could be used to determine sample concentrations of cDNA.

SYBR green I is an intercalating dye that can insert between the bases of nucleic acids, and thus is double-strand DNA specific. Increasing the temperature to levels which permit denaturation of the double strand therefore results in loss of fluorescence. Each amplicon varies in G + C content and in size; therefore, melting temperatures (Tm) values can be used to distinguish between amplicons. Since the SYBR green I stain cannot differentiate specific amplicons from other PCR-generated contaminants (such as primer-dimers), all amplicons generated by qPCR were subjected to melt-curve analysis (example provided in Figure 21). Amplicons in this study (*rplJ*, *ahpC1*, and *ahpC2*) showed Tm values of 83°C, 87°C, and 89°C, respectively. Presence of multiple peaks or loss of symmetry of the typical bell-shaped distribution would signify contaminating PCR products (non-specific amplification or primer-dimers). All amplicons generated by qPCR were also subjected to agarose gel electrophoresis to confirm the accuracy of the melt-curve analysis.

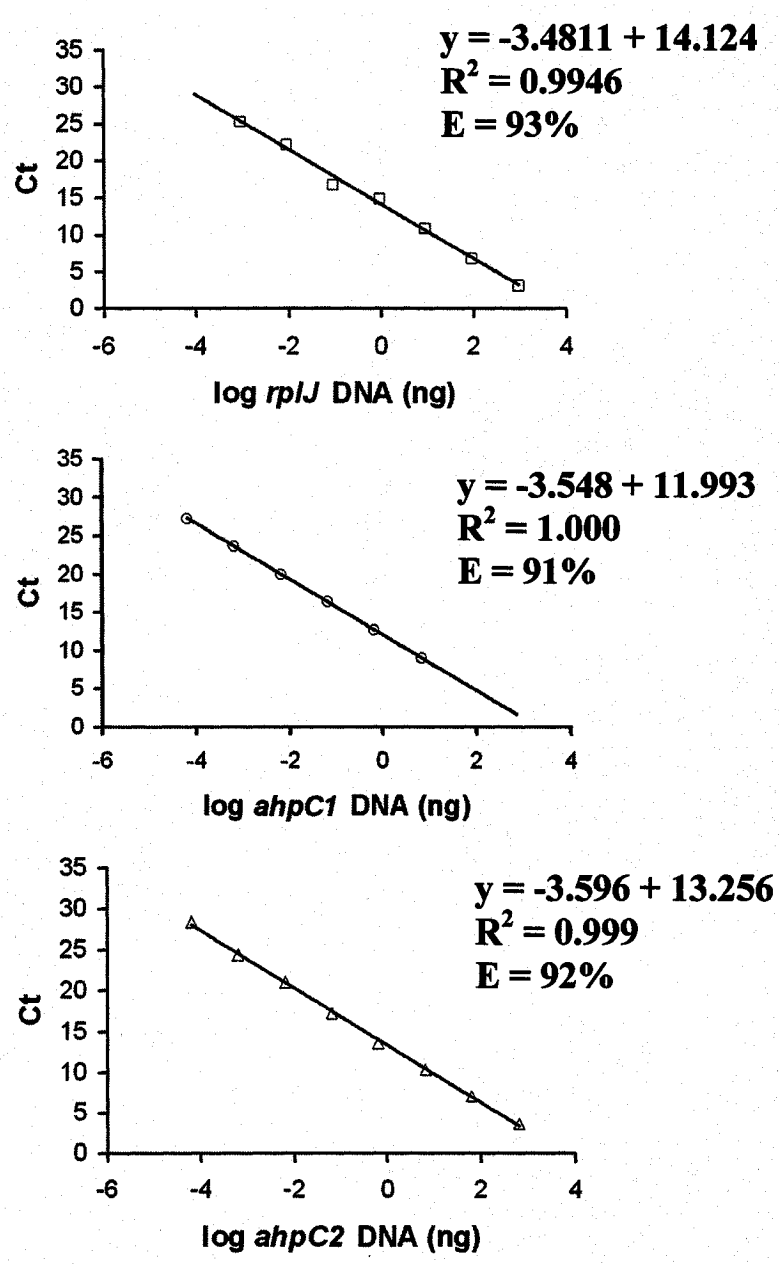



**Figure 20** Standard curves for *rplJ*, *ahpC1*, and *ahpC2*. Standard curves were generated by 10-fold serial dilutions of amplicons generated using genomic DNA as template and primers specific for *rplJ*, *ahpC1*, and *ahpC2*. The PCR efficiency (E) is illustrated along with the equation and regression. Ct is defined as the PCR cycle where the level of fluorescence exceeds that of the background levels.

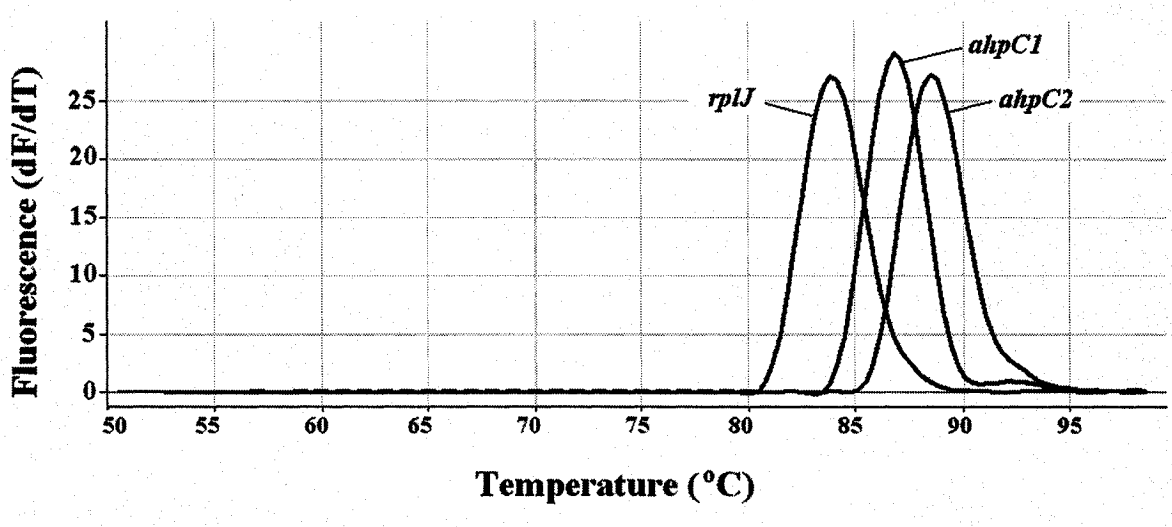



**Figure 21** Melt-curve analysis of qPCR products. Overlap of melt curves for *rplJ*, *ahpC1*, and *ahpC2*. For visualization of melting temperatures (Tm), fluorescence melting peaks were obtained by plotting the negative derivative of fluorescence over temperature (-dF/dT) versus temperature.

Relative quantification of cDNA levels was obtained from equations derived from gene-specific standard curves generated by qPCR using genomic DNA (Figure 20), normalized to the internal control (*rplJ*), and calculated as fold increase (or decrease) relative to the calibrator sample (*ahpC2* levels in wild-type designated as 1×). Consistent with results depicted in Figure 18, qPCR analysis confirmed a significantly higher level of *ahpC1* than *ahpC2* mRNA in the wild-type Lp02 strain (Figure 22). However, *ahpC2* mRNA levels increased over 15-fold in the *ahpC1* mutant compared to levels expressed in the wild-type strain, whereas *ahpC2* levels were restored to wild-type in the *ahpC1*::*km* pC1 strain (complemented *ahpC1* mutant). These results suggest that increases in *ahpC1* transcription (and supposedly AhpC1 levels) may suppress or negatively regulate the expression of *ahpC2D*, which is consistent with the GFP-reporter fluorescence profiles observed in Figure 18. Slightly elevated *ahpC1* and *ahpC2* mRNA levels were consistently observed in the *ahpC1*::*km* pC1 and *ahpC2D*::*km* pC2D strains, respectively; however, differences were not significant from wild-type levels. It should also be noted that, in all cases, *grlA* and *ahpD* mRNA levels were similar to those observed for *ahpC1* and *ahpC2*, respectively, which further confirms the operon structure cartooned in Figure 12 (). The pJB908 empty vector had no influence on *ahpC1* or *ahpC2* expression when present in wild-type or mutant cells (data not shown).

### 4.5. Phylogenetic Analysis of *L. pneumophila* OxyR

Comparative genomics revealed that *L. pneumophila* possesses a homolog of the peroxide-inducible oxidative-stress regulator OxyR found in numerous other organisms (Figures 23 and 24). In contrast to other documented loci where *oxyR* is can be found

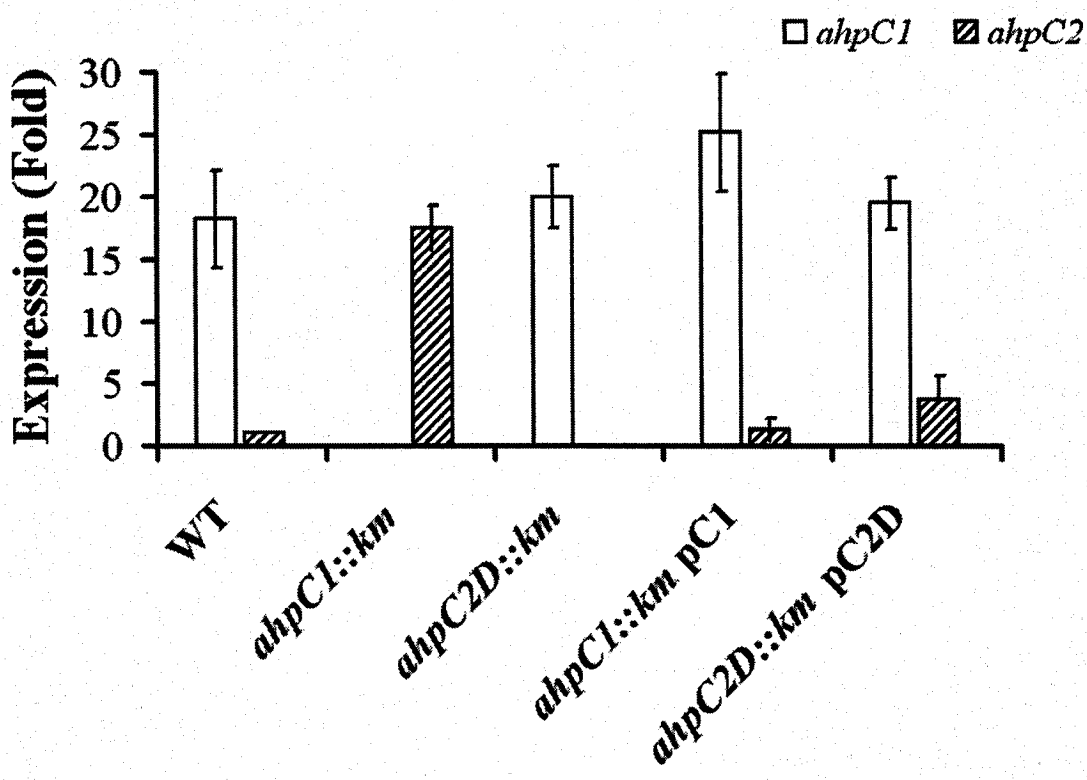



**Figure 22** Compensatory expression between *ahpC1* and *ahpC2*. Real-time PCR (qPCR) of cDNA amplified from wild-type, *ahpC* mutants and complemented *ahpC* mutant strains. Quantification of samples was performed using standard curves generated using DNA as template. Expression levels were normalized to *rplJ* (internal control) and plotted as a fold increase relative to the calibrator sample (*ahpC2* levels in the wild-type strain) designated arbitrarily as 1×. Illustrated are *ahpC1* (open) and *ahpC2* (hatched) levels in wild-type or mutant strains. Results are the mean ± SD of quadruplicate samples from three independently grown cultures.

adjacent to *katA* (Nakjarung *et al.*, 2003), *katG* (Kim *et al.*, 2000; Loprasert *et al.*, 2002, Sigaud *et al.*, 1999), *ahpC* (Dandayuthapani *et al.*, 1997; Pagan-Ramos *et al.*, 2006; Hahn et al, 2002; Lopasert *et al.*, 1997), *dps* (Rocha *et al.*, 2000) or the regulatory-RNA gene *oxyS* (Christman *et al.*, 1989), analysis of sequences flanking *L. pneumophila oxyR* indicated that a gene of unknown function (predicted to encode a MFS transporter) may be divergentlytranscribed at this locus. *L. pneumophila* OxyR shares approximately 44-50% identity and 64-66% similarity with *C. burnetii*, *X. campestris* and *R. grylli* and 42-44% identity and 62-66% similarity with the well-characterized *E. coli* and *S. typhimurium* OxyR. Nakjarung *et al.* (2003) generated a phylogenetic tree where OxyR proteins could be grouped into four clades. Clade I contains *Mycobacteria* spp. and *S. coelicolor*, clade II mostly α-proteobacteria including *C. cresentus* and *Rhizobiacae*, clade III mostly members of the β-proteobacteria such as *Neisseria* spp. and *Burkholderia* spp. and some γ-proteobacteria including *P. aeruginosa*; and finally, clade IV groups γ-proteobacteria including the *Enterobacteriaceae*, *Pasturellaceae*, and some from the genera *Vibrio*, *Pseudomonas*, and *Xanthomonas*. The findings shown in Figure 23 indicate the same phylogeny as described by Nakjarung *et al.* (2003), with the exception of clade IV, where *X. campestris* and other organisms such as *L. pneumophila*, *C. burnetii*, and *R. grylli* could be further divided from *E. coli* and *Salmonella* spp. Into clade IVB and IVA, respectively.

Multiple sequence alignments of various OxyR proteins reveal that LpOxyR contains all the typical features of the OxyR proteins. These include high conservation in the helix-turn-helix motif of the DNA-binding domain in the N-terminal region and the two conserved cysteine residues (Cys199 and Cys208) that form the disulfide bond

**Figure 23** Phylogenetic analysis of *L. pneumophila* OxyR. Abbreviations, organisms, and GenBank accession numbers are as follows: Aci, *Acinetobacter* sp. (CAA86928); Aeh, *Alkalilimnicola ehrlichei* (ZP_00866572); Atu, *Agrobacterium tumefaciens* (AAK88806); Bfr, *Bacteroides fragilis*, (AAG02620); Bme, *Brucella melitensis* (AAD00508); Bps, *Burholderia pseudomallei* (AAK72465); Ccr, *Caulobacter cresentus* (AAK25659); Cbu, *Coxiella burnetii* (AA090973); Eco, *Escherichia coli* (NP_418396); Hha, *Halorhodospira halophila* (NZ_AAOQ01000002); Hin, *Haemophilus influenzae* (NP_438728); LpLens, LpPhil, and LpParis for *L. pneumophila* Lens (CAH16018), Philadelphia-1 (AAU27894), and Paris (CAH12930), respectively; Mav, *Mycobacterium avium* (AAA79918); Mle, *Mycobacterium leprae* (P52678); Mma, Mlo, *Mesorhizobium loti* (BAB53129); *Mycobacterium marinum* (AAC61302); Ngo, *Neisseriae gonorrheae* (AAM51822); Nmo, *Nitrococcus mobilis* (NZ_AAOF01000002); Psy, *Pseudomonas syringue* (AAO53620); Pmu, *Pasteurella multocida* (NP_246285); Rgr, *Rickettsiella grylli* (ZP_01301253); Rle, *Rhizobium leguminosarum* (CAD27227); Sco, *Streptomyces coelicolor* (NP_629185); Sen, *Salmonella enterica* (NP_457935); Sme, *Sinorhizobium meliloti* (NP_384869); Vch, *Vibrio cholerae* (AAF95777); Xca, *Xanthomonas campestris* (AAC45427); Xfa, *Xylella fastidiosa* (A82669); Ype, *Yersinia pestis* (CAC93381). Clades I to IV were previously classified by Nakjarung *et al.* (2003).

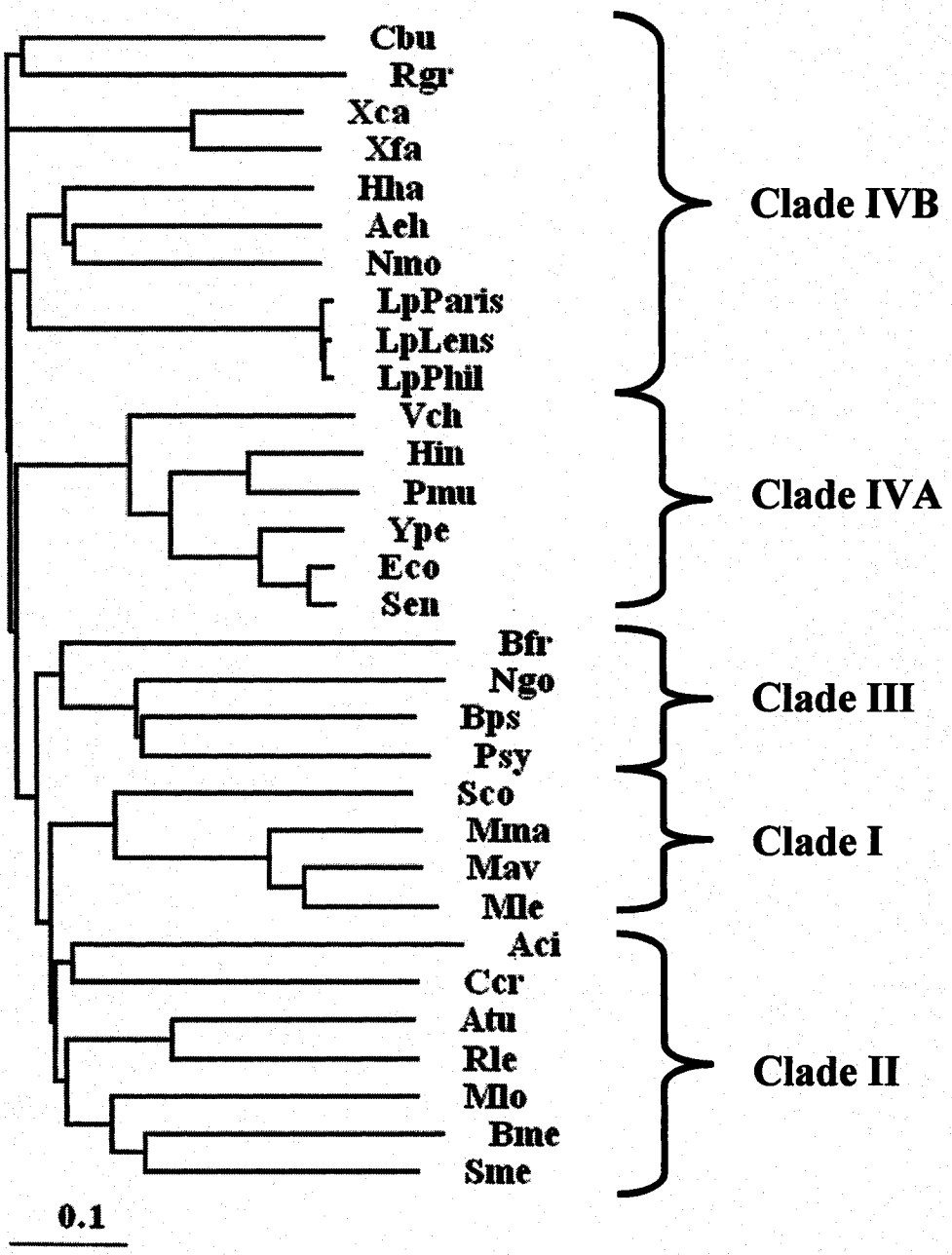

Cbu
Rgr
Xca
Xfa
Hha
Aeh
Nmo
LpParis
LpLens
LpPhil
Vch
Hin
Pmu
Ype
Eco
Sen
Bfr
Ngo
Bps
Psy
Sco
Mma
Mav
Mle
Aci
Ccr
Atu
Rle
Mlo
Bme
Sme
0.1
Clade IVB
Clade IVA
Clade III
Clade I
Clade II

**Figure 24** Multiple sequence alignments of *L. pneumophila* OxyR homologs. Abbreviations, organisms, and GenBank accession numbers are as follows: Cbu, *Coxiella burnetii* (AA090973); Eco, *Escherichia coli* (NP_418396); Hha, *Halorhodospira halophila* (NZ_AAOQ01000002.1); Hin, *Haemophilus influenzae* (NP_438728); LpLens, LpPhil, and LpParis, for *L. pneumophila* Lens (CAH16018), Philadelphia-1 (AAU27894), and Paris (CAH12930), respectively; Nmo, *Nitrococcus mobilis* (NZ_AAOF01000002); *Pasteurella multocida* (NP_246285); Rgr, *Rickettsiella grylli* (ZP_01301253); Sen, *Salmonella enterica* (NP_457935); Vch, *Vibrio cholerae* (AAF95777); Xca, *Xanthomonas campestris* (AAC45427); Xfa, *Xylella fastidiosa* (A82669); Ype, *Yersinia pestis* (CAC93381). The helix-turn helix DNA-binding domain is indicated, as well as Cys199 and Cys208 which have been implicated in OxyR activation.

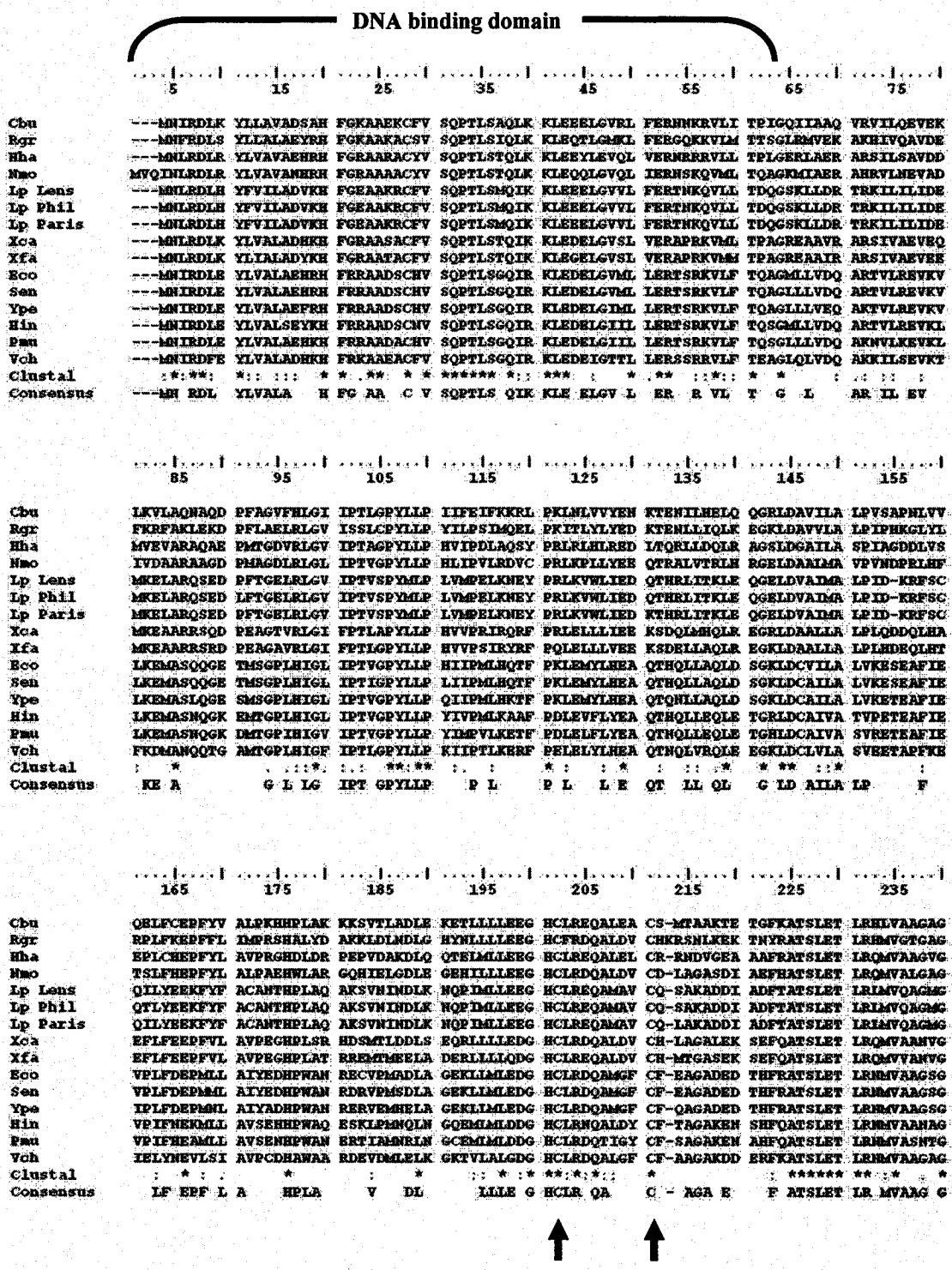
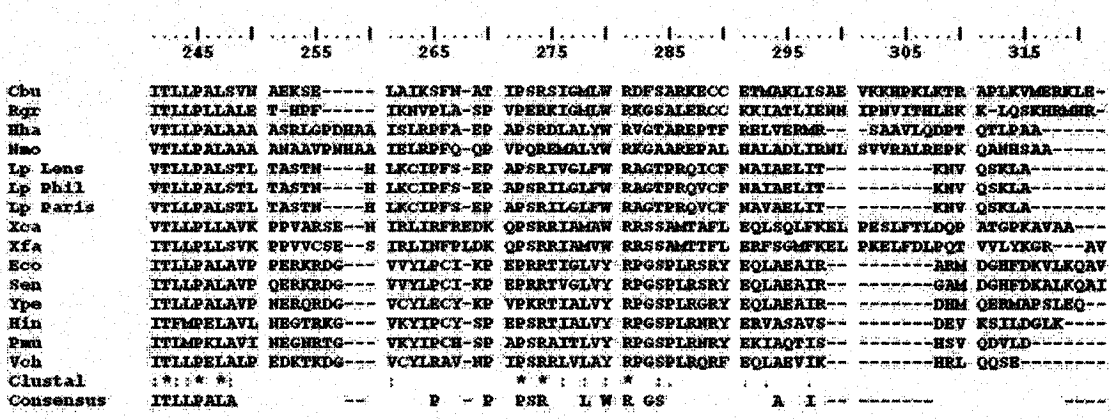

DNA binding domain
Cbu
Rgr
Hha
Hmo
Lp Lens
Lp Phil
Lp Paris
Xca
Xfa
Eco
Sen
Ype
Hin
Pmu
Vch
Clustal
Consensus
Cys199
Cys208

Figure (continued)



involved in activation of OxyR by oxidation (Figure 24). Other previously identified crucial residues implicated in OxyR oligomerization, DNA binding, and trancriptional activation are also conserved (Kullik *et al.*, 1995a and b; Choi *et al.*, 2001). It should be noted that *L. pneumophila* possesses a second LysR regulator exhibiting weak similarity to OxyR. However, this homolog displays substitutions in multiple crucial residues (R4K, L32V, T100V, I110F, H114R, H198F, R201H, C208A, E225D, R266W, and others), suggesting that it is non-functional (Kullik *et al.*, 1995a and b; Choi *et al.*, 2001).

### 4.6. Overproduction and Purification of LpOxyR

To examine the possible regulation by LpOxyR of *ahpC1*, *ahpC2*, or *oxyR* (autoregulation), the LpOxyR protein was first overexpressed using the IPTG-inducible promoter found in pET29b. Following IPTG-mediated induction of *E coli* BL21 Codon Plus cells harboring the petLpOxyR construct and enrichment on a $Ni^{2+}$-NTA column, sufficient amounts of a highly purified $His_6$-LpOxyR were recovered (Figure 25). The apparent molecular mass of the protein was 30-35 kDa, consistent with the expected molecular mass (33.4 kDa) of the product of *oxyR* found in the *L. pneumophila* genome. The purified protein was excised from the silver stained polyacrylamide gel and submitted for sequencing. Peptides identified by mass spectroscopy corresponded to 181/296 amino acids (61% sequence coverage) of the hydrogen peroxide-inducible gene-activator OxyR of *L. pneumophila* subsp. *pneumophila* str. Philadelphia-1.

### 4.7. Interaction between LpOxyR and the Promoter Region of *ahpC2* ($P_{ahpC2}$)

To investigate whether LpOxyR could act as a DNA-binding protein that binds the *ahpC1*, *ahpC2*, and *oxyR* promoter regions ($P_{ahpC2}$, $P_{ahpC1}$ and $P_{oxyR}$, respectively), a

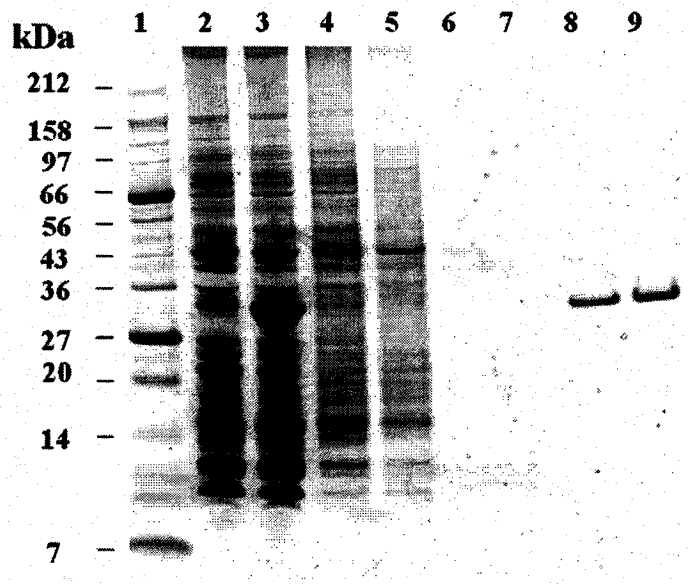



**Figure 25** Overproduction and purification of LpOxyR. *E. coli* BL21 Codon Plus cells harboring petLpOxyR were grown in LB broth and expression was induced with IPTG. Cells were disrupted by sonication and protein lysates were subject to purification on a $Ni^{2+}$-NTA column. $His_6$-LpOxyR enrichment was monitored by SDS-PAGE and silver staining. Lanes: 1) Protein Marker, Broad Range (InVitrogen); 2) whole cell extract without induction; 3) whole cell extract after a 1 h induction with 1 mM IPTG; 4) supernatant of sonicated lysate after ultracentrafugation; 5) flow-through from $Ni^{2+}$-NTA column; 6) and 7) washes; 8) $His_6$-LpOxyR eluate from $Ni^{2+}$-NT column; 9) $His_6$-LpOxyR purified by dialysis (1:10 dilution).

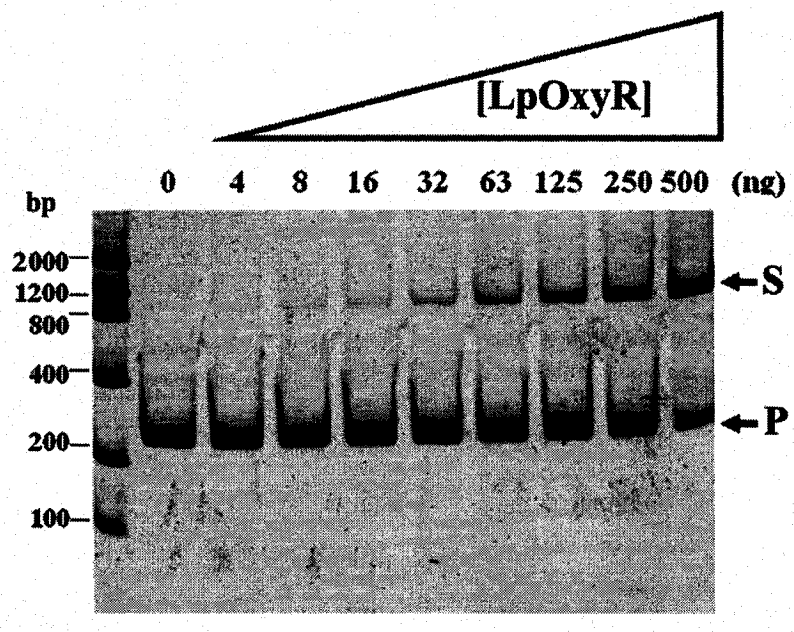
non-radioactive EMSA was performed using SYBR green I nucleic-acid stain. Since SYBR green I is an intercalating agent, this dye only fluoresces when double-stranded DNA is present. Therefore, in the assay, alterations (caused by proteins) of DNA mobility in non-denaturing polyacrylamide gel electrophoresis can be attributed to protein-DNA interaction. A 226-bp DNA fragment containing $P_{ahpC2}$ (-199 to + 27 relative to *ahpC2* coding sequence) was incubated in the presence of purified $His_6$-LpOxyR and formation of protein-DNA complexes was observed (Figure 26A). With increasing amounts of protein appeared a retarded band ("shift") representing the DNA-protein complex. This is evident in the sample containing 500 ng of $His_6$-LpOxyR, where 84% of the DNA probe was found in the bound state. No differences in binding were observed under oxidizing or reducing conditions (Figure 26B). Similar experiments were also carried out with $P_{ahpC1}$ and $P_{oxyR}$; however, no protein-DNA interactions could be observed (data not shown).

### 4.8. Mapping of the LpOxyR Binding Site within the $P_{ahpC2}$

To delimit the minimum promoter region necessary for *ahpC2* expression, PCR-generated 5'-deletion fragments of $P_{ahpC2}$ were cloned upstream of *gfpmut3* (encoding GFP) (Figure 27A). These promoter fragments shared a common 3' end at +27 (relative to the *ahpC2* initiation codon) and a variable 5' end ranging from nucleotides -199 to -66. The ability of the $P_{ahpC2}$ fragments to drive the expression of GFP was assessed both in the wild-type Lp02 and the *ahpC1* mutant (Figure 27B and C), since *ahpC2* levels are increased in the *ahpC1* mutant. Although higher levels of promoter activity were observed in the *ahpC1* mutant, both strains displayed similar growth phase-dependent profiles of *ahpC2*. The 5'-deletions of $P_{ahpC2}$ eliminating base pairs -179 to -109 showed

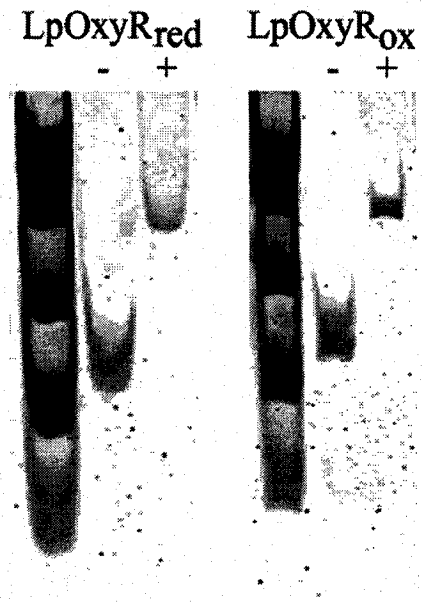
**Figure 26** LpOxyR interactions with the *ahpC2* promoter region ($P_{ahpC2}$). A) Mobility shift assay performed using 50 nanograms of a 226-bp $P_{ahpC2}$ fragment spanning the region -199 to +27 (relative to the *L. pneumophila ahpC2* intiation codon) were incubated with indicated amounts of $His_6$-LpOxyR. Lanes: 1) Low Mass Ladder (InVitrogen); 2-10) DNA in the presence of 0, 3.9, 7.8, 15.6, 31.3, 62.5, 125, 250 or 500 ng of $His_6$-LpOxyR, respectively. Arrows indicate bands representing the DNA probe (P) and mobility shift (S). Densitometry analysis is reported as a percentage of DNA probe (%P) or shift (%S) relative to the total intensity of each lane (with background correction). B) Mobility-shift assay performed in the presence of 25 ng $P_{ahpC2}$ with (+) or without (-) LpOxyR under reducing ($LpOxyR_{red}$) or oxidizing ($LpOxyR_{ox}$) conditions.

A)




| Lane | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|---|
| S (%) | 0 | 1 | 1 | 2 | 3 | 7 | 12 | 36 | 84 |
| P (%) | 100 | 99 | 99 | 98 | 97 | 93 | 88 | 64 | 16 |

B)



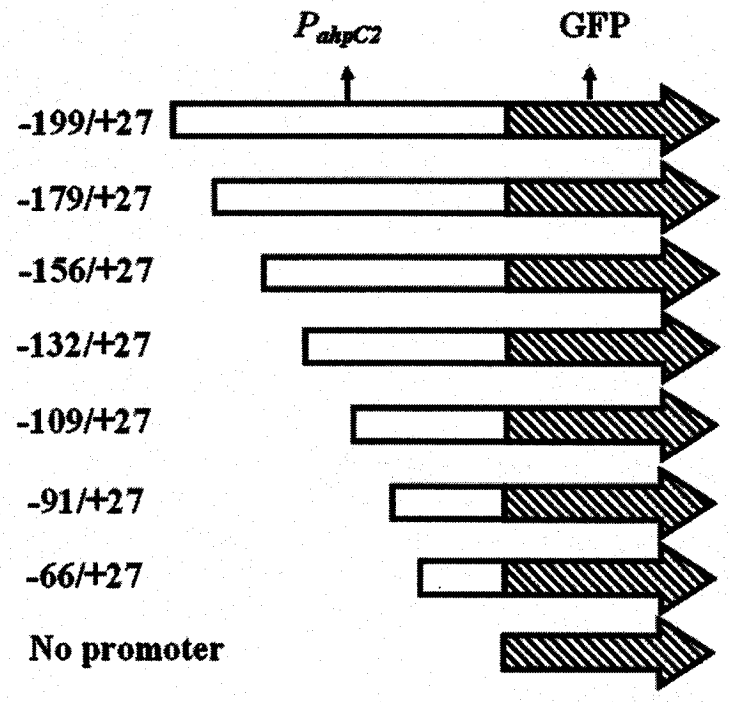
transcriptional activities similar to that of the full-length $P_{ahpC2(-199/+27)}$ fragment. When $P_{ahpC2}$ sequences were deleted from -109 to -91 or -66 there was complete loss of transcriptional activity (Figure 27B). A complementary set of EMSA experiments was performed with various $P_{ahpC2}$ fragments (Figure 27C). Deletion of nucleotides -199 to -109 had no effect on LpOxyR binding, whereas increased amounts of DNA were left unbound when nucleotides -91 to –66 were removed. These data are consistent with the abrogation of promoter activity in the GFP-reporter assay. Sequence comparison for this region with well-documented OxyR-binding sequences found upstream of *ahpC* of *P. aeruginosa* (Ochsner *et al.*, 2000) and *X. campestris* (Charoenlap *et al.*, 2005; Loprasert *et al.*, 2000; Mongkolsuk *et al.*, 2000), or of OxyR-regulated genes (*ahpC*, *katG* and *oxyR*) of *E. coli* (Tartaglia *et al.*, 1989; Toledano *et al.*, 1994), revealed a putative LpOxyR binding consensus 50 to 90 bp upstream of the *ahpC2* initiation codon (Figure 28).

### 4.9. DNase I Footprinting

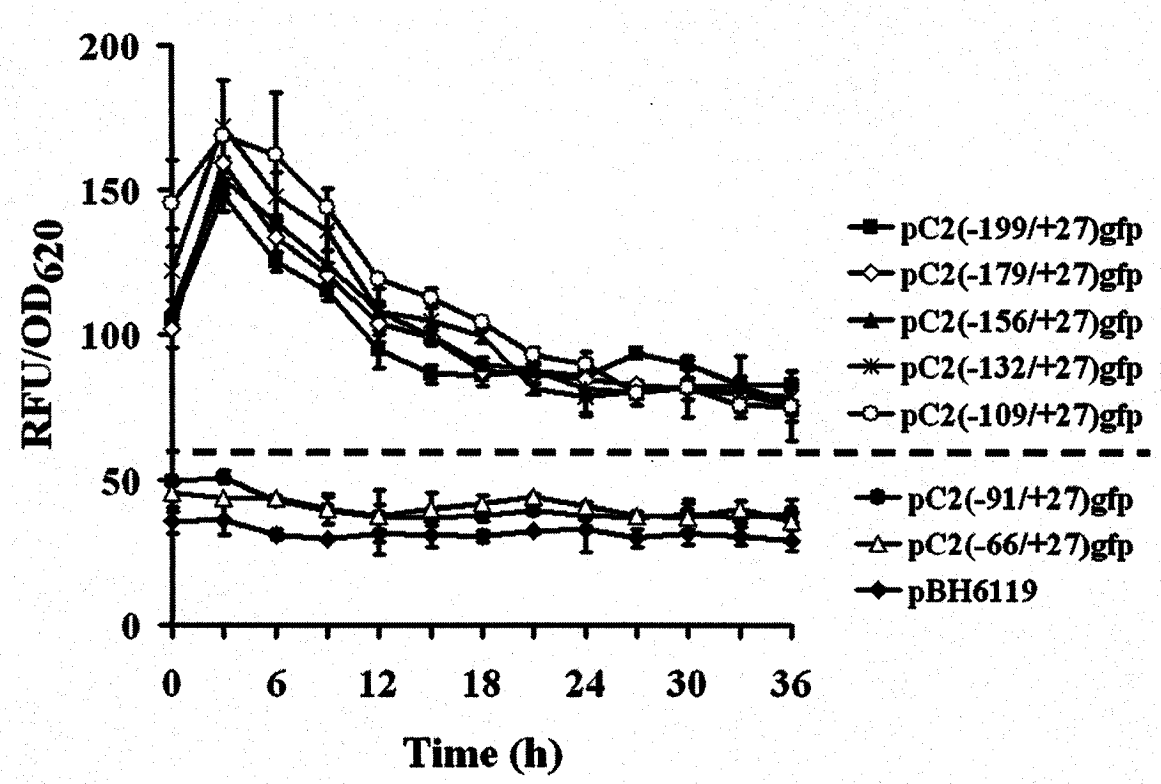
To further delineate the specific sequence of $P_{ahpC2}$ recognized by LpOxyR, oxidized and reduced forms of LpOxyR were used in a standard DNase I protection (DNA footprinting) assay. These experiments were all performed in collaboration with Dr. Karen Brassinga in the laboratory of Dr. Paul Hoffman (University of Virginia, Charlottesville, VA). Oxidized and reduced forms of LpOxyR ($LpOxyR_{ox}$ and $LpOxyR_{red}$, respectively) protected different regions of $P_{ahpC2}$ from DNase I treatment (Figure 29A). $LpOxyR_{red}$ showed an extended footprint (-55 to -106 bp upstream of *ahpC2*) whereas $LpOxyR_{ox}$ protected a shorter region (-61 to -106 bp upstream of *ahpC2*). Furthermore, the DNA footprint defined under reducing conditions indicated

**Figure 27** Promoter deletion analyses f or $P_{ahpC2}$. Fluorescence from various $P_{ahpC2}$ fragment-GFP constructs in (B) wild-type or (C) *ahpC1::km* cells was measured. pBH6119-derived constructs are numbered relative to *ahpC2* initiation codon. For example, the pC2(-199/+27)gfp construct harbors the -199 to +27 region of $P_{ahpC2}$ region cloned upstream of *gfpmut3* of pBH6119. D) Mobility shift assay performed with the 5'end-deletions of $P_{ahpC2}$. Each lane contained 50 ng of the DNA fragments incubated with (right pannel) or without (left pannel) 500 ng of $His_6$-LpOxyR. Lane represents the Low Mass Ladder (Invitrogen). DNA fragments are annotated relative to the *ahpC2* initiation codon.

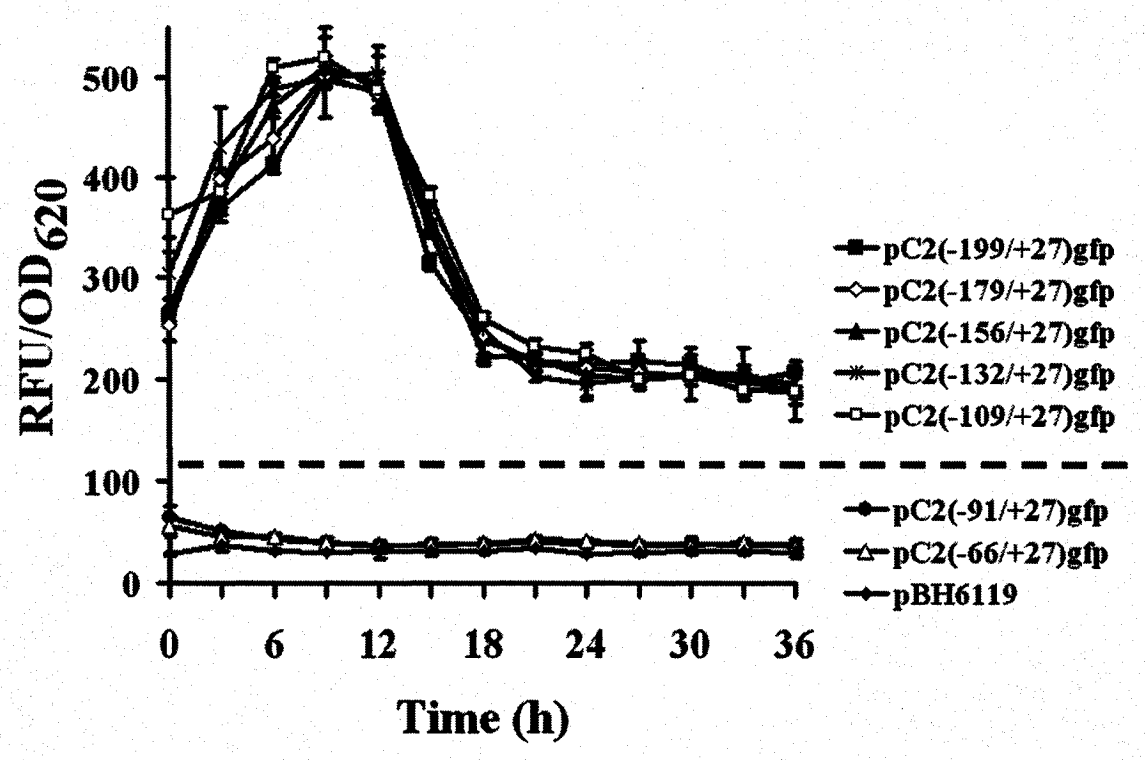
A)


$P_{ahpC2}$
GFP
-199/+27
-179/+27
-156/+27
-132/+27
-109/+27
-91/+27
-66/+27
No promoter

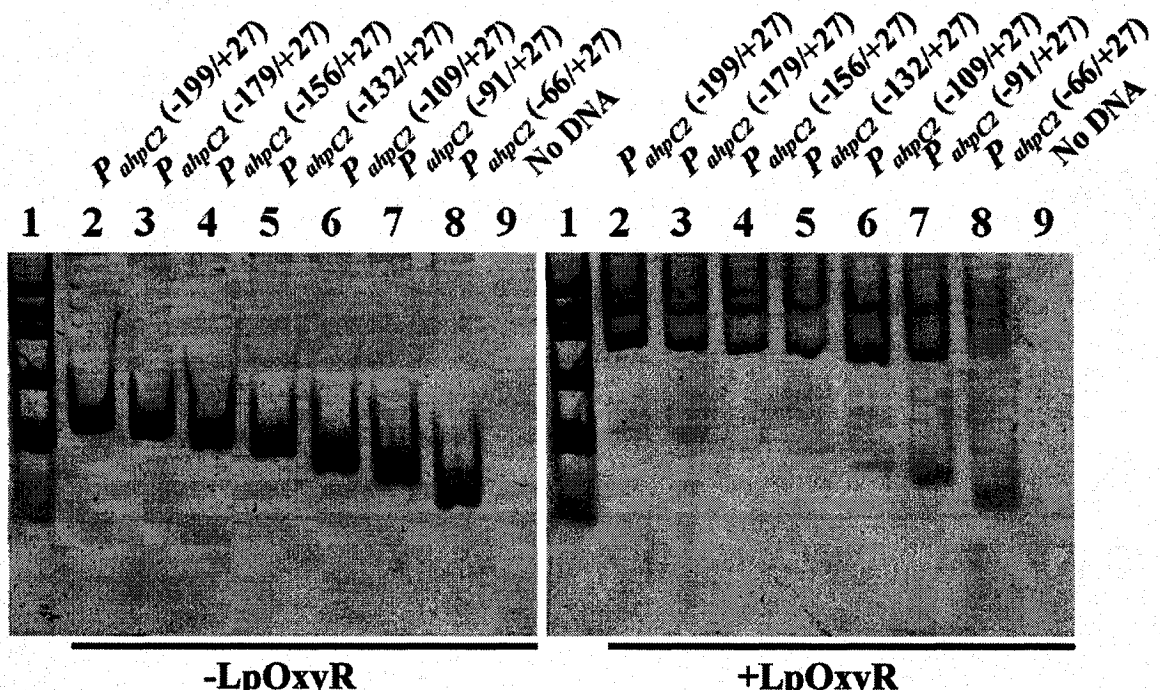
B)


RFU/OD620
200
150
100
50
0
0
6
12
18
24
30
36
Time (h)
pC2(-199/+27)gfp
pC2(-179/+27)gfp
pC2(-156/+27)gfp
pC2(-132/+27)gfp
pC2(-109/+27)gfp
pC2(-91/+27)gfp
pC2(-66/+27)gfp
pBH6119

C)


RFU/OD620
500
400
300
200
100
0
0
6
12
18
24
30
36
Time (h)
pC2(-199/+27)gfp
pC2(-179/+27)gfp
pC2(-156/+27)gfp
pC2(-132/+27)gfp
pC2(-109/+27)gfp
pC2(-91/+27)gfp
pC2(-66/+27)gfp
pBH6119

D)


P ahpC2 (-199/+27)
P ahpC2 (-179/+27)
P ahpC2 (-156/+27)
P ahpC2 (-132/+27)
P ahpC2 (-109/+27)
P ahpC2 (-91/+27)
P ahpC2 (-66/+27)
No DNA
1 2 3 4 5 6 7 8 9
1 2 3 4 5 6 7 8 9
-LpOxyR
+LpOxyR

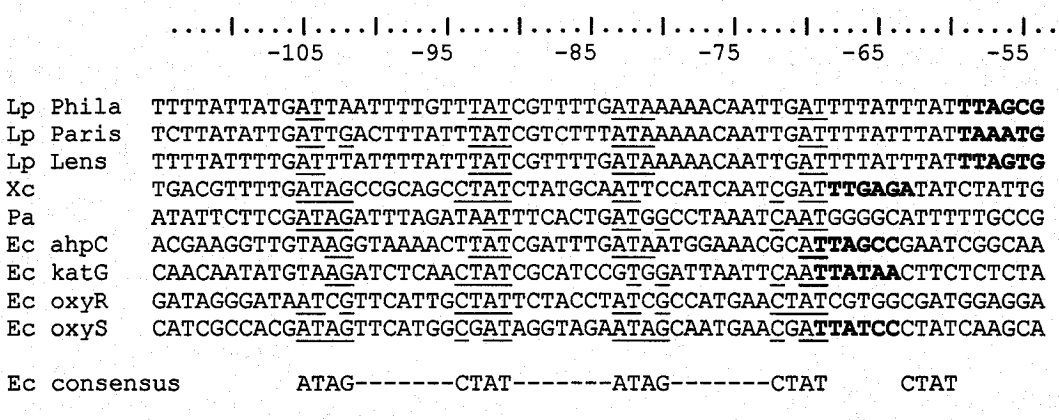


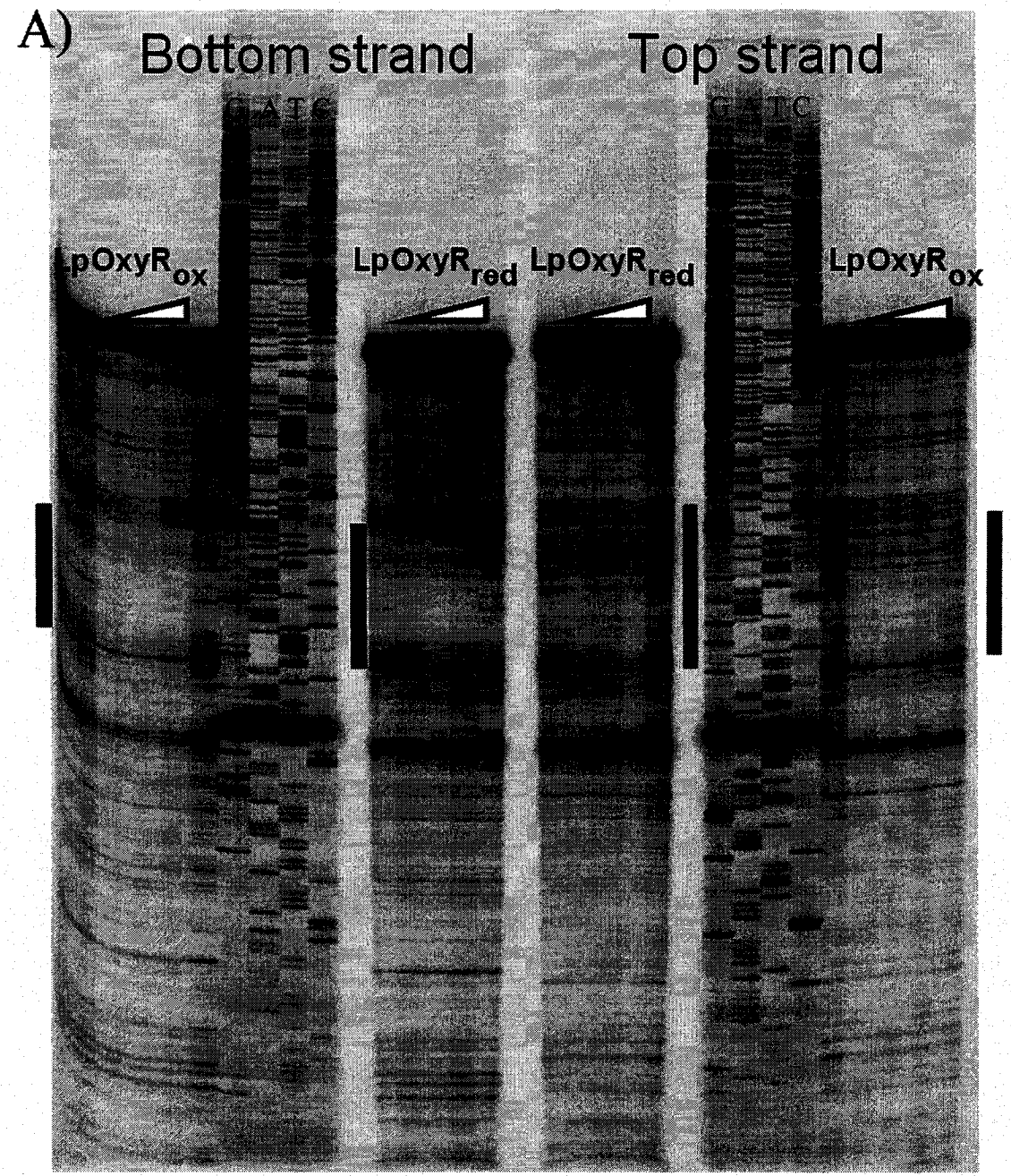

**Figure 28** Putative LpOxyR binding consensus. Shown are the sequences representing equivalent positions upstream of *ahpC* from *L. pneumophila* Philadelphia-1 (*L. pneumophila* Phila), Paris and Lens, *P. aeruginosa* (Pa), *X. campestris* (Xc), and *E. coli* (Ec). OxyR-binding sites for *E. coli katG*, *oxyS*, and *oxyR* genes are also indicated. Nucleotides identical to the *E. coli* OxyR-binding consensus (Toledano *et al.*, 1994) are underlined. Dashes in *E. coli* consensus represent positions of lesser or no conservation. The CTAT in parenthesis represents the fifth DNA contact with reduced OxyR (Toledano *et al.*, 1994). The fifth site in *X. campestris* is 4 bp dowstream (Loprasert *et al.*, 2000). Numbering is relative to the initiation codon of *L. pneumophila ahpC2*. Bold sequences represent well-characterized or putative -35.

that the $LpOxyR_{red}$-binding site overlaps with the putative -35 sequence corresponding to the RNA polymerase-binding site (TTAGCG, located -55 to -60 bp upstream of *ahpC2D*), while this region would be fully accessible under oxidizing conditions (Figure 29B).

### 4.10. Complementation of an *E. coli oxyR* Mutant

Since LpOxyR DNA footprints revealed that oxidation and reduction influenced the protection provided by LpOxyR binding, efforts were made to determine if LpOxyR could function as a peroxide sensor and transcriptional activator. If so, it was hypothesized that expression of *L. pneumophila oxyR* in the *E. coli* MG1655 (K-12)-derived *oxyR::km* mutant GS077 should restore peroxide resistance to these cells. Since *E. coli oxyR* mutants are known to grow poorly under aerobic conditions, strains were grown under anaerobic conditions. When challenged with $H_2O_2$ in a standard disk diffusion assay, *E. coli* GS077 cells exhibited stronger peroxide sensitivity than did wild-type MG1655 cells. Indeed, the zones of inhibition (Appendix 2) in these assays were 32 ± 1 mm for wild-type and 54 ± 1 mm for GS077. The presence of the pBAD22 empty-vector control did not influence peroxide sensitivity in these strains. In the presence of inducing levels of arabinose, GS077 cells harboring pbadEcOxyR showed fully restored peroxide resistance to levels indistinguishable from that of wild-type *E. coli* MG1655, whereas the presence of glucose-mediated repression caused a peroxide-sensitive phenotype similar to that of GS077 lacking plasmid (Figure 30). In contrast, pbadLpOxyR could only partially alleviate the peroxide sensitivity of *E. coli* GS077 cells under inducing conditions. Since SDS-PAGE confirmed high-level expression of EcOxyR and LpOxyR in presence of arabinose (Figure 31) and disk diffusion assays

**Figure 29** DNaseI protection of $P_{ahpC2}$ by LpOxyR. A) Shaded bars indicate LpOxyR DNA footprints for top and bottom DNA strands of a 226-bp fragment of $P_{ahpC2}$ for both oxidized and reduced LpOxyR ($LpOxyR_{ox}$ and $LpOxyR_{red}$, respectively). B) Schematic summary of LpOxyR footprints. Regions corresponding to RNA polymerase (-35) and ribosome-binding sites (RBS) are single- or double-underlined, respectively. Thicker lines represent the *E. coli* consensus.




B)

REDUCED

OXIDIZED

-35

ATTATGATTAATTTTGTTATCGTTTTGATAAAAACAATTGATTTTATTTATTTAGCGGT

TTCACCTAAATTTAACAATGAAAATGGTGTTAATTTAATGAGGAGAAAACTTatgattacggtagg

RBS

M-I-T-V ...

AhpC2

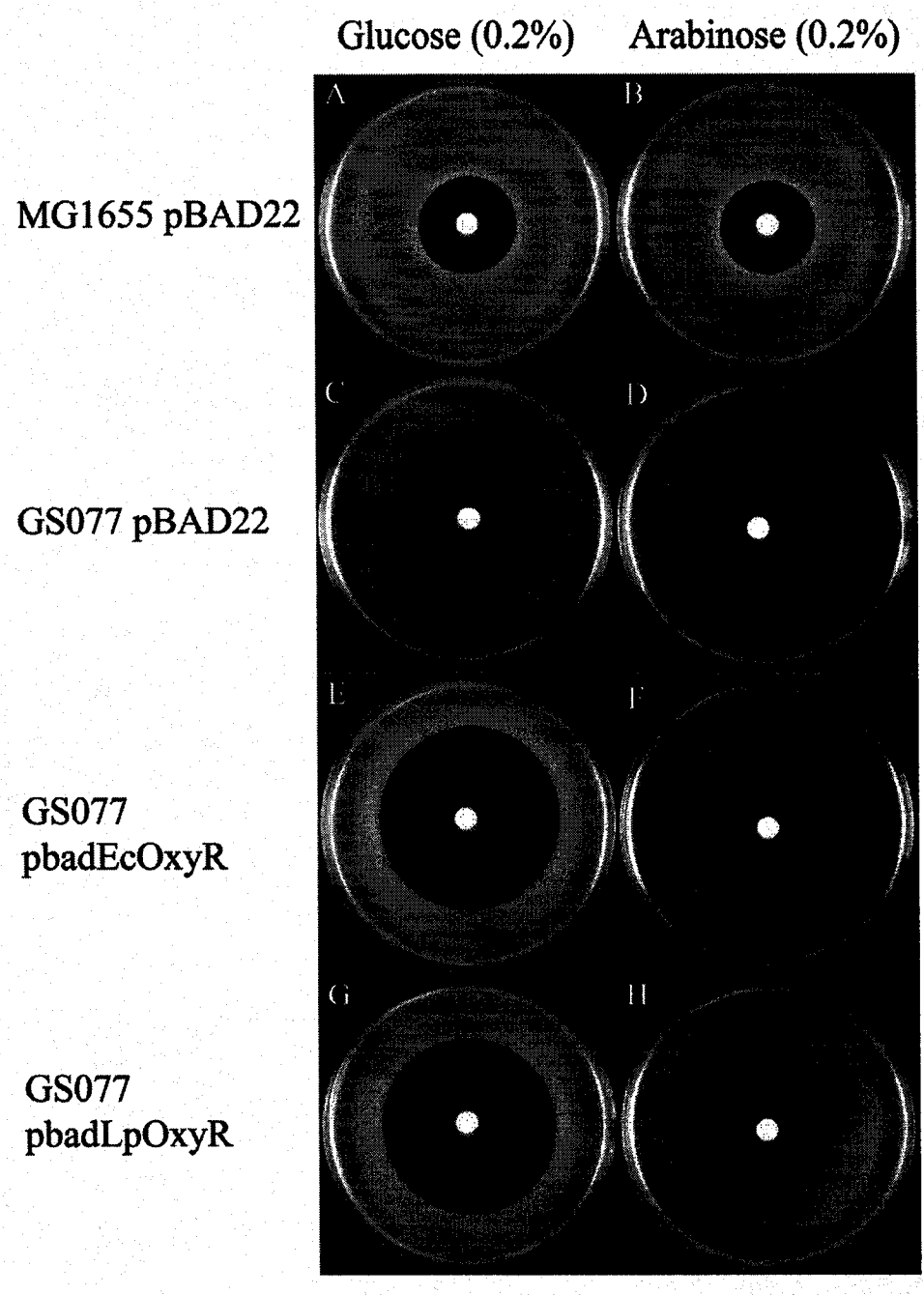



**Figure 30** Effects of *E. coli* and *L. pneumophila oxyR* expression in *E. coli* GS077. Illustrated are representative disk diffusion assays performed under repressing (0.2% glucose) or inducing (0.2% arabinose) conditions. Sterile disks containing 10 µl of 30% $H_2O_2$ were placed in the center of each plate and bacterial growth was observed after 24 h incubation under anaerobic conditions.

performed on media containing increasing concentrations of arabinose gave similar results (Appendix 2), mobility shift assays were performed with $His_6$-LpOxyR and promoters of known members of the *E. coli* OxyR regulon (*ahpCF, katG*, and *oxyR*). Consistent with the findings in Figure 30, $His_6$-LpOxyR had only affinity for *E. coli* promoters under oxidizing conditions (Figure 32).

### 4.11. Growth-Phase Dependent Expression of *oxyR*

To determine the temporal expression of *oxyR* in *L. pneumophila*, a GFP-reporter assay was performed. As illustrated in Figure 33, *oxyR* expression increased during exponential and decreased in stationary phases. Similar trends were also observed in assays where the pOxyRgfp contruct was introduced into the peroxide-sensitive *ahpC1* and *ahpC2D* mutants. Since some OxyR homologs are known to increase their own expression in response to peroxides, GFP fluorescence was monitored following addition of a sub-lethal (50 μM) concentration of $H_2O_2$. No differences were observed (data not shown). Since LpOxyR does not bind $P_{oxyR}$ as seen with other OxyR homologs, attempts were also made to identify possible regulators of *oxyR* expression using the GFP-reporter assay. The pOxyRgfp construct was introduced by electroporation into strains mutant for known regulators of *L. pneumophila* virulence: *rpoS*, *letA*, and *himAB* (encoding integration host factor, IHF). No differences in *oxyR* expression were observed in any of the mutants analyzed (Figure 33).

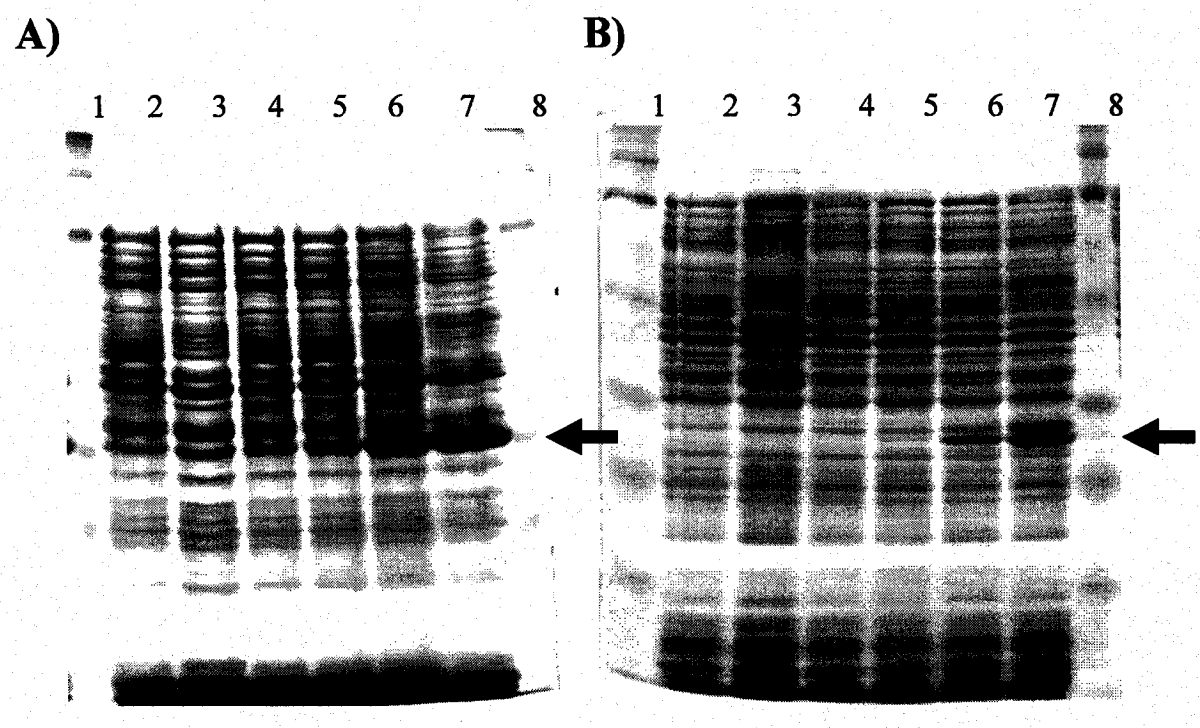



**Figure 31** Arabinose-dependent expression of *E. coli* and *L. pneumophila oxyR*. SDS-PAGE was performed to evaluate expression of A) EcOxyR or B) LpOxyR in *E. coli* DH5α cells harboring pbadEcOxyR and pbadLpOxyR, respectively. Cells were grown to mid-exponential phase in MH broth containing 0.2% glucose or increasing concentrations (0, 0.0002, 0.002, 0.02, 0.2%) of arabinose. After 60 min of incubation at room temperature, cells were harvested and analyzed by SDS-PAGE. Lanes are as follows: 1) and 8) Pre-stained protein markers (InVitrogen); 2) no induction; 3) 0.2% glucose; 4) 0.0002% arabinose; 5) 0.002% arabinose; 6) 0.02% arabinose; 7) 0.2% arabinose. Arrows indicate bands that correspond to the correct size for A) EcOxyR or B) LpOxyR.

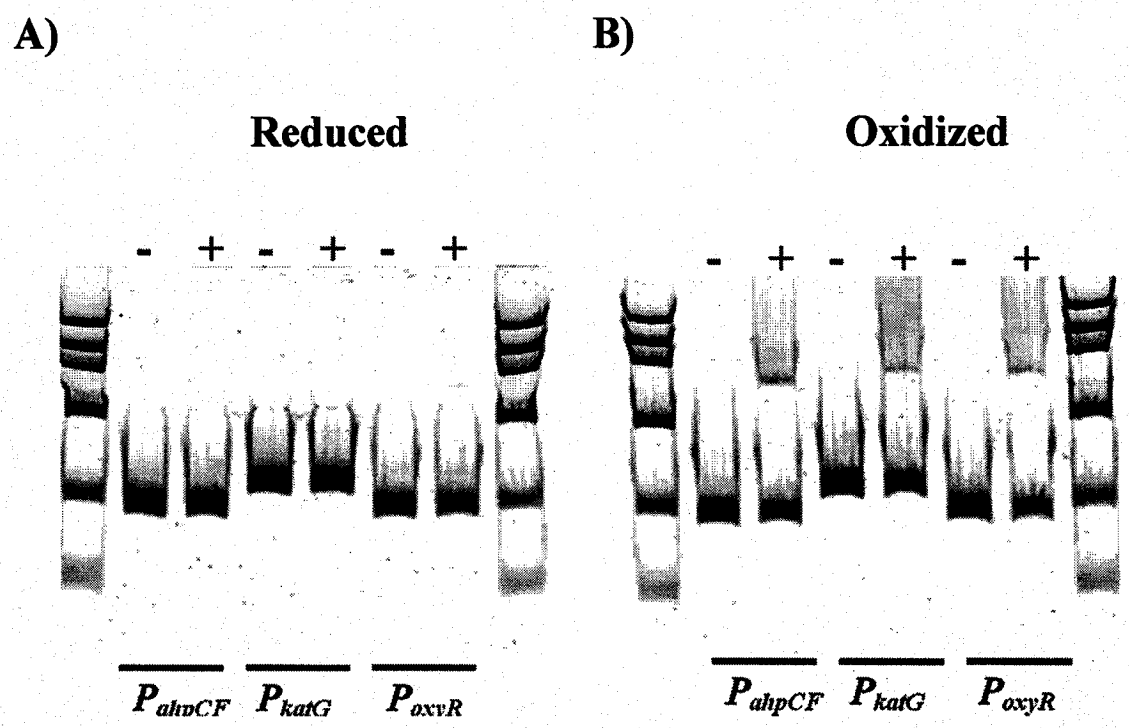



**Figure 32** Interaction of LpOxyR with *E. coli* OxyR-regulated promoters. Fifty nanograms of *E. coli ahpCF*, *katG* or *oxyR* promoter DNA ($P_{ahpCF}$, $P_{katG}$, and $P_{oxyR}$, respectively) were incubated in the absence (-) or presence (+) of 500 ng of $His_6$-LpOxyR under (A) reducing conditions (200 mM DTT) or (B) oxidizing conditions.

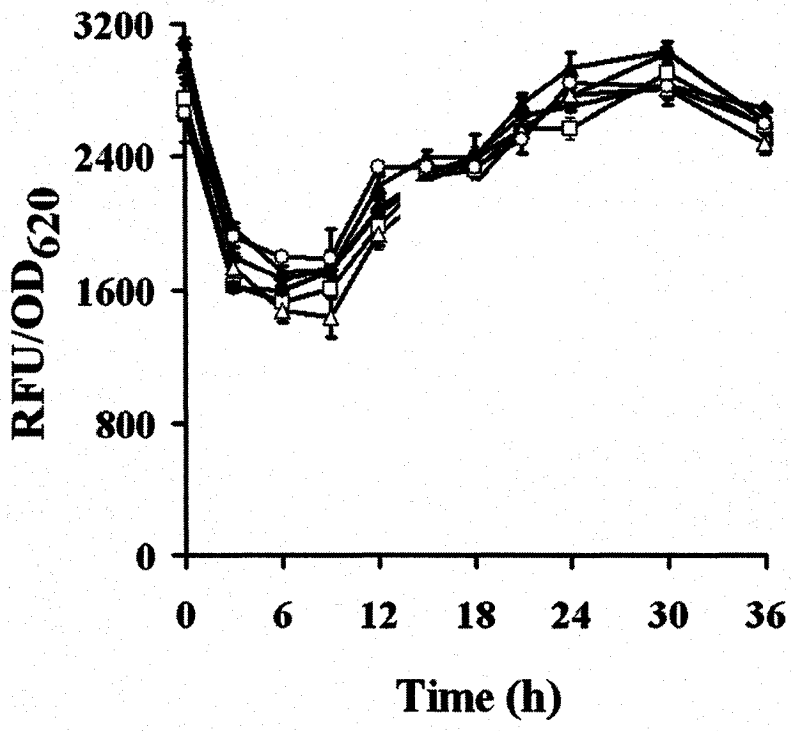
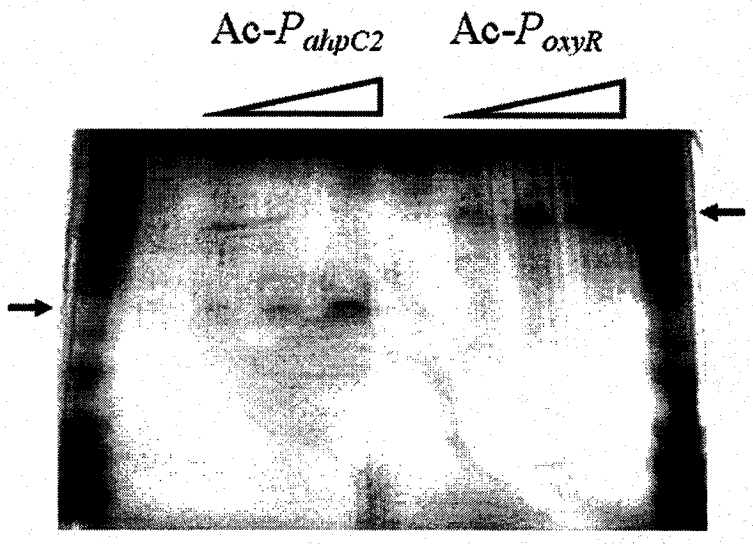



**Figure 33** Expression of *L. pneumophila oxyR*. GFP-reporter assays for expression of $P_{oxyR}$ cloned into pBH6119 and harbored by strains decribed below. Fluorescence was determined for samples taken every 3 h following the growth of wild-type (♦) or mutant strains *ahpC1::km* (□), *ahpC2D::km* (●), MB379 [*rpoS::km*] (Δ), MB414 [*letA-22*] (▲), and IHF DKO [*himA::gm/himB::km*] (○) in BYE broth. Relative fluorescence units (RFU) were normalized to 1.0 $OD_{620}$. Results are the mean ± standard deviation of three independent experiments.

### 4.12. Acrylamide Capture of DNA-Bound Complexes

Since direct GFP-reporter assays failed to identify regulators of *oxyR* expression, an acrylamide capture technique was optimized to perform this task. After a binding reaction between protein(s) from a cell lysate of wild-type Lp02 cells and PCR-amplified DNA that has been immobilized into the polyacrylamide matrix by its Acrydite moiety, the method relies on the electrophoretic removal of contaminant proteins. Since the binding reaction was polymerized into the wells of a non-denaturing gel, Acrydite-DNA-captured proteins were excised and analyzed by SDS-PAGE. After silver staining, a distinct band (relative to the no-DNA control) was observed for each promoter region ($P_{ahpC2}$ and $P_{oxyR}$) (indicated in Figure 34). These bands were excised and submitted for mass spectrometry analysis. To validate this technique, reactions were first performed using Ac-$P_{ahpC2}$. The protein captured by Ac-$P_{ahpC2}$ corresponded to LpOxyR, validating it use to determine transcription factors. However, the only proteins captured by Ac-$P_{oxyR}$ in the lysate were the β and β'subunits of RNAP. No sigma factors have yet been identified.




| Lane | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|---|---|
| Lysate (µg) | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 |
| Ac-DNA (µg) | 0 | 0 | 2 | 4 | 8 | 0 | 2 | 4 | 8 | 0 |

**Figure 34** Acrylamide capture of regulators of *ahpC2* and *oxyR* expression. The acrylamide capture of DNA-bound complexes was performed in reactions containing 1 µg of a French press protein lysate obtained from BYE-grown *L. pneumophila* and indicated amounts of Acrydite-linked $P_{ahpC2}$ or $P_{oxyR}$-specific DNA. After electrophoretic removal of non-specific proteins by non-denaturing electrophoresis through a 0.5 × TBE 5% polyacrylamide gel, wells were excised and heated in SDS sample buffer containing β-mercaptoethanol. This material was subjected to SDS-PAGE followed by silver staining revealed distinct proteins (indicated by arrows) captured by respective promoter regions. Lane 1 and 10 contain Broad Range Protein Markers (BioRad) that were added prior to SDS-PAGE.

# Chapter 5: Discussion

## 5.1. Oxidative Stress and *L. pneumophila* Antioxidant Defenses

From natural aquatic environments to human alveolar macrophages, *L. pneumophila* must respond and adapt to many harsh conditions, including oxidative stress (Abu Kwaik *et al.*, 1997; Hales and Shuman, 1999; Lynch *et al.*, 2003). As described above, *L. pneumophila* defences used to cope with ROIs include both periplasmic (KatA and SodC) and cytoplasmic antioxidant enzymes (KatB, SodB, and AhpC) (Bandyopadhyay and Steinman, 1998 and 2000; Bandyopadhyay *et al.*, 2003; Sadosky *et al.*, 1994; St John and Steinman, 1996; Rankin *et al.*, 2002). Those studies have led to a model where *L. pneumophila* KatA and KatB might provide $H_2O_2$-scavenging activity to detoxify the phagosomal milieu to promote intracellular growth (Amemura-Maekawa *et al.*, 1999; Bandyopadhyay *et al.*, 2003). However, *L. pneumophila katA*, *katB*, and *katAkatB* mutants are no more susceptible to $H_2O_2$ than is the wild-type strain, suggesting that other enzymes provide $H_2O_2$-scavenging function (Bandyopadhyay and Steinman, 1998 and 2000). AhpCs are well-established detoxifiers of $H_2O_2$, organic (lipid) peroxides, and peroxynitrite (Baker *et al.*, 2001; Bryk *et al.*, 2000; Bsat *et al.*, 1996; Master *et al.*, 2002; Poole and Ellis, 1996; Seaver and Imlay, 2001; Tartaglia *et al.*, 1990), and are thus likely candidates for peroxide scavenging by *L. pneumophila*. Whole-genome analyses of *L. pneumophila* reveal two homologs of alkyl hydroperoxide reductase (*ahpC1* and *ahpC2*) (Cazalet *et al.*, 2004; Chien *et al.*, 2004). Though both AhpC1 and AhpC2 were shown to be up-regulated during intracellular growth (Brüggemann *et al.*, 2006; Rankin *et al.*, 2002) and may play important roles in detoxifying the phagosome of ROIs, no effort had yet been made to investigate whether

these AhpCs indeed protect *L. pneumophila* against ROIs. We initiated this study to determine whether AhpC enzymes are primarily responsible for scavenging peroxides in the apparent absence of measurable catalase activity in *L. pneumophila*. We have characterized the two phylogenetically distinct alkyl hydroperoxide reductase systems that provide essential and partially redundant peroxide-scavenging function in *L. pneumophila*. Here we show that AhpC2 AhpD (AhpC2D) and AhpC1 protect *L. pneumophila* from peroxide challenge and that expression of each gene is growth-phase dependent, with *ahpC1* expressed during post-exponential phase and *ahpC2D* expressed during early-exponential phase. Mutational studies indicated that at least one functional AhpC is required for viability and that both genes are required for full resistance to $H_2O_2$ and organic peroxides. Finally, the increased expression of *ahpC2D* in an *ahpC1* mutant background is most likely due to an OxyR-mediated compensatory response following the increased oxidative stress caused by loss of AhpC1 function (discussed below).

Like numerous factors that contribute to the virulence of *L. pneumophila* (Cirillo *et al.*, 1994), growth of *L. pneumophila* in protozoa has been shown to enhance the pathogen's resistance to peroxide stress (Abu Kwaik *et al.*, 1997; Bandyopadhyay *et al.*, 2004). Protection against peroxide stress could play a crucial role in pathogenesis, since *L. pneumophila* is likely faced with an oxidative burst during phagocytosis by neutrophils, macrophages, and protozoa (Davies *et al.*, 1991; Halablab *et al.*, 1990; Jacobs *et al.*, 1994). While antioxidant enzymes generally respond to endogenous ROIs produced during aerobic respiration (Gonzalez-Flecha and Demple, 1995; Imlay, 2003) they have also been used effectively as survival strategies for intracellular pathogens (Braunstein *et al.*, 2003; De Groote *et al.*, 1997; Gee *et al.*, 2005; Krishnakumar *et al.*,

2004; Master *et al.*, 2002; Manca *et al.*, 1999; Piddington *et al.*, 2001). Detoxification of peroxides from the LCV might be particularly vital to ensure conditions favoring intracellular growth of *L. pneumophila*. For example, oxidation of the cysteine to cystine by ROIs in the LCV could prevent uptake of this essential amino acid (Ewann and Hoffman, 2006). Damage to iron-sulfur clusters found in aconitase and fumarase could inactivate catabolic pathways like the TCA cycle, which is needed to support *L. pneumophila* growth. Lipid peroxidation of membranes and propagation of free-radical reactions would be deleterious to the cell (Wang *et al.*, 2006). And finally, interactions of $H_2O_2$ with iron could promote Fenton reaction-mediated damage to DNA (hydroxyl radical production), leading to potentially lethal mutations. We have focussed on the two *L. pneumophila* AhpCs, since these enzymes are known to prevent ROIs-mediated damage by scavenging $H_2O_2$, organic (lipid) peroxides, and peroxinitrite (Baker *et al.*, 2001; Bryk *et al.*, 2000; Bsat *et al.*, 1996; Master *et al.*, 2002; Poole and Ellis, 1996; Seaver and Imlay, 2001; Tartaglia *et al.*, 1990).

### 5.2. Two Distinct Alkyl Hydroperoxide Reductase Systems in *L. pneumophila*

Since phylogenetic analysis indicates that both *L. pneumophila* and *C. burnetii* contain two AhpCs, it would be logical to question why these two systems are conserved in these two intracellular pathogens. AhpC1 shows some similarity to the well-characterized system of *H. pylori*, where oxidized AhpC is reduced by NADPH-dependent reactions involving Trx/TR (Baker *et al.*, 2001). Conversely, AhpC2 shows similarity to the AhpC system of *M. tuberculosis* and *S. coelicolor* (Hillas *et al.*, 2000; Jaeger *et al.*, 2004; Hahn *et al.*, 2002; Koshkin *et al.*, 2004; Tian *et al.*, 2005). Although AhpC1 and AhpC2 are presumed to be functionally redundant, expression of both AhpC

systems in *L. pneumophila* was found to be growth-phase dependent. During growth, *ahpC1* was expressed during mid- and post-exponential phases while *ahpC2D* was expressed during early-exponential phase. An increase in AhpC1 expression during mid- and post-exponential phases could respond to increased levels of peroxides generated during the transition period in which respiratory metabolism slows down and oxidation of redox-active metabolites increases (Abu Kwaik *et al.*, 1997; Bandyopadhyay *et al.*, 2004). Conversely, AhpC2 might play a role similar to that of *ahpC* in *M. tuberculosis*, in which levels increase during early-exponential growth in macrophages (even in absence of a functional OxyR), and was suggested to function during reactivation from latent infection or during transmission to a new host (Springer *et al.*, 2001). Therefore, the two AhpCs might play redundant functions in *L. pneumophila*, but are required at distinct stages of growth.

Another notable difference between AhpC1 and AhpC2 is the fact that *ahpC1* and *ahpC2* are found in operons with *grlA* and *ahpD*, respectively. Genes encoding disulfide reductases, like *ahpD* and *ahpF*, and involved in the reduction of oxidized AhpC by NAD(P)H-dependent reactions, are generally found immediately adjacent to *ahpC* and are transcribed as a polycistronic message. RT-PCR analysis supports the hypothesis that the reduction of oxidized AhpC1 might be performed by the Grx-like activity of GrlA, and driven by the glutathione redox system. Glutaredoxin-like proteins are been used by other organisms to reduce members of the Prx family, as observed for *Chromacium gracile* (Vergauwen *et al.*, 2001) and *Clostridium pasteurianum* (Reynolds *et al.*, 2002). However, *L. pneumophila* GrlA might not be functional since it lacks a crucial cysteine residue (CXXS in GrlA versus CXXC in functional motifs) required for disulfide

reductase function. The function of GrlA could be tested by deletion of *grlA* (leaving *ahpC1* functional) and testing the resultant cells for changes in susceptibility to peroxides. Alternatively, AhpC1 could be reduced by thioredoxin and thioredoxin reductase, as seen with *H. pylori* (Baker *et al.*, 2001). Using the purified AhpC and other components, enzymatic analysis could be used to reconstruct the complete system and test these possibilities.

As observed for *M. tuberculosis* and *S. coelicolor*, *L. pneumophila ahpC2* was found in an operon with *ahpD*, suggesting that *L. pneumophila* AhpC2 might be reduced by a similar multi-component system involving AhpD, DlaT, and LpdA (Hillas *et al.*, 2000; Jaeger *et al.*, 2004; Hahn *et al.*, 2002; Koshkin *et al.*, 2004; Tian *et al.*, 2005). In fact, all of these components are encoded by the *L. pneumophila* genome. Interestingly, DlaT was characterized as the E2 component of pyruvate dehydrogenase, directly linking antioxidant defences to general metabolism (Tian *et al.*, 2005). One could speculate that exponentially growing *L. pneumophila* would exploit catabolic (pyruvate or α-ketoglutarate oxidation) sources of reducing power to drive AhpC2D activity when energy sources are abundant, but then switch to a more direct NAD(P)H-driven AhpC1 system during post-exponential phase when energy sources are limiting (Nyström, 2004). In *E. coli*, stationary-phase protection against peroxides is ensured by increased cellular expression of the $\sigma^S$-regulated monofunctional catalase (KatE or HPII) (Mulvey *et al.*, 1990). Due to a lack of a monofunctional catalase in *L. pneumophila*, the catalase activity of AhpCs (discussed below) might provide a crucial role in different growth phases. How oxidative stress drives the expression of *ahpC1* and *ahpC2* remains to be determined.

### 5.3. Peroxidatic Functions of *L. pneumophila* AhpC1 and AhpC2

Though AhpCs are well-established scavengers of peroxides in other organisms, no study had addressed whether *L. pneumophila ahpC1* and *ahpC2* could provide peroxidatic activity to protect *L. pneumophila* from $H_2O_2$. Our complementation studies, where these genes were expressed in a catalase/peroxidase *E. coli* mutant (J1377), support the hypothesis that AhpC1 and AhpC2 provide peroxidase activity to protect against $H_2O_2$. To further confirm the role of AhpC1 and AhpC2D as peroxide scavengers in *L. pneumophila*, *ahpC1* and *ahpC2D* chromosomal deletion mutants were constructed and shown to be significantly more sensitive to peroxides than the wild-type strain. In contrast, *katA, katB* and the *katAkatB* double-deletion mutants do not exhibit significant increases in susceptibility to $H_2O_2$ (Bandyopadhyay and Steinman, 1998 and 2000). Though *L. pneumophila* KatA and KatB are annotated as bifunctional catalase-peroxidases, they seem to exhibit only peroxidatic activity. Early work by Pine *et al.* (1984a and b) demonstrated that *L. pneumophila* strains are catalase negative and peroxidase positive, whereas other species (such as *L. micdadei, L. jordanis* and *L. bozemanii*) were catalase positive and peroxidase negative. The weak catalase activity noted in extracts of *L. pneumophila* cells could in retrospect be attributed to residual metabolic activities driving AhpC activity. Similarly, the weak NAD(P)H catalase activity reported in studies of *L. pneumophila katA* and *katB* expressed in an *E. coli katG* and *katE* mutant (UM383) is probably due to the *E. coli* AhpCF activity (Bandyopadhyay and Seinman, 1998). The *E. coli* strain used in our studies contains the *ahpCF* deletion as well as those for *katG* and *katE* (Seaver and Imlay, 2001); therefore our studies indicate that AhpC1 and AhpC2 display hydrogen peroxide-scavenging

activity that is inconspicuous in *L. pneumophila*. Furthermore, *L. pneumophila ahpC1* and *ahpC2D* chromosomal deletion mutants were more susceptible to various peroxides and to the superoxide-generating compound paraquat, suggesting that AhpC1 and AhpC2D can, as found in other organisms, contribute to peroxidase functions.

### 5.4. AhpC Function is Essential in *L. pneumophila*

Since loss of *ahpC1* and *ahpC2D* function was accompanied by an increase in peroxide sensitivity, the *ahpC* double mutant might be expected to create a physiological state that does not support growth. The challenging task to recover *L. pneumophila* ROI-sensitive mutants is exacerbated by the fact that *L. pneumophila* is unable to grow under anaerobic conditions and even wild-type *L. pneumophila* requires ROI-scavenging components in its culture medium. This later view is supported by early work demonstrating formation of inhibitory levels of $H_2O_2$ and $\bullet O_2^-$ the growth medium (Hoffman *et al.*, 1983) and the growth-promoting detoxification properties of supplements such as activated charcoal and α-ketoglutaric acid that formulate the BCYE isolation medium (Pine *et al.*, 1986). In our assays, the *ahpC1ahpC2D* double mutant could not be constructed even in presence of ROI-scavenging compounds found in BCYE solid medium or upon supplementation with an excess of catalase. The failure to construct this mutant supports the hypothesis that at least one AhpC is essential for viability. One could propose that AhpC function in *L. pneumophila* might be relieved by expression of *E. coli ahpC* (and not catalase) in the *ahpC* mutants.

The reason that an excess of catalase in BCYE might not suffice in promoting growth of the *ahpC* double mutant is that catalases exhibit a relatively low affinity for $H_2O_2$ (high $K_m$) whereas AhpC systems exhibit a much higher affinity (low $K_m$);

therefore, AhpC, and not catalase, is considered to be responsible for scavenging most of the peroxide generated in bacteria (Seaver and Imlay, 2001). An alternative possibility would be that AhpCs might provide other functions not attributed to catalases or catalase-peroxidases, such as molecular chaperone functions (Chuang *et al.*, 2006). In *H. pylori*, chaperone functions are observed during conditions of oxidative stress, where AhpC forms high-molecular-weight complexes that are able to refold proteins (Chuang *et al.*, 2006), and dimeric, decameric and dodecameric crystal structures of AhpC have been reported in many other organisms (Guimaraes *et al.*, 2005; Hofmann *et al.*, 2002; Wood *et al.*, 2002). However, the essentiality of *L. pneumophila* AhpCs is likely attributed to protection against ROIs, since this organism is known to be particularly susceptible to oxidative stress (Domingue *et al.*, 1990; Hoffman *et al.*, 1983; Kim *et al.*, 2002; Locksley *et al.*, 1982; Pine *et al.*, 1983). The reasons for this sensitivity to oxidative stress likely include: the fact that *L. pneumophila* contains lower levels of glutathione when compared to *E. coli* (Locksley *et al.*, 1982), innapropriate oxidation of cysteine residues in outer membrane proteins might prevent interchain disulfide bonding that is essential for maintenance of porin function and outer-membrane integrity (Butler *et al.*, 1985), or autooxidation of respiratory chain components (Hoffman and Pine, 1982) that produce ROIs.

### 5.5. AhpCs and Intracellular Growth

It is noteworthy that the two AhpCs of *L. pneumophila* are homologous to those found in *C. burnetii*, a bacterium that is closely related to *Legionella* (Seshadri *et al.*, 2003). This could suggest that these two intracellular pathogens might employ common defense strategies to detoxify the intracellular milieu. Consistent with a putative

protective role in detoxification of ROIs in phagosomes, *L. pneumophila ahpC1* levels are shown to increase during intracellular growth of *L. pneumophila* in macrophages (Rankin *et al.*, 2002) and *ahpC2* and *ahpD* levels were elevated during the replicative phase in protozoa (Brüggemann *et al.*, 2006). We confirmed an earlier report by Rankin *et al.* (2002) indicating that an *ahpC1* mutant displays similar intracellular growth kinetics in PMA-differentiated U937 cells as the wild-type strain. In addition, we extended these findings by showing that an *ahpC2D* mutant also exhibits wild-type intracellular growth kinetics. In contrast, Bandyopadhyay *et al.* (2003) proposed that KatA and KatB are crucial for pathogenesis by maintaining a critically low level of $H_2O_2$ between the periplasm and cytosol to protect macromolecular targets required for invasion or survival within macrophages, or to maintain a redox state necessary for metabolic changes accompanying a transition from an extracellular transmissible form to an intracellular replicative state. Though the catalase-peroxidases may provide some important functions necessary for intracellular survival, further investigations would be necessary to validate such a model. The peroxide sensitivity of *ahpC* mutants and the absence of growth defects suggest that *L. pneumophila* may not be exposed to toxic concentrations of peroxides in macrophages as previously believed. Perhaps *L. pneumophila* pathogenesis also includes mechanisms associated with inactivation of respiratory burst signals. Three scenarios can be envisioned: 1) *L. pneumophila* inhibits activation of the NADPH oxidase; 2) the NADPH oxidase is not activated by *L. pneumophila*; or 3) induction of compensatory responses among antioxidant enzymes may be sufficient to cope with the assault of ROIs generated by the NADPH oxidase.

Our data are consistent with observations that *L. pneumophila* generates a unique replicative phagosome that escapes the signal-transduction cascades associated with phagolysosomal fusion and activation of the respiratory burst. Since *L. pneumophila* establishes an ER-like organelle that diverges from endocytic maturation, the oxidative burst would only be in the early events following uptake. While *L. pneumophila* was shown to trigger NADPH oxidase activation by a PLC-dependent signaling cascade leading to activation of PKC in neutrophils, this response might be inhibited shortly after uptake by toxins, proteases, protein kinases, lipases, or other unidentified factors (Friedman *et al.*, 1982; Lochner *et al.*, 1985; Saha *et al.*, 1985; Sahney *et al.*, 1990; Szeto and Shuman, 1990). Over the years, much has been learned about the cell biology of an *L. pneumophila* infection through the study of avirulent mutants. The Dot/Icm T4SS is thought to generate a replication-permissive niche in the host cell that avoids being trafficked toward the endocytic pathway. However, *L. pneumophila dot/icm* mutants are not killed in macrophages, even if they fail to multiply. These mutants remain viable in a vacuole that still evades endocytic maturation (Joshi *et al.*, 2001; Fernandez-Moreira, 2006). Though these studies have suggested a role for LPS in inhibition of phagolysosomal fusion, other factors may play a valuable role in *L. pneumophila* virulence. Such factors could include T2SS-secreted proteins (described above), some of which have been shown to inhibit the oxidative burst (Friedman *et al.*, 1982; Lochner *et al.*, 1985; Saha *et al.*, 1985; Sahney *et al.*, 1990; Szeto and Shuman, 1990). While other studies have suggested that *L. pneumophila* may be exposed to ROIs following phagocytosis in macrophages (Jacobs *et al.*, 1984; Kura *et al.*, 1994; Saito *et al.*, 2001),

no studies have investigated whether components of NADPH oxidase are recruited to the *Legionella*-containing phagosome.

An alternative explanation for the lack of intracellular growth for *L. pneumophila ahpC* mutants would be a failure to stimulate an oxidative burst. For example, in non-stimulated macrophages where the levels of ROIs generated by the oxidative burst are low, the phenotype of a *M. tuberculosis sodC* mutant is absent, whereas the *sodC* mutant (and not the wild-type strain) is readily killed by macrophages activated with IFN-γ (Piddington *et al.*, 2001). Our intracellular growth analysis should be repeated with other cells types or with different stimuli triggering activation of the NADPH oxidase. One could assume that a more suitable model would be *L. pneumophila* infections using GM-1 cells, a clone of the U937 phagocytes that expresses a larger respiratory burst upon IFN-γ stimulation (Garotta *et al.*, 1991). However, IFN-γ is known to restrict growth of *L. pneumophila* in macrophages, presumably due to an increased production of ROIs and decreased access to iron (Byrd and Horwitz, 2000), where the LCV is redirected towards the endocytic pathway (Santic *et al.*, 2005).

It is possible that antioxidant enzymes might be important under conditions not evaluated in this study. Edelstein *et al.* (1999) isolated *L. pneumophila* mutants that are not defective for macrophage infection but are dramatically impaired for virulence in guinea pigs. ROIs released from the plasma-membrane-localized NADPH oxidase of activated macrophages and neutrophils could play a role in the clearance of *L. pneumophila*. In such a case, oxidative-stress defenses could be important during transmission to a new phagocyte, particularly after the onset of the inflammatory response. *L. pneumophila* would thus have to survive for at least a minimal amount of

time in the extracellular mileu of alveoli following egress from a spent host (Chandler *et al.*, 1979; Rodgers *et al.*, 1978). Intratracheal inoculation of *L. pneumophila* in guinea pigs is an attractive and well-documented model that could be used for such studies (Edelstein *et al.*, 1999; Weeratna *et al.*, 1994). The intracellular growth kinetics for wild-type and mutant strains could also be compared using cells known to be defective in the NADPH oxidase, such as a murine model of chronic granulomatous disease (CGD). Parallel studies should also be performed in numerous cell lines using inhibitors of the NADPH oxidase, such as apocynin (Gee *et al.*, 2005).

On the other hand, antioxidant enzymes might not be required for human infection per se, but could be important for survival of *L. pneumophila* in natural environments. By analogy to the case for *C. crescentus*, ROIs scavenging enzymes might play an important role in environmental survival by protecting against the toxic effects of redox-cycling agents shed into pond water by plants and other organisms (Schnell and Steinman, 1995). Second, bacterial factors like catalase decrease the susceptibility of the cell to antimicrobial agents including oxidative biocides such as monochloramine and $H_2O_2$ in biofilms (Cochran *et al.*, 2000). In fact, catalase protects aggregated bacteria by preventing full penetration of $H_2O_2$ into the biofilm (Stewart *et al.*, 2000). In mycobacteria, *ahpC* was shown to be up-regulated during static growth by an OxyR-independent mechanism, which could play an important role in biofilm formation (Springer *et al.*, 2001). One could imagine a similar situation with *L. pneumophila*, where resistance to oxidizing biocide would ensure survival of the organisms in biofilms (Domingue *et al.*, 1990; Hassett *et al.*, 1999; Kim *et al.*, 2002). However, this scenario is unlikely since it is generally accepted that *L. pneumophila* does not replicate in biofilms

in absence of protozoa (Greub and Raoult, 2004). Furthermore, the cyst-like form that is thought to enable survival of *L. pneumophila* for extended periods of time in biofilms is metabolically dormant. Since AhpC would only be able to sustain peroxide-scavenging functions in presence of electron carriers like NADH or NADPH, its antioxidant functions might thus only be promoted during intracellular growth in protozoa. Interestingly, microarray data show that *ahpC2D* is up-regulated during replication in *Acanthamoebae castellanii* (Brüggemann *et al.*, 2006) and catalase activity increases with respect to the growth rate in continuous culture (Berg *et al.*, 1985). Though protozoan host cells are known to produce an oxidative burst during phagocytosis (Davies *et al.*, 1991; Halabab *et al.*, 1990), little attention has been paid to the involvement of antioxidant defenses during protozoan infection (Bandayopadhyay *et al.*, 2004). It is likely that *L. pneumophila* would only be faced with ROIs in the phagosome of protozoa early in the infection process but, as observed in macrophages, the evasion of the phagolysosomal fusion might shield *L. pneumophila* against an otherwise toxic assault from ROIs. Since both *ahpC1* and *ahpC2* are highly conserved in *L. pneumophila* and *C. burnetii*, two organisms that display developmental cycles, it is also possible that these enzymes might be involved in the transition from the vegetative to the replicative form. Further studies should analyze the role of the *L. pneumophila* AhpCs during protozoan infection.

### 5.6. Compensatory Expression of *ahpC2* in *ahpC1::km*

Though the lack of intracellular growth defects with *L. pneumophila ahpC* mutants could be attributed to other factors (discussed above), our results suggest that this situation could be attributed to increased expression of other antioxidant enzymes that

accompanies loss of AhpC function. Compensatory responses are not unique to *L. pneumophila*. Catalase-deficient cells of *M. tuberculosis* (Sherman *et al.*, 1996) and *Burkholderia pseudomallei* (Loprasert *et al.*, 2003) showed enhanced *ahpC* expression. Increased catalase levels are also observed following inactivation of *ahpC* in *E. coli* (Seaver and Imlay, 2001), *X. campestris* (Charoenlap *et al.*, 2005; Mongkolsuk *et al.*, 2000), and *B. subtilis* (Bsat *et al.*, 1996). In *P. aeruginosa*, an enhanced activity of the organic hydroperoxide-resistance protein (Ohr) was detected in an *ahpC* mutant, suggesting compensatory functions between Ohr and AhpC (Oschner *et al.*, 2001). While no direct evidence was provided, previous studies suggested that the loss of one catalase-peroxidase (KatA or KatB) in *L. pneumophila* might lead to compensation by the other to prevent the increased doubling time observed in the *katAkatB* double mutant (Bandyopadhyay and Steinman, 2000). Based on GFP reporter fusions and qPCR data, and since the growth rates of the *ahpC* mutants were similar to that of the wild-type strain, our results indicate that *ahpC2D* expression increases to compensate for a deficiency in AhpC1 production. To our knowledge, this is the first report of a compensatory link between two AhpCs. We propose that the *ahpC2D* operon of *L. pneumophila* might act as a secondary "back-up" system that is up-regulated with the loss of AhpC1 function (perhaps in response to increased oxidative stress), whereas high basal levels of AhpC1 would be sufficiently protective in the absence of AhpC2D.

While the qPCR data suggest that *ahpC2* levels regain wild-type levels in the *ahpC1* mutant, the microdilution susceptibility assays and peroxide-challenge analyses revealed that *L. pneumophila ahpC1* and *ahpC2D* mutants are sensitive to peroxides. GFP reporter assays suggested that expression levels for *ahpC2* were consistently

elevated in the *ahpC1* mutant, yet never attained levels observed for *ahpC1* in the post-exponential phase of growth. This might explain why the *ahpC1* mutant was more sensitive to peroxides than the *ahpC2D* mutant in the microdilution susceptibility assays. Peroxide challenges should thus be repeated with bacteria grown to various phases of growth. Secondly, one should be aware of situations leading to misinterpretations, such as post-transcriptional regulation that is known to occur in *L. pneumophila*, where protein levels might not always correlate with mRNA profiles. A western analysis might provide a more accurate outlook on the extent of compensation between the two AhpCs. Nonetheless, the GFP-reporter assay and qPCR analysis uncovered that *ahpC2* expression levels were consistently higher in the *ahpC1* mutant, suggesting that AhpC2D activity might be sufficient to sustain a viable state in absence of AhpC1 function.

The compensatory response by AhpC2D might be particularly important, since AhpC1 contains a motif that has been linked to a susceptibility to over-oxidation in eukaryotic Prxs (Wood *et al.*, 2003). The sensitivity to inactivation by over-oxidation of the peroxidatic cysteine (to sulfinic and sulfonic acids) is due in part to the Gly-Gly-Leu-Gly (GGLG) motif (residues 94-97) (Wood *et al.*, 2003) found in *L. pneumophila* AhpC1 (but not in AhpC2). Other homologs of *L. pneumophila* AhpC1 also possess this motif. Though our study indicated that *ahpC2D* levels increased with loss of AhpC1 function, we can not exclude the possibility that AhpC2D acts in concert with other antioxidant enzymes which might compensate for the loss of AhpC1 function. In mycobacteria, AhpC and KatG (catalase-peroxidase) are both required for full virulence in human macrophages (Manca *et al.*, 1999). An inverse correlation between the amount of peroxide-detoxifying activity and bacterial killing was observed (Manca *et al.*, 1999).

This interrelationship between resistance by $H_2O_2$ and survival within human macrophages is also likely to occur for *L. pneumophila*. However, analysis of antioxidant enzymes might be complicated by functional redundancy that often occurs in *L. pneumophila*. A lesson learned from the effectors of the Dot/Icm system, the contribution of multiple genes encoding peroxide-scavenging enzymes or other protective strategies might impede analysis of single factor. Previous studies have highlighted difficulties with trying to correlate catalase and peroxidase activity with virulence due to differing activities among members of the *Legionella* family (Pine *et al.*, 1986). These conclusions could be further complicated by our data suggesting that compensatory responses might mask the role of individual factors in virulence. To observe synergy amongst antioxidant enzymes, subsequent studies must utilize genetic techniques to create strains lacking multiple antioxidant enzymes, such as combinations between AhpC1, AhpC2D, KatA, and KatB or other putative peroxide-scavenging factors like MsrA.

### 5.7. Regulation of the *L. pneumophila ahpC2D* Operon

A more effective strategy to investigate the role of oxidative-stress defences in *L. pneumophila* pathogenesis might be to create loss-of-function mutants affecting global regulators of the oxidative-stress response. In *L. pneumophila*, mechanisms that govern expression of numerous factors in response to extra- or intracellular cues have been described in some detail, yet account for only some of its virulence-associated phenotypes (Molofsky and Swanson, 2004). It is surprising that there is a paucity of data regarding the regulation of antioxidant defenses in *L. pneumophila*, since the transmissive form is known to become more resistant to oxidative stress (Bachman and Swanson,

2001; Hales and Shuman, 1999; Hammer *et al.*, 2002; Lynch *et al.*, 2003; Molofsky *et al.*, 2003). Although numerous antioxidant enzymes have been characterized in *L. pneumophila*, the mechanism(s) by which oxidative stress drives expression of these genes has not been addressed. In *E. coli*, deletion of genes involved in antioxidant functions or the addition of extracellular peroxides is known to activate the global oxidative-stress regulator OxyR to increase expression of its target genes like catalase and *ahpC* (Altuvia *et al.*, 1994; Christman *et al.*, 1985 and 1989; Storz *et al.*, 1990; Toledano *et al.*, 1994). Since compensatory expression among peroxide-detoxifying enzymes is usually mediated by the peroxide sensor/transcriptional regulator OxyR (Charoenlap *et al.*, 2005; Mongkolsuk *et al.*, 2000) and since OxyR has been shown to activate transcription of *ahpC* in response to oxidative stress in other organisms (Charoenlap *et al.*, 2005; Ochsner *et al.*, 2000; Loprasert *et al.*, 2003; Morgan *et al.*, 1986; Mongkolsuk *et al.*, 2000; Storz *et al.*, 1990), it was likely that the up-regulation of the *ahpC2D* operon in *L. pneumophila* could be mediated by an OxyR-mediated response to a rise in the level of intracellular peroxides in the *ahpC1* null background.

In *C. burnetii* (Seshadri *et al.*, 2003), *S. coelicolor* (Hahn *et al.*, 2002), and some mycobacteria (Dandayuthapani *et al.*, 1997; Pagan-Ramos *et al.*, 2006), *oxyR* is divergently transcribed from the *ahpCD* operon. Neither the *ahpC1* or *ahpC2D* loci in *L. pneumophila* display an *oxyR-ahpC* genomic organization. However, a previously uncharacterized homolog of the oxidative-stress regulator OxyR was identified as the product of a separate locus in the *L. pneumophila* genome and was designated LpOxyR. Our data suggested that *L. pneumophila* OxyR might be responsible for the regulation of the *ahpC2D* operon. By analogy to *E. coli* OxyR, EMSA showed that the oxidized form

of LpOxyR is as a DNA-binding protein that specifically recognizes $P_{ahpC2}$. The apparent dissociation constant (K = $1.5 \times 10^{-11}$ M, see supplemental information in Appendix 1) between LpOxyR and the *ahpC2* promoter region was consistent with those obtained in *E. coli* (Tartaglia *et al.*, 1992). Sequence alignment of *ahpC* promoter regions from different organisms and $P_{ahpC2}$ promoter deletion analysis revealed an OxyR-binding site 55 to 90 base pairs upstream of *ahpC2*. This position is typical of that for LysR-type transcription factors that bind on average 65 bp upstream of their target genes (Schell, 1993). This consensus binding motif contained the signature core sequence T-$N_{11}$-A of LysR-type transcriptional regulators (Schell, 1993) embedded in a larger motif containing 11 of 16 base pairs of the *E. coli* OxyR-recognition site (Toledano *et al.*, 1994). This degree of similarity is consistent with the OxyR-binding sites found upstream of *ahpC* in *E. coli* (Toledano *et al.*, 1994; Tartaglia *et al.*, 1992), *P. aeruginosa* (Ochsner *et al.*, 2000), *X. campestris* (Charoenlap *et al.*, 2005; Loprasert *et al.*, 2000; Mongkolsuk *et al.*, 2000), and other *E. coli* OxyR-regulated genes (Tartaglia *et al.*, 1989; Toledano *et al.*, 1994). The LpOxyR-binding site is analogous to that of *E. coli* OxyR (Toledano *et al.*, 1994) but differs markedly from the mycobacterial OxyR-binding site described by Dhandayuthapani *et al.* (1997) and Pagan-Ramos *et al.* (1998). This observation was particularly surprising since the *ahpC2D* operon shares strong similarity with the equivalent genes of mycobacteria (LeBlanc *et al.*, 2006). To formally address if binding of LpOxyR to $P_{ahpC2}$ implies that it acts as a transcriptional activator of the *ahpC2D* operon, 5'-deletion fragments of $P_{ahpC2}$ were cloned upstream of GFP. Promoter fragments with partial or full deletion of the putative LpOxyR-binding site showed altered LpOxyR binding in the mobility-shift assays and completely lost promoter

activity in the GFP-reporter assay. Thus, the inability of LpOxyR to bind $P_{ahpC2}$ results in absent *ahpC2D* expression.

*E. coli* OxyR is known to bind as a dimer of dimers to DNA sequences presenting a duplication of the ATAG-$N_7$-CTAT palindrome (Toledano *et al.*, 1994). In many cases, binding of the second half-site by LysR-type regulators depends on external signals that alter interactions between the regulator monomers and subsequent protein-DNA contacts (Schell, 1993). As for OxyR, it is known to bind in both reducing and oxidizing conditions, but only the latter promotes expression of its target genes. For example, treatment of *E. coli* OxyR with millimolar amounts of DTT was shown to promote binding of OxyR to contact sites that block the -35 of the promoter region of *oxyS*, whereas addition of peroxides alters the conformation of OxyR to free the -35 sequence and leads to strong induction of *oxyS* (Storz *et al.*, 1990; Toledano *et al.*, 1994; Tartaglia *et al.*, 1992). Since the DNA-binding domain, the cysteine residues involved in activation (Cys199 and Cys208), and other residues shown to mediate a redox-dependent conformational change in OxyR are highly conserved in LpOxyR, we propose that this protein is likely to exhibit similar properties (Choi *et al.*, 2001; Kullik *et al.*, 1995; Zheng *et al.*, 1998). EMSA and DNaseI-protection assays suggest that LpOxyR is able to bind to $P_{ahpC2}$ under both reducing and oxidizing conditions with similar affinities as shown by *E. coli* OxyR for its targets (Tartaglia *et al.*, 1992). Reduced LpOxyR displayed an extended DNA footprint, whereas oxidized LpOxyR resulted in a shorter protected region. Moreover, the reduced LpOxyR footprint overlaps the potential -35 sequence of *ahpC2* (TTAGCG with respect to the -35 of *E. coli ahpC*, TTAGCC) (Tartaglia *et al.*, 1989), whereas this region is freely accessible with oxidized LpOxyR. These findings

are consistent with the model (Loprassert *et al.*, 2000; Storz *et al.*, 1990; Toledano *et al.*, 1994) where oxidation induces a conformational change in OxyR that alters DNA-binding specificity, and interactions with RNA polymerase, and leads to transcription activation of its target genes. Reduced LpOxyR would bind to a more extended area, possibly bending DNA and preventing the recruitment and activation of RNA polymerase (Storz *et al.*, 1990; Toledano *et al.*, 1994). As observed for the regulation of *ahpC* in *E. coli* (Storz *et al.*, 1990; Toledano *et al.*, 1994), mycobacteria (Dandayuthapany *et al.*, 1997; Pagan-Ramos *et al.*, 1998) and *X. campestris* (Loprassert *et al.*, 2000), our data seems to indicate that oxidized LpOxyR activates the *ahpC2D* promoter whereas reduced LpOxyR represses the operon. It is interesting that LpOxyR displays characteristics that are similar to *ahpC* regulation in *E. coli*, but the LpOxyR footprint extends farther than those observed for *E. coli* OxyR (Toledano *et al.*, 1994). In fact, the putative -35 sequence of *L. pneumophila ahpC2* is displaced by 9 base pairs compared to that of the *ahpC* of *E. coli*. The reason behind this discrepancy remains elusive, yet this might explain why reduced LpOxyR was unable to bind to *E. coli* promoters (*ahpC*, *katG*, and *oxyR*).

### 5.8. LpOxyR Functions as a Peroxide Sensor/Transcriptional Activator in *E. coli*

To further evaluate whether LpOxyR is able to function as a peroxide sensor and transcriptional activator, *E. coli* OxyR and LpOxyR were used to complement an *E. coli oxyR::km* mutant strain (GS077) using the arabinose-inducible system of pBAD22. Under inducing conditions, expression of *E. coli oxyR* was able to restore peroxide resistance for GS077 to levels of the wild-type *E. coli* strain MG1655. In contrast, expression of *L. pneumophila oxyR* could only partially restore this resistance. Mobility-

shift assays using LpOxyR and *E. coli* DNA at concentrations described for binding to $P_{ahpC2}$ showed that a relatively high amount of DNA remained unbound, suggesting LpOxyR may have weak affinity for *E. coli ahpCF*, *katG*, and *oxyR* promoters. The weak association with promoters for the peroxide-detoxifying enzymes AhpCF and catalase (KatG) may explain why LpOxyR was unable to fully complement the peroxide-sensitive phenotype of the *E. coli oxyR* mutant. Nonetheless, the partial complementation of the *E. coli* GS077 phenotype by expression of *L. pneumophila oxyR* suggests that LpOxyR likely functions as a peroxide sensor and transcriptional activator. In *L. pneumophila*, LpOxyR likely activates *ahpC2D* expression in response to oxidative stress, where absence of oxidative stimuli would repress the operon. This model could explain the compensatory up-regulation of *ahpC2D* with loss of AhpC1 function. We believe that increased intracellular concentrations of peroxides or changes in the redox status of the cell with loss of AhpC1 function would be accompanied by OxyR-mediated increased expression of *ahpC2D*. To fully validate such a hypothesis, *ahpC2D* expression levels could be evaluated in an *oxyR* mutant of *L. pneumophila*. However, the *L. pneumophila oxyR* mutant could not be constructed using conditions described in this thesis (discussed below). It would also be of interest to determine the levels of *ahpC2D* expression in *L. pneumophila* mutants lacking other antioxidant proteins including KatA, KatB, SodB, SodC, MsrA, and Ohr.

### 5.9. Induction of OxyR-Regulated Genes

In *E. coli*, OxyR is responsible for the peroxide-inducible response in exponentially growing cells (Gonzalez-Flecha and Demple, 1997). Induction by low concentrations of $H_2O_2$ is accompanied by a subsequent increased resistance to higher

concentrations of $H_2O_2$ arising from activation of the OxyR regulon (Demple and Halbrook, 1983; Storz and Altuvia, 1994). Preliminary attempts to induce the expression of *L. pneumophila ahpC2D* during exponential growth with exogenous peroxides were not successful, suggesting a slow regulatory response. Similar results have been reported for *katA* and *katB* (Bandyopadhyay and Steinman, 1998 and 2000). It is possible that *ahpC2D* expression is induced by other peroxide concentrations (or by other compounds) not evaluated in this study.

If LpOxyR cannot respond to exogenous peroxides, then why is the *ahpC2D* operon up-regulated in the *ahpC1* mutant? And how is *ahpC2* and *ahpD* expression increased during the replicative phase in protozoa? OxyR has been shown to be activated by numerous stimuli, such as changes in the redox status of the cell (Aslund *et al.*, 1999; Kim *et al.*, 2002). Differences between activation of OxyR in *E. coli* and in *L. pneumophila* could be attributed to difference in the redox status of the cell. In *E. coli*, the redox potential for OxyR has been estimated at -185 mV, whereas the cytoplasm is -280 mV (Aslund *et al.*, 1999; Zheng *et al.*, 1998). Under these conditions, OxyR would be reduced and deactivated. Activation of OxyR would be achieved by the high reactivity of the protein with $H_2O_2$ or by a shift in the redox status of the cell (Aslund *et al.*, 1999). For example, some *E. coli* mutants allowing disulfide bond formation in the cytoplasm were found to have a constitutively active OxyR (Aslund *et al.*, 1999; Zheng *et al.*, 1998). *L. pneumophila* is known to have lower concentrations of glutathione than *E. coli* (Locksley *et al.*, 1982), suggesting that OxyR could be constitutively active or inducible by low concentrations of peroxide. Multiple thioredoxins, glutaredoxins, and other factors (like GrlA) are likely to influence the redox status in *L. pneumophila*. It

would be of interest to investigate the contribution of GrlA, since the *ahpC2D* operon is expressed at a stage during growth when GrlA and AhpC1 are absent.

Kim *et al.* (2002) showed that several post-translational modifications of the single regulatory thiol (SH) occur, such as nitrosylation (S-NO), hydroxylation (S-OH), and oxidation to form a mixed disulfide with glutathione (S-SG). Overall, these changes result in different structures, cooperative properties, DNA-binding affinities, and promoter activities. The activation of OxyR by nitrosylation should be pursued further since reactive nitrogen intermediates are likely to occur in the phagosomal milieu of macrophages during *L. pneumophila* infection and AhpCs are known to scavenge reactive nitrogen intermediate-derived compounds like peroxynitrite (Bryk *et al.*, 2000; Loprasert *et al.*, 2003; Master *et al.*, 2002). Future studies should also investigate the possible relationship between the thiol-disulfide ratio and the expression of LpOxyR-regulated genes. Treatment with diamide, a compound known to promote oxidation of glutathione, could be particularly useful for such analysis (Zheng *et al.*, 1998). In fact, treatment with diamide was shown to increase expression of *ahpC* in mycobacteria (Dosanjh *et al.*, 2005). The hypothesis that OxyR might respond to endogenously produced ROIs is supported by others where catalase activity (shown in this study to be attributed to *L. pneumophila* AhpCs) was shown to increase with respect to the growth rate and levels of aeration of the culture (Berg *et al.*, 1985). Since the doubling time of *L. pneumophila* is approximately 6-fold longer than that of *E. coli*, the rate of endogenous $H_2O_2$ production might not require a rapid and extensive peroxide-inducible response (Gonzalez-Flecha and Demple, 1997). *L. pneumophila* might have evolved a more redundant approach for dealing with peroxide stress by constitutively producing multiple antioxidant enzymes.

The lack of an observable inducible response by *L. pneumophila* could also be attributed to mutations in LpOxyR. For example, in *E. coli*, a H218D mutation of OxyR results in wild-type DNA-binding ability but causes an increase in the sensitivity to peroxide due to a partially impaired activation of OxyR by $H_2O_2$ (Choi *et al.*, 2001; Wang *et al.*, 2006). The LpOxyR residue in this position corresponds to a H218D mutation compared to *E. coli* OxyR, suggesting that LpOxyR might be partially impaired for peroxide activation. Another substitution, H125I, in LpOxyR is associated with lack of induction in *E. coli* (Wang *et al.*, 2006). The presence of the sequence variations in LpOxyR is consistent with the partial complementation the *oxyR* mutant of *E. coli* (GS077) by *L. pneumophila oxyR*. It should be noted that the two substitutions in LpOxyR (H125I and H218D) are also found in *X. campestris* (H125P and H218E), yet a peroxide-inducible activation of OxyR-regulated genes is still observed in this organism (Loprasert *et al.*, 1997). Since LpOxyR shares closer similarity to *X. campestris* OxyR than *E. coli* OxyR, these sequence vriations do not exclude its role as an oxidative-stress regulator.

The partial complementation of an *E. coli oxyR* mutant by LpOxyR and lack of induction of *ahpC2D* by $H_2O_2$ in *L. pneumophila*, suggest that the regulation of *L. pneumophila ahpC2* might be more complicated than the model proposed in Figure 7. Using BPROM software (www.softerry.com), a second promoter region was identified for *ahpC2*. The -35 sequence (TTGCAA) was found upstream from the OxyR binding site, where OxyR would bind adjacent to and upstream of the -10 sequence (TATTAT). Using primer-extension analysis, Loprasert *et al.* (2000) located two promoters for *ahpC* while providing no explanation for this finding. This second *ahpC2* promoter region in

*L. pneumophila* could provide a mechanism of regulation similar to that seen for *agn43* of *E. coli* (encoding an outer-membrane protein involved in biofilm formation and autoaggregation), where expression is regulated both by OxyR and by Dam-mediated methylation of three GATC sequences in the promoter region (Wallecha *et al.*, 2002). The maximal level of transcription was observed with methylated sequences, where binding of OxyR was abrogated (Henderson and Owen, 1999; Wallecha *et al.*, 2002). A lower level of transcriptional regulation was observed in the absence of both Dam-methylation and OxyR. Binding of OxyR to unmethylated DNA resulted in repression of *agn43*. DNA methylation is known to regulate a number of processes in *E. coli*, such as DNA replication, repair and transcription of other genes (Boye and Lobner-Olesen, 1990; Braaten *et al.*, 1994; Schlagman *et al.*, 1986). In *S. typimurium*, DNA methylation regulates virulence-gene expression, where *dam* mutants are avirulent (Balbontin *et al.*, 2006; Heithoff *et al.*, 1999). Another example is found in *Caulobacter cresentus*, where the cell cycle-regulated methyltransferase (CcrM) coordinates multiple aspects of the developmental cycle (Reisenauer and Shapiro, 2002; Reisenauer *et al.*, 1999). The importance of methylation in this organism is highlighted by the fact that *ccrM* is essential for growth (Reisenauer *et al.*, 1999). Since *L. pneumophila ahpC2D* possesses GATT sequences (in positions relative to the *agn43* promoter region of *E. coli*), the mechanism of regulation of *L. pneumophila ahpC2D* is likely more complex than a two-state model of regulation where expression is controlled by reduction or oxidation of OxyR. Therefore, induction of *ahpC2* by oxidized LpOxyR might only occur during a stage of growth when the promoter region is not methylated.

Another possible scenario is that expression of *ahpC2* could be regulated by an OxyR-independent process. The reciprocal expression pattern of *ahpC1* and *ahpC2D* during growth suggests the presence of a second regulator for the oxidative-stress response in *L. pneumophila*. No information could be inferred from the regulatory control of other antioxidant enzymes in *L. pneumophila* (*ahpC1*, *katA*, *katB*, *sodB*, *sodC*, and possibly others) since their mechanisms of regulation remain elusive. Genome analysis indicates that, unlike other organisms, *L. pneumophila* does not possess other known regulators of oxidative stress (like SoxRS or OhrR), and its RpoS has not been linked to protection against ROIs. However, *L. pneumophila* possesses an essential Fur homolog (Hickey and Cianciotto, 1997). Fur homologs such as PerR are known to confer peroxide resistance by activating the expression of genes encoding antioxidant enzymes such as AhpC (Baillon *et al.*, 1999; Bsat *et al.*, 1996; Tardat and Touati, 1993; van Vliet *et al.*, 1999). In *M. tuberculosis* where *oxyR* is a pseudogene, a second level of OxyR-independent regulation has also been proposed for *ahpC2D* expression (Springer *et al.*, 2001). Interestingly, the lack of a functional OxyR in *M. tuberculosis* results in increased resistance (and not sensitivity) to peroxides (Pagan-Ramos *et al.*, 2006). This paradox has been attributed to the de-repression of *katG* by the mycobacterial Fur homolog (encoded by *furA*), a characteristic crucial for activation of the anti-tuberculosis pro-drug isoniazid (also called isonicotinyl hydrazine, isonicotinic acid hydrazide, or simply INH) (Wengenack *et al.*, 1998; Zahrt *et al.*, 2001). FurA appears to be a dominant regulator of oxidative stress and intracellular survival in mycobacterial species lacking a functional *oxyR* (Zahrt *et al.*, 2001). Whether *L. pneumophila* uses its Fur homolog to regulate oxidative stress remains to be determined. However, it should be noted that direct

comparison of *ahpC* expression levels between wild-type and a *fur* mutant is not possible, since Fur function is essential in *L. pneumophila* (Hickey and Cianciotto, 1997).

### 5.10. Regulation of *L. pneumophila oxyR*

Like other members of the LysR family, OxyR is generally found to positively control expression of target genes while playing a negative autoregulatory role (Schell, 1993). Surprisingly, GFP reporter assays revealed that *L. pneumophila oxyR* expression is growth-phase dependent. However, no differences in *oxyR* expression were observed between wild-type and the *ahpC* mutants, suggesting that the compensatory response of *ahpC2D* in the *ahpC1*-deficient background is not due to differences in *oxyR* expression. GFP-reporter data also suggested that sub-lethal concentrations of $H_2O_2$ have no influence on LpOxyR levels. Logically, this result was expected since *E. coli* OxyR represses its own transcription in both oxidized and reduced forms by binding to its own promoter. However, in *S. coelicolor*, increased levels of OxyR are observed in response to peroxides (Hahn *et al.*, 2002). In *L. pneumophila*, a putative OxyR-binding site was found upstream of and overlapping the coding sequence of *L. pneumophila oxyR*, suggesting a possible autoregulatory process. However, EMSA analysis indicated that LpOxyR is unable to bind to its own promoter under both reducing and oxidizing conditions. To date, no other examples have been reported where OxyR does not bind to its own promoter, with the exception of *X. campestris* (Mongkolsuk *et al.*, 1997). In that organism, *ahpC* is expressed as a monocistronic mRNA whereas *ahpF*, *oxyR*, and *orfX* are expressed as a polycistronic message. It is curious that LpOxyR displays closer similarity to *X. campestris* OxyR than to those from *E. coli* or mycobacteria. However, no *ahpF* gene is found in the *L. pneumophila* genome and no genes are orientated at the

same direction as *L. pneumophila oxyR* in its chromosomal locus. However, some sort of a regulatory mechanism must be involved since changes in *L. pneumophila oxyR* expression were observed during growth. In fact, GFP-reporter assays indicated that LpOxyR levels increased during exponential and post-exponential phase of growth.

What could regulate the expression of *oxyR*? Our data indicates that *oxyR* expression was unchanged in strains mutant for known regulators of *L. pneumophila* virulence (RpoS, LetA, and IHF), suggesting that the growth-phase-dependent expression of *oxyR* is mediated by RpoS-, LetA-, and IHF-independent processes. To identify possible regulators of *oxyR* expression, an ACDC technique was used. Since this technique removed non-specific DNA-binding proteins by electrophoresis, only specific proteins should be captured by Acrydite-bound DNA. This technique was first validated with the promoter region of *ahpC2*, where the DNA captured a single protein identified as OxyR. When assays were performed using the promoter region of *L. pneumophila oxyR*, two proteins were identified as the β and β' subunits of the core RNA polymerase. No sigma factor or transciptional regulator has yet been identified for *oxyR*. Two scenarios could thus be envisioned. One is where conditions for the assay were not optimal to capture possible regulators, or that regulation of *oxyR* involves other regulatory mechanisms such as methylation (as discussed above). To ensure response regulators would be captured, the ACDC technique was repeated with addition of acetyl phosphate. No differences were observed. Such assays should also be repeated with a binding buffer lacking EDTA. Transcription factors like Fur can only bind and repress expression from target genes when bound to iron, where EDTA would chelate this metal. Though the ACDC technique did not reveal the mechanisms of *oxyR* regulation in *L.*

*pneumophila*, it was successful in identifying OxyR as the specific regulator for *ahpC2* (though the regulation of *ahpC2* by Fur can not be excluded). Furthermore, the protein lysate tested was obtained and purified from a *L. pneumophila* broth culture growth to a single time point. Additional factors might be expressed under different experimental conditions or at other time points during growth. As described above, there is an obvious need to repeat these assays under many different conditions. Nonetheless, careful consideration should be taken when interpreting acrylamide capture results since this assay can also capture regulators of divergently transcribed genes. In the *L. pneumophila oxyR* locus, a homolog of a major facilitator family transporter is located in this orientation.

Despite not knowing the mechnisms of *oxyR* regulation, we believe that LpOxyR acts as a peroxide sensor or redox-sensitive transcription factor where oxidation leads to transcriptional activation of the *ahpC2D* operon during oxidative stress. Identification of LpOxyR as an oxidative stress regulator in *L. pneumophila* is of fundamental value where improving our knowledge of the defense strategies used by intracellular pathogens faced with oxidative-stress might help delineate the complex regulatory network involved during both survival in natural environments and evasion of the immune defenses during human infection. Furthermore, the delineation of a consensus binding motif should aid in identification of additional OxyR-regulated genes. For example, using the "pattern search" option on the *L. pneumophila* Paris and Lens genome website (http://genolist.pasteur.fr/LegioList/), putative OxyR-binding sites were found upstream of many genes including some members of the *E. coli* OxyR regulon (*dps*, *fur*, *grx*, *trx*), but were not observed for others (*gorA* or the catalase-peroxidases). The *Legionella dps*

homologue has recently been characterized in *L. pneumophila*, and was shown to protect against $H_2O_2$ by sequestering iron and prevent Fenton-mediated DNA damage (Park *et al.*, 2006). In addition, promoters of other genes also contained putative OxyR-binding sites, such those encoding for antioxidant enzymes (*ahpC2D*, *sodB* and *msrA*), efflux pumps (that could exclude redox-cycling compounds and organic solvents), metal-ion transporters (that could modulate rates of Fenton chemistry), components of the respiratory chain such as cytochrome oxidases (that could modulate rates of endogenous $\bullet O_2^-$ production), DNA repair or modification enzymes (such as endonucleases or methylases), known *L. pneumophila* virulence factors such as the zinc metalloproteinase (shown to inhibit the oxidative burst), virulence factors like substrates of the Dot/Icm T4SS (SidG, Sdhb, and SdeA), and numerous transcriptional regulators like FleQ, FleN, and members of the LysR family (possibly providing cross-talk with other regulatory pathways). The possible presence of several OxyR-binding sites suggests that this regulator might be involved in processes other than oxidative stress. Though purely speculative, a reason why the *oxyR* mutants could not be obtained could be that OxyR regulates the expression of an essential gene. It is interesting that there is a paucity of *L. pneumophila* mutants that evade lysosomal fusion but are defective for intracellular replication, suggesting that we might not be able to isolate such mutants on artificial media.

### 5.11. Conclusions

From the natural aquatic environments to human, *L. pneumophila* monitors its environment to replicate when possible, and promotes survival traits when conditions deteriorate. Human *L. pneumophila* infection is resolved by cell-mediated and adaptive

immune responses in most individuals. Therefore, conditioning of *L. pneumophila* in the intracellular milieu of protozoa prime the organism with virulence strategies, despite these strategies eventually fail in most humans. However, immunocompromized individuals must rely on proper antibiotic treatment or face potentially life-threatening complication of Legionnaires' disease. The lack of cost-effective prevention strategies and current failures to eliminate the organism from aquatic man-made environments subjects us all potentially to infection. Therefore, investigations into the molecular mechanisms permitting adaptation to oxidative stress might provide insight on more effective means for detection, treatment, and prevention of this respiratory disease.

Since *L. pneumophila* might be faced with an oxidative burst during the early stages of macrophage, neutrophil, and protozoan infection, delineation of antioxidant mechanisms used by *L. pneumophila* to detoxify ROIs during phagocytosis might be of fundamental importance to understand both *L. pneumophila* pathogenesis and environmental survival. As a step forward in determining the role of antioxidant defenses during *L. pneumophila* infection and environmental survival, the major findings in this study can be summarized briefly as the identification and characterization of two AhpCs which are responsible for peroxide-scavenging activity in *L. pneumophila*, the possibility of compensatory responses such as observed for *ahpC2D* upon loss of AhpC1 function, and the identification of OxyR as the first oxidative stress regulator in *L. pneumophila*. Since previous work by Bandayopadhyay and Steinman (1998 and 2000) had proposed a model where *L. pneumophila* KatA and KatB would catalyze detoxification of $H_2O_2$ in the phagosomal lumen and a *katAkatB* double mutant is no more susceptible to $H_2O_2$, our results suggest that the peroxidase activity of *ahpC1* and *ahpC2D* might also contribute

to the generation of a replication-permissive vacuole in host cells. However, the peroxide sensitivity observed for *L. pneumophila ahpC* mutants and lack of intracellular growth defects brings into question whether compensatory responses are involved in protecting *L. pneumophila* from the oxidative burst in macrophages, or whether this organism uses strategies acquired in natural environments to prevent, inhibit, or evade ROIs produced by the NADPH oxidase. To avoid being consumed by protozoa, it would be effortless to imagine that *L. pneumophila* would want to mount antioxidant defenses long enough to generate its replication-permissive niche or perhaps immediately avoid ROIs by inhibiting or preventing activation of the respiratory burst. One could also envision the use of antioxidant enzymes to cope with endogenous metabolic by-products of aerobic respiration. Further investigations will surely reveal the roles (if any) of antioxidant enzymes like AhpC, oxidative-stress regulators like OxyR, and other factors implicated in antioxidant defenses during human infection and environmental survival.

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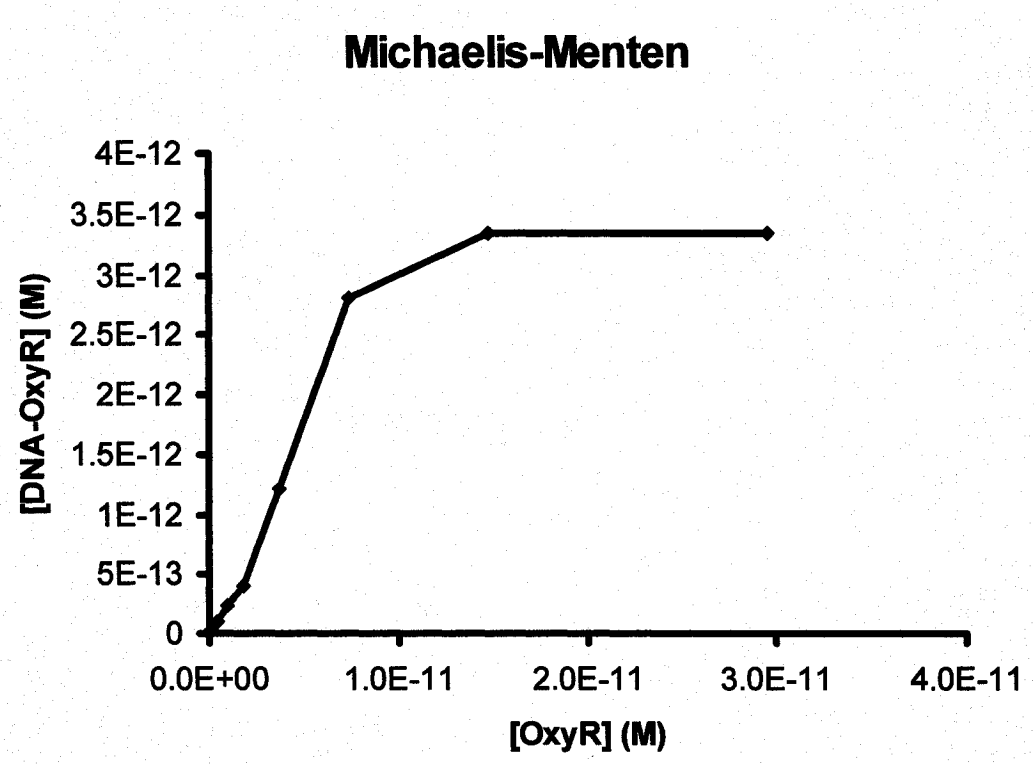
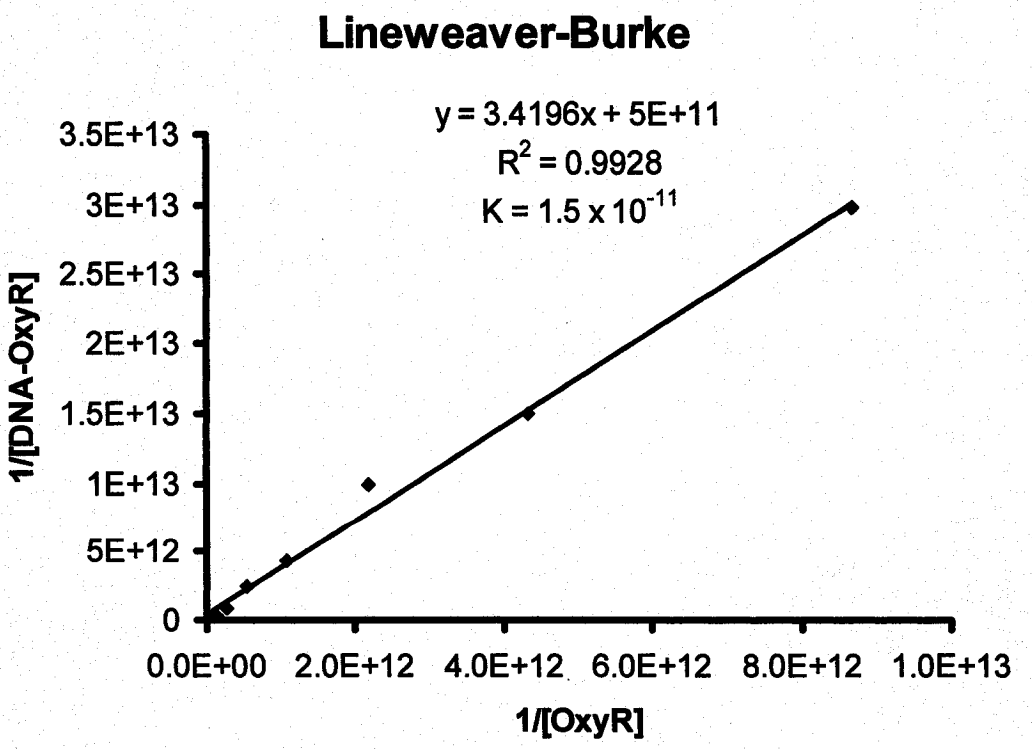
**Appendix 1**: Binding Affinity of LpOxyR for the Promoter of *ahpC2*



**Appendix 2**: Complementation of *E. coli* GS077

**Table A1** Zones of inhibition in mm using 10 µl of 3% $H_2O_2$

| Strain | Glucose (%) | Arabinose (%) | | | | |
|---|---|---|---|---|---|---|
| | 0.2 | 0 | 0.0002 | 0.002 | 0.02 | 0.2 |
| K12[a] | 23 ± 1 | 21 ± 1 | 22 ± 1 | 21 ± 1 | 21 ± 1 | 21 ± 1 |
| K12 pbad[b] | 22 ± 1 | 21 ± 1 | 23 ± 1 | 20 ± 1 | 22 ± 1 | 22 ± 1 |
| GS077[c] | 40 ± 1 | 38 ± 2 | 39 ± 2 | 38 ± 2 | 38 ± 1 | 37 ± 2 |
| GS077 pbad[b] | 41 ± 1 | 39 ± 1 | 37 ± 2 | 39 ± 2 | 38 ± 1 | 38 ± 1 |
| GS077 pbadEcoxyR[d] | 40 ± 1 | 27 ± 2 | 18 ± 1 | 18 ± 0 | 17 ± 1 | 18 ± 1 |
| GS077 pbadLpoxyR[d] | 39 ± 1 | 37 ± 1 | 32 ± 1 | 31 ± 3 | 31 ± 2 | 30 ± 2 |

Experiment were performed using Mueller-Hinton (MH) solid medium [a]without antibiotics, or supplemented with [b]ampicillin (100 µg/ml), [c]kanamycin (40 µg/ml), or [d]ampicillin (100 µg/ml) and kanamycin (40 µg/ml).