Environmental and Genetic Factors that Contribute to the Adaptation and Survival of *Legionella pneumophila* in Water Systems

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TABLE OF CONTENT

ABSTRACT

Respiratory infections, including community-acquired pneumonia, are most common among the elderly and immunocompromised individuals. *Legionella pneumophila* (*Lp*) contributes to this disease burden as the leading cause of Legionnaires' disease (LD), an atypical and potentially life-threatening pneumonia whose incidence rates have seen an increase in the last decade. This water-borne bacterium is transmitted through contaminated aerosols that are emitted from a variety of water distribution systems. *Lp* is ubiquitous in freshwater, and notoriously persistent in anthropogenic systems, surviving a slew of disinfectants. An important knowledge gap in *Legionella* research is the identification of genetic factors contributing to the long-term survival of the bacterium in water, and in response to the environmental stresses encountered within aquatic systems. As a first step, the development of a reproducible proxy for a freshwater model was required. We present Fraquil as an appropriate medium for the study of *Lp*. Using this freshwater medium; we found that *Lp* is able to survive for over 6 months in 15^{o}C water while retaining its ability to infect host cells. Another finding that merits further study is entry of *Lp* into a viable-but-non-culturable state at 4^{o}C. Fraquil was further used to study a set of regulators that contribute to the bi-phasic lifestyle of *Lp*. As a result, we report that the LetA/S two-component system is crucial for a general transcriptomic shutdown that allows *Lp* to survive in water. After probing the mechanism of activation, we report that while dependent on ppGpp for expression, LetS is activated independently of this global response in water. Finally, we identified a second regulatory protein, OxyR, as a contributor to surviving oxidative stress in water. For the first time, the work presented here offers a reproducible medium to study *Legionella* in water, allowing better comparisons between studies and the ability to control the complexity of the water medium when exploring the effect of environmental factors on bacterial behaviour. It has been successfully used in the work presented here and elsewhere to study genetic factors that contribute to the survival of *Legionella* in water, an area of research that has only recently begun to be explored. Furthermore, we have identified a major contributor to the overall survival in water, the LetA/S two-component system. Finally, we have characterized OxyR which was previously found to be dispensable for responding to exogenous oxidative stress. We report that it is essential for *Lp* to survive oxidative stress in the water environment, likely through non-traditional mechanisms. The work presented herein broadens our knowledge

about the regulatory mechanisms used by this aquatic pathogen, potentially leading the way to innovative detection and/or control methods.

RESUMÉ

Les infections respiratoires, y compris la pneumonie acquise dans la communauté, sont les plus fréquentes chez les personnes âgées et les personnes immunodéprimées. *Legionella pneumophila* (*Lp*) contribue à ce fardeau de la maladie en tant que principale cause de la maladie du légionnaire (LD), une pneumonie atypique et potentiellement mortelle dont les taux d'incidence ont augmenté au cours de la dernière décennie. *Lp* est transmise par des aérosols contaminés qui sont émis dans l'environnement par une variété de systèmes hydriques. *Lp* est omniprésent dans l'eau douce, et notoirement persistant dans les systèmes anthropiques, étant résistant à de nombreux traitement désinfectants. Les gènes qui contribuent à sa survie dans l'eau et à sa résistance aux stresses environnementaux rencontrées dans les systèmes hydriques sont peu connus. Dans un premier temps, le développement d'un modèle d'eau douce reproductible était nécessaire. Je présente le Fraquil comme modèle d'eau pour l'étude de *Lp*. En utilisant ce modèle, j'ai constaté que *Lp* est capable de survivre pendant plus de 6 mois dans cet eau à une température de 15°C tout en conservant sa capacité d'infecter les cellules hôtes. De plus, il semble que *Lp* entre dans un état viable-mais-non-cultivable à 4°C. D'autres études seront nécessaires pour bien comprendre ce processus. Le Fraquil a ensuite été utilisé pour étudier un ensemble de régulateurs qui contribuent au mode de vie bi-phasique de *Lp*. En conséquence, j'ai déterminé que le système à deux composants LetA/S est crucial pour un arrêt transcriptomique général qui permet à *Lp* de survivre dans l'eau. Après avoir sondé le mécanisme d'activation, j'ai trouvé que LetS est activé indépendamment de la réponse stringente. Enfin, j'ai identifié que le régulateur global OxyR contribue à la résistance au stress oxydatif dans l'eau. Pour la première fois, le travail présenté ici offre un moyen reproductible pour étudier la légionelle dans l'eau, permettant de meilleures comparaisons entre les études et la capacité de contrôler la complexité du milieu de l'eau lors de l'exploration de l'effet des facteurs environnementaux. Il a été utilisé avec succès dans le travail présenté ici et ailleurs pour étudier les facteurs génétiques qui contribuent à la survie de *Legionella* dans l'eau. En outre, nous avons identifié un facteur majeur de la survie globale dans l'eau, le système à deux composants LetA/S. Nous avons également caractérisé OxyR qui, auparavant, était considéré non-essentiel pour répondre au stress oxydatif exogène. Nous signalons qu'il est essentiel pour la survive de *Lp* au stress oxydatif dans l'eau, probablement grâce à la régulation des gènes non-traditionnels. Le travail présenté ici élargit

notre connaissance des mécanismes utilisés par ce pathogène aquatique, ouvrant la voie à des méthodes novatrices de détection et/ou de contrôle.

CONTRIBUTIONS TO KNOWLEDGE

This doctoral work aims to expand the understanding of the relationship between *Legionella pneumophila* (*Lp*), the nutrient-poor water environment and the genetic factors that contribute to its successful persistence when faced with environmental stresses in water. The highlights of the research presented here are as follows:

i. I have established a suitable freshwater model to study the behaviour of *Lp* in its natural aquatic environment. Using this model, I show that the bacterium is able to retain its virulence potential over long periods of time in water. In addition, I have determined that 15ºC is the optimal temperature for long-term survival in water. The survival is negatively affected by low pH, but is not affected by the concentration of trace metals. I also show the entry of *Lp* in to a viable-but-non-culturable state at 4^{o}C.

ii. The LetA/S two-component system was found to be a major determinant in the survival of *Lp* in water. My data show that the transcriptomic shutdown elicited by exposure to water is under LetS control in *Lp*. The regulatory pattern under LetS control was also found to be distinct from that observed in rich broth, suggesting that the bacterium can fine-tune its response to a given environment. I also show that the LetA/S system acts in parallel to the stringent response pathway.

iii. Functional characterization of the putative oxidative stress response regulator, OxyR, reveleaed, for the first time, a role for this protein in overcoming oxidative stress in water. Previously reported to be insensitive to oxidative stress, this regulator seems to mediate oxidative insults through novel pathways.

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

α-KG: α-ketoglutarate

ACP: Acyl-carrier protein

AHK: α-hydroxyketone

AYE: ACES-buffered yeast extract broth

BCYE agar: Buffered charcoal yeast extract agar

BSA: Bovine serum albumin

c-di-GMP: cyclic di-GMP

CAP: Community-acquired pneumonia

CDC: Centers for Disease Control

CDD: Conserved Domain Database

CFU: Colony forming unit

Cm: Chloremphenical

COG: Clusters of orthologous groups

CYE agar: Charcoal yeast extract agar

DL: Detection limit

Dot: Defective in organelle trafficking

E phase: Exponential phase

ED: Entner-Doudoroff

EEA1: Early endosome antigen 1

Enh: Enhanced entry

EPA: Environmental Protection Agency

ER: Endoplasmic reticulum

EWGLI: European Working Group for *Legionella* Infections

GFP: Green fluorescent protein

Gm: Gentamicin

H_2O_2: Hydrogen peroxide

HPC: Heterotrophic plate count

HPT: Histine phosphotransfer domain

ICM: Intracellular multiplication

IPTG: Isopropyl-β-D-thiogalactopyranoside

Kn: Kanamycin

LAI-1: *Legionella* autoinducer 1

LAMP: Lysosome-associated membrane glycoproteins

LB: Luria-Bertani

LCV: *Legionella*-containing vacuole

LD: Legionnaires' disease

LetA/S: *Legionella* transmission activator and sensor

Lp: *Legionella pneumophila*

Lp1: *Legionella pneumophila* serogroup 1

LPS: Lipopolysaccharide

Lqs: *Legionella* quorum sensing

Lsp: *Legionella* secretion pathway

LTTR: LysR Type Transcriptional Regulator

Lvh: *Legionella vir* homolog

Mab: Monoclonal antibody

MIF: Mature intracellular form

MLST: Multi-locus sequence typing

MOI: Multiplicity of infection

mRNA: messenger RNA

Na-pyruvate: Sodium pyruvate

NCBI: National Center for Biotechnology Information

NSERC: National Sciences and Engineering Research Council of Canada

OD: Optical density

ORF: Open reading frame

PAGE: Polyacrylamide gel electrophoresis

PBS: Phosphate-buffered saline

PCR: Polymerase chain reaction

PE phase: Post-exponential phase

PF: Pontiac fever

PHB: Poly-3-hydroxybutyrate

PMA: Phorbol 12-myristate 13-acetate

ppGpp: Guanosine tetraphosphate

PPP: Pentose phosphate pathway

pppGpp: Guanosine pentaphosphate

PYG: Peptone yeast extract glucose

qPCR: quantitative polymerase chain reaction

QS: Quorum sensing

R domain: Receiver domain

RNAP: RNA polymerase

ROS: Reactive oxygen species

RPMI medium: Roswell Park Memorial Institute Medium

RR: Response regulator

SBT: Sequence-based typing

SD: Standard deviation

SK: Sensor kinase

SR: Stringent response

sRNA: small regulatory RNA

Str: Streptomycin

T domain: Transmitter domain

Tat: Twin-arginine translocation

TCA: Tricarboxylic acid

TCS: Two-component system

TOC: Total organic carbon

U.S.: United States of America

USD: United States Dollars

VBNC: Viable-but-non-culturable

WHO: World Health Organization

WT: Wild-type

CHAPTER 1: Introduction

In 1675, Antoni van Leeuwenhoek, a Dutch draper, discovered "wee animalcules" in rain water, a finding that he shared with the Royal Society two years later in his famous Letter 18 (Friedman and Friedland 2000). Eventually, this led to the identification of microorganisms as major agents of disease that claim millions of lives. The astronomical advances in scientific expertise and sanitation over the past 300 years have led to the eradication of smallpox and the reduction in the burden of many diseases caused by microorganisms, but also to acknowledging the beneficial role of bacterial communities to human health. Nonetheless, bacterial infections remain a concern around the world.

Legionella pneumophila (*Lp*) is an emerging bacterial pathogen that was first identified in 1976 as the etiological agent of a pneumonic epidemic at an American Legion convention in Philadelphia (Brenner et al. 1979). While this was the first reported *Legionella* outbreak, the bacterium has been retrospectively identified as the cause of cases and outbreaks as early as 1947 (Ager and Tickner 1983, McDade et al. 1979, Terranova et al. 1978, Thacker et al. 1978). To date, over 50 *Legionella* spp. and 70 serogroups have been identified (Burillo et al. 2017, DSMZ 2015). Among these, *Lp* contributes most to the disease burden in the Western hemisphere, representing over 85% of reported cases worldwide (Berjeaud et al. 2016, Yu et al. 2002). The only exception being Australia and New Zealand, where *L. longbeachae* causes up to 50% of total infections (Whiley and Bentham 2011). *L. longbeachae* is commonly found in potting soils, in contrast to *Lp,* which resides in aquatic environments (Fields et al. 2002). Given the disease burden attributed to *Lp,* it has been and remains the subject of the majority of scientific research.

Lp is a ϒ-proteobacterium that is a resident of natural environments and can be found in most freshwater sources. In natural aquatic environments, *Lp* parasitizes and replicates within various protozoa, mainly amoeba (Abu Kwaik et al. 1998, Borella et al. 2005a). However, it is the colonization of man-made water environments that allows *Lp* to infect human hosts. Indeed, it is a common contaminant of built water environments, the source of human infections (Fields et al. 2002). *Lp* is transmitted to humans through aerosols generated from contaminated man-made water systems, including water cooling towers, showers, hot springs, decorative fountains and spas (Borella et al. 2005a, Fields et al. 2002). Infection by *Legionella*

results in Legionnaires' disease (LD), a severe and life-threatening pneumonia, and Pontiac fever, a milder flu-like illness (Fields et al. 2002). Healthcare related costs associated with LD are estimated to be as high as $430 million USD per year (Collier et al. 2012). The most vulnerable to *Lp* infections include the elderly, males, smokers and the immunocompromised (Hilbi et al. 2010). In fact, 60% of U.S. LD outbreaks between 2011 and 2012 occurred in hospital or healthcare settings (Beer et al. 2015).

According to a survey sponsored by the Environmental Protection Agency (EPA), it is estimated that we spend an estimated 90% of time indoors (Klepeis et al. 2001). As a result, exposure to pathogenic organisms as well as contaminants via water distribution systems of the built environment is of increased interest, as are strategies for their prevention. *Lp* is a prime example of how modern infrastructures create niche environments where previously benign microorganisms may increase in concentration or even acquire virulent traits so as to pose a significant threat to human health. In support of *Lp* being an increasing health risk, one study identified 114 respiratory disease outbreaks at mass gatherings between 2009 and 2013. Of these outbreaks, 96 were Legionellosis (Rainey et al. 2016). A CDC report found that 21 out of 32 drinking water-associated outbreaks between 2011-2012 were caused by *Lp* (Beer et al. 2015). The reported incidence rate of LD is still quite low, in the order of 1.3-3.5 cases/100 000 people in Canada (Doebbeling and Wenzel 1987). However, the actual LD burden within the population is considered to be higher due to severe underreporting of cases. In addition, while LD receives the most attention during community outbreaks, a Canadian study estimates that 80% of *Lp* infections are sporadic cases (Ng et al. 2008). This is in line with trends seen in other atypical pneumonia cases (Cunha 2006). With increasing awareness, recent years have seen a rise in the numbers of LD cases being reported in Europe and the U.S., further highlighting the importance of understanding how this bacterium persists in water distribution systems despite some of the preventative measures currently in place (Beer et al. 2015, WHO 2007). In the 40 years since its discovery, only one case of transmission between humans has been reported, suggesting that the human host is an evolutionary dead end for *Lp*. In 2016 the first alleged case of person-to-person transmission was reported in Spain (Correia et al. 2016). Nonetheless, the current knowledge indicates that managing *Lp* infections is inherently dependent on managing the contamination of water systems.

To date, there have been no reported cases of humans contracting Legionnaires' disease from natural water sources, which suggests that either the contamination of man-made water sources allows the bacterium to multiply to a density that allows human colonization, and/or that the conditions that it encounters within these built environments selects for strains that are transmissible to human hosts. Over the years, numerous epidemiological studies have reported correlations between temperature and trace metal concentrations on the presence of *Lp* in plumbing and water distribution systems around the world (Bargellini et al. 2011, Borella et al. 2004, Edagawa et al. 2008, Leoni et al. 2005, Marrie et al. 1994). Nonetheless, these environmental factors have never been studied using a reproducible medium, leaving variations that have been reported regarding their influence on *Lp* open for interpretation.

The past 40 years of *Legionella* research has allowed insight into the genetic basis of the mechanism of pathogenicity of this bacterium. In contrast, genes that allow successful and prolonged survival in aquatic systems have been largely ignored. Therefore, this dissertational work aims to bridge the existing knowledge gap in two ways; 1) identifying important environmental factors influencing (positively or negatively) the survival of *Lp* in water using a reliable and reproducible freshwater model, 2) identifying and characterizing regulatory pathways that determine the successful survival of *Lp* when faced with the nutritionally meagre water environment and when challenged with exogenous insults such as oxidative stress.

In a more global perspective, the work presented here pushes forward our understanding of water-borne pathogens in general. I present a working model to study how freshwater microorganisms adapt to their environment. As such, *Lp* has been proposed as a model organism for studying the host immune response to intracellular bacterial infection, vesicular transport, studying the evolution of bacterial pathogens and bacteria-amoeba interactions (Comas 2016, Harb et al. 2000, Mascarenhas and Zamboni 2016). Through this work, I also re-enforce *Legionella* as a suitable model organism to study the most important survival mechanisms employed by water-borne pathogens. Given its persistence in anthropogenic water systems, I believe it is also an excellent model to study the persistence of water-borne bacteria in general. Genetic elements that are crucial for survival in water have been understudied for many pathogens. In support of this, Dr. Faucher's lab has already conducted seminal work to identify genes which are critical for water survival (Li and Faucher 2016, Li et al. 2015, Trigui et al. 2014).

CHAPTER 2: Literature Review

2.1 Disease Characteristics and Epidemiology

The transmission of *Legionella* occurs through aerosols that are emitted from various water distribution systems, with the exception of *L. longbeachae* whose method of transmission remains unclear (Borella et al. 2005a, WHO 2007, Zimmerman 1979). Inhalation of *Legionella*-contaminated aerosols by susceptible individuals grants the bacterium access to the lower respiratory tract where it can infect and grow inside alveolar macrophages by evading the endocytic pathway, thereby causing Legionnaires' disease (LD) or Pontiac fever (PF), covered by the umbrella term Legionellosis (Fields et al. 2002, Prashar and Terebiznik 2015). Risk factors for contracting LD is similar to those of other community acquired pneumonia (Torres et al. 2013). An increased incidence of LD is well established among older individuals, with a higher infection rate in males compared to females (Phares et al. 2007, Principe et al. 2017, Torres et al. 2013).

LD is a severe and life-threatening atypical pneumonia. In addition to inflammation of the lower respiratory tract, atypical pneumonia distinguishes itself from its typical counterpart by extra-pulmonary involvement of other organs, producing symptoms that include headaches, diarrhea, mental confusion, bradycardia and, rarely, renal failure (Cunha 2006, Cunha et al. 2016, Phin et al. 2014). Early treatment of LD with macrolides, tetracyclines, ketolides or quinolones can successfully control disease progression (Cunha et al. 2016, Phin et al. 2014). In contrast to LD, PF causes a mild pathology, but has a higher attack rate and shorter incubation time (36 hours *vs.* 7-10 days) (Muder et al. 1986, Tossa et al. 2006). It is a self-limiting, flu-like illness that often resolves without recourse to antibiotic therapy (Mercante and Winchell 2015, Tossa et al. 2006). Two schools of thought debate the cause of PF; one argues that it is a mild form of LD, and the other affirms that it is a distinct illness caused by the inhalation of the bacterium in the viable-but-non-culturable (VBNC) state (section 2.6.2), inhalation of *Legionella*-containing amoeba or by excessive endotoxin production (Edelstein 2007, Mercante and Winchell 2015). A recent study compared two *Legionella* isolates, one involved in PF and another in LD with the goal of teasing out differences between bacteria causing the respective pathology (Wang et al. 2015). The authors report that the PF-associated strain has a significantly lower growth rate in human cells, and is non-motile compared to the LD-causing strain. While it is a compelling report that is the first to perform such a

comparative characterization, it is important to be cognizant that the PF strain was isolated from an environmental source, and the LD strain, from a patient. As such, the former strain has not been definitively proven to be the cause of PF; it has merely been isolated from an infection source.

Most LD cases are attributed to *Lp* infection (Lesnik et al. 2016, Mercante and Winchell 2015, Ng et al. 2008), despite a slew of sero-epidemiological studies reporting exposure of human subjects to a variety of *Legionella* species and serogroups (Mercante and Winchell 2015). The discrepancy between disease burden and environmental exposure can be explained in two ways: 1) Some members of the *Legionellae* family are inherently more virulent to the human host or 2) The overrepresentation of certain members as disease causing agents can be attributed to their capacity to colonize and successfully withstand the harsh water distribution systems. It is likely that both factors contribute to produce a successful *Legionella* strain. In support of the first hypothesis, an 18-year study originating from the United Kingdom reports that *Lp* dominates clinical isolates, while representing less than 50% of environmental isolates (Harrison et al. 2007). A French study found that while *Lp* dominated both the environmental and clinical isolates, *Lp* serogroup 1 was disproportionately identified in clinical samples (Doleans et al. 2004). A Puerto Rican study testing various water sources found that different test sites were dominated by specific *Legionella* species; *L. gormanii* in pristine river water and *L. micadadei* in saline environments (Ortiz-Roque and Hazen 1987). This observation supports the axiom that some *Legionella* species may be better adapted to surviving anthropogenic water systems; thereby, explaining the overwhelming dominance of *Lp* in human disease. *Lp* is also highly represented in water samples originating from modern water distribution systems (Borella et al. 2005b, Doleans et al. 2004, Kao et al. 2013, Kruse et al. 2016). It is, however, prudent to keep in mind that the isolation and detection methods used favour *Lp*, especially in the clinical setting; thereby, under-representing other species.

Like other community acquired pneumonias (CAPs), LD is seasonal, with the highest incidence occurring during late summer to early fall (Cilloniz et al. 2017). This hints to the conditions that favour the growth of *Legionella* in water systems. While the etiological agent of up to 70% of CAPs remains unidentified, *Lp* is estimated to be the cause of 4-28% of the remaining bacterial and viral CAPs (Borella et al. 2005a, Mercante and Winchell 2015, Restrepo and Anzueto 2005, Rusin et al. 1997). An early study used a guinea pig model to

isolate naturally-occurring *Legionella* strains from a North Carolina lake receiving continuous warm water from a nuclear plant (Fliermans et al. 1981). Interestingly, this year-long study observed that when guinea pigs were infected with an equivalent inoculum, higher infection rates were observed during the months of May, June and July (Fliermans et al. 1981). More recent studies have found that *Lp* populations thrive especially during warmer months and that natural populations are affected by anthropogenic activity (Kao et al. 2015, Parthuisot et al. 2010, Wery et al. 2008).

2.2 Physiology and Growth Requirements

Legionella are aquatic, rod-shaped microorganisms of approximately 0.3-0.9 μm width and 2-20 μm length (Fliermans 1996). Several artificial media support the growth of *Legionella*; however, buffered charcoal yeast extract (BCYE/CYE) agar is the most successful and widely used media (Devos et al. 2005, Mercante and Winchell 2015). *Legionella* are fastidious, chemoorganotrophic bacteria, utilizing amino acids as their major source of carbon and energy (Fliermans 1996, Price et al. 2014, Tesh et al. 1983). Members of the *Legionellaceae* family are unable to reduce nitrate or use ammonium as a nitrogen source (Keen and Hoffman 1984). CYE medium calls for cysteine supplementation, one of the amino acid auxotrophies of *Legionella*; however,, *L. oakridgensis, L. jordanis* and *L. nagasakiensis* are known to lose their cysteine auxotrophy upon serial passaging (Mercante and Winchell 2015). Interestingly, *Francisella tularensis* is one of few other bacteria that have a reported cysteine requirement similar to *Legionella* (Edelstein 2007, Price et al. 2014). Moreover, the bacterium is an auxotroph for seven amino acids, and acquires them from the host cell (Eylert et al. 2010, Fliermans 1996, Pine et al. 1979, Price et al. 2014, Tesh et al. 1983).

Under laboratory conditions, various *Legionella* species and isolates has been shown to replicate *in vitro* at temperatures between 17-51.6^{o}C, with the optimal growth temperature being approximately 37^{o}C (Kusnetsov et al. 1996, Söderberg et al. 2004, Yee and Wadowsky 1982). Nonetheless, cell growth is significantly reduced at temperatures above 45^{o}C (Kusnetsov et al. 1996). While lab-adapted *Legionella* spp. produce isolated colonies in three days at 37^{o}C, environmental *Legionella* may grow more slowly, taking up to 14 days to form colonies, making detection and isolation difficult. Some *Legionella* species isolated from groundwater wells were found to only grow on CYE at 30^{o}C, indicating that common usage of

37°C as the incubation temperature for isolation may exclude some environmental species (Riffard et al. 2001). Similarly, an earlier study also reports *Legionella* strains originating from a hot water tank grew better at 37°C and 45°C, but not at 25°C (Stout et al. 1985). In contrast a strain that was isolated from a cold water system only produced colonies efficiently at 25°C, but not at 37°C or 45°C (Stout et al. 1985). While current standardized isolation methods require incubation of selective media at 37°C (Dunne Jr et al. 2017), the aforementioned data highlights the importance of factoring the temperature of the water source when isolating *Legionella* from the environment. Yet another adversity to culturing environmental *Legionella* is variation in colony morphology; while most species form white colonies with a blue-violet hue, some species are described as being pigmented yellow-green, blue-white or red-pink (Mercante and Winchell 2015).

2.3 Natural Environment of Epidemiologically Relevant *Legionella*

Since its discovery and isolation in the late 1970's there has been a vested interest in identifying the ecological niches of legionellae. A diverse array of natural aquatic sources harbours members of the *Legionella* genus. A variety of *Legionella* species have been isolated and/or detected using culture-independent methods from water sources ranging from rivers, lakes, hypersaline lakes, ground water, hot springs, polar lakes, ocean water, estuarian water and sewage (Carvalho et al. 2008, Fliermans et al. 1981, Gast et al. 2011, Lin et al. 2007, Ortiz-Roque and Hazen 1987, Pagnier et al. 2012, Palmer et al. 1993, Riffard et al. 2001). In Quebec, members of the *Legionellaceae* family were detected in 43% of freshwater samples tested (Joly et al. 1983). As such, *Legionella* are considered to be ubiquitous in freshwater sources, albeit at low concentrations, representing less than 1% of the total bacterial population (Borella et al. 2005a, Fliermans et al. 1981). Their widespread presence in natural waters inadvertently introduces *Legionella* into water used for human consumption and activities. Even though several other pathogenic *Legionella* species have been recovered from soils, *L. longbeachae* is the only species whose mode of infection is directly associated to potting soils and compost mixes (Conza et al. 2014, Currie and Beattie 2015, Koide et al. 1999, Schalk et al. 2014, Steele et al. 1990, Travis et al. 2012, van Heijnsbergen et al. 2014, van Heijnsbergen et al. 2015, van Heijnsbergen et al. 2016). Notwithstanding the increasing awareness about non-*pneumophila* species contributing to disease burden, *Lp* remains the major public health

concern in most parts of the world, including Canada (Campèse et al. 2015, Ng et al. 2008, Phares et al. 2007, Yu et al. 2002).

2.4 Taxonomy

Members of the *Legionella* genus are ɣ-proteobacteria belonging to the Legionellaceae family of the Legionellales order (NCBI Taxonomy Database). With over 50 species identified to-date, *Lp* is the most widely studied thus far, mainly due to its dominance as the etiological agent in the vast majority of reported Legionnaires' disease cases (Diederen 2008, DSMZ 2015, Yu et al. 2002). Nonetheless, *L. longbeachae* causes a considerable number of LD cases in Australia, New Zealand, Japan and Thailand (Isenman et al. 2016, Li et al. 2002, Phares et al. 2007, van Heijnsbergen et al. 2015, Whiley and Bentham 2011). Cases of LD attributed to *L. longbeachae* are garnering more attention in North America and Europe as well (Martin et al. 2016, Picard-Masson et al. 2016, Potts A 2013). Other species commonly associated with disease include *L. micdadei, L. bozemanae* and *L. dumoffii* (Burillo et al. 2017, Mercante and Winchell 2015).

Identifying a new species involves a combination of morphological characteristics, fatty acid and ubiquinone profiles, protein analysis, DNA hybridization patterns, rRNA restriction patterns, serological testing, as well as DNA sequence analysis (Benson et al. 1991, Brenner et al. 1979, Grimont et al. 1989, Lo Presti et al. 2001, Saunders et al. 1988, Wilkinson et al. 1990). The ease and low cost of sequencing technology has led to the use of 16S rRNA, 5S rRNA, the spacer between the 23S and 5S rRNA genes, and the Mip gene, among others, as the basis for molecular typing of *Legionella* species and subgroups (Cloud et al. 2000, Hayden et al. 2001, Lück et al. 2010, Ratcliff et al. 1998, Yang et al. 2010). As the primary cause of disease world-wide, serogrouping of *Lp* is also commonly used, with serogroups 1 (responsible for more than 75% of disease) and 6 causing the most cases of LD (Burillo et al. 2017, Mathys et al. 2008, Mercante and Winchell 2015). The Lag-1 gene codes for a virulence epitope of LPS and serves as the basis for traditional serogrouping (Zou et al. 1999). Serogroup 1 is recognized by monoclonal antibody 2 (Mab2) or Mab3/1 of the international and Dresden monoclonal panels respectively (Burillo et al. 2017, Mercante and Winchell 2015). LPS modification is strongly associated with clinical disease and predominates in outbreak strains, but is less frequently found in environmental *Lp* serogroup 1 (Lp1) isolates (Mercante and

Winchell 2015). More recently, Sequence Based Typing (SBT) is becoming the accepted method of classifying *Lp*1 clinical and environmental isolates (Gaia et al. 2003, Ginevra et al. 2009, Lévesque et al. 2016, Montagna et al. 2016, Ratzow et al. 2007). SBT is based on Multi-locus sequence typing (MLST) that was first used to type *Streptococcus pneumoniae,* which sequences and compares 7 housekeeping genes (Gaia et al. 2003). The European Working Group for *Legionella* Infections (EWGLI) pioneered the development of SBT on the heels of a large scale study evaluating the genotypic methods used for *Lp* typing (Fry et al. 1999). SBT was proposed and is currently used as a simpler and more rapid typing method, which uses a combination of 7 genes (*flaA, pile, asd, mip, mompS, proA* and *neuA*), some of which are under selective pressure, while others are not (Gaia et al. 2005, Gaia et al. 2003, Ratzow et al. 2007).

2.5 Contamination of Anthropogenic Water Systems by *Legionella*

Despite their ubiquity in natural waters (80% detection rate in freshwater sources), *Lp* infections have only been reported from man-made water systems (Borella et al. 2005a). It is widely believed that concentrations of *Lp* in natural water sources are too low to cause disease in humans, and that anthropogenic water distribution systems, once contaminated, allow for optimal survival and growth conditions for *Lp* to replicate to titers that cause human disease in susceptible individuals (Marrie et al. 1992). Therefore, water distribution systems in built environments remain the main concern for the spread of legionellosis. Reported reservoirs and sources of *Legionella* infection include, but are not limited to, cooling towers (Essig et al. 2016, Maisa et al. 2015, Scaturro et al. 2015), spa pools (Coetzee et al. 2012, Euser et al. 2010, Kuroki et al. 2017, Sánchez-Busó et al. 2015), water fountains (Haupt et al. 2012, O'Loughlin et al. 2007, Smith et al. 2015), humidifiers (Moiraghi et al. 1987, Moran-Gilad et al. 2012, Yiallouros et al. 2013), misters (Barrabeig et al. 2010, Mahoney et al. 1992), potable water systems on naval vessels (Ahlen et al. 2016, Goutziana et al. 2008), domestic hot water systems linked to taps or showers (Alary and Joly 1991, Dufresne et al. 2012, Erdoğan and Arslan 2016), and hospital hot water distribution systems (Bédard et al. 2016b, Brûlet et al. 2008, Oren et al. 2002). In general, *Legionella* contaminations are commonly associated with hot water systems (Borella et al. 2005a).

Between 6-32% of residential waters are reportedly contaminated with *Legionella*, the occurrence varying depending on location and type of home (Arnow et al. 1985, Borella et al.

2004, Mathys et al. 2008, Stout et al. 1992, Straus et al. 1996). An interesting case study is that of a domestic water sampling from single-family home dwellings in Quebec City which concluded that 32% of homes were *Legionella* positive (Alary and Joly 1991). Importantly, the study is one with a high sample size (211 homes tested). This high level of contamination was correlated to the use of electric water heaters (instead of gas or oil heaters), which allow sections of cooler water within the tank, providing a suitable niche for *Legionella* replication. This characteristic of electric heaters, and its subsequent effect on *Legionella* counts, was corroborated in other studies (Dewailly and Joly 1991, Lee et al. 1988). Larger structures such as water distribution systems, hot water tanks and cooling towers of hospitals, public buildings and hotels are more frequently contaminated with *Legionella* than domestic water systems: contamination rates ranging from 20 to 94% in the establishments tested have been reported (Alary and Joly 1992, Borella et al. 2005b, Ikedo and Yabuuchi 1986, Kruse et al. 2016, Leoni et al. 2005, Lück et al. 1993, Marrie et al. 1994, Mouchtouri et al. 2007, Vickers et al. 1987). The relatively low contamination rates observed in residential plumbing compared to larger infrastructure have been attributed to sedimentation allowing a niche for *Legionella* growth, and temperature stratification that is more common within large systems (Alary and Joly 1992, Bédard et al. 2016a, Flannery et al. 2006, Schwake et al. 2016, Vickers et al. 1987). In fact, the higher rates of *Legionella* positive samples in some of the domestic water samples are partially attributed to sampling apartment buildings, with larger water distribution systems, compared to single family dwellings.

Undoubtedly, contaminated aerosols generated by man-made water systems, whether domestic or industrial, is the vehicle for *Legionella* that causes LD and Pontiac fever. Higher concentrations of culturable *Legionella* have been reported in anthropogenic systems compared to the original water sources feeding them (Buse et al. 2012, Lesnik et al. 2016, Stout et al. 1992). This data can be interpreted in two ways; either the man-made systems allow proliferation of *Legionella* at concentrations below the detection limits used in these studies, or engineered systems promote culturability by providing favorable conditions for *Legionella*. A recent German study found that *Lp* numbers increased in domestic water samples compared to source water, while total bacterial count dropped, suggesting that the physical and chemical treatments that were used in these water systems reduced the general bacterial load allowing *Lp* to thrive (Lesnik et al. 2016). *L. anisa, L. dumoffii* and *L. micadadei* are pathogenic species

of *Legionella* that have only been found in engineered water systems, raising the possibility that these particular species have adapted to man-made systems (Buse et al. 2012). Alternatively, it is possible that conditions found in water distribution systems favour the growth of *Lp* on standard media, while conditions found in the natural environment repressed culturability, inducing a viable-but-non-culturable (VBNC) state (See Section 2.6.2). These data hint that water distribution systems provide the conditions favourable for *Legionella* growth, where it is known to proliferate to levels that allow human colonization.

2.6 Life Cycle

In its natural water environment, proliferation of *Legionella* occurs when the bacterium parasitizes eukaryotic host cells, multiplying intracellularly (Declerck et al. 2007). *Legionella* can infect and successfully multiply within freshwater protozoa, namely *Acanthamoeba* spp., *Naegleria* spp., and *Hartmenella* spp. (Abu Kwaik et al. 1998, Borella et al. 2005a, Fields 1996). Other natural hosts include ciliated protozoa, the slime mold *Dictyostelium discoideum* and the soil nematode *Caenorhabditis elegans* (Brassinga et al. 2010, Fields et al. 1984, Hägele et al. 2000, Watanabe et al. 2016). In the human host, *Legionella* can infect alveolar macrophages, but also alveolar epithelial cells to a lesser degree (Chandler et al. 1977, Chiaraviglio et al. 2008, Garduño et al. 1998b, Horwitz and Silverstein 1981, Prashar et al. 2012, Winn Jr 1988). The bacterium can also successfully infect primate macrophages and guinea pigs, but not mice with the exception of the A/J strain (Kishimoto et al. 1979, Kishimoto et al. 1981, Winn Jr 1988, Yamamoto et al. 1988). Amino acid substitutions in the Naip5 gene of the A/J strain impair the ability of macrophages to recognize intracytoplasmic flagellin, which is essential to mount a successful pro-inflammatory immune response, making A/J mice susceptible to *Legionella* infection (Diez et al. 2003, Molofsky et al. 2006).

Since *Legionella* can grow on laboratory media, it has been termed a facultative intracellular parasite. Given its capacity to grow on artificial media, some authors have alluded to the possibility that *Legionella* may be able to grow planktonically in nature if given sufficient nutrients (Steinert et al. 2002, Temmerman et al. 2006); however, no reports have convincingly shown this to be true. In fact, in the rare event that sufficient nutrients are available in an aquatic environment, members of the microbial community with a more rapid

doubling time would overtake the *Legionella* population. Thus, the data at hand would support the theory that legionellae exhibit strictly intracellular multiplication in nature.

In the nutrient-poor water environment, *Legionella* can also exist as planktonic, free-living cells or as part of a microbial community in biofilms (Fig 2.1) (Steinert et al. 2002). As a response to environmental stresses, *Legionella* are also able to form VBNC cells (Fig 2.1) that do not form colonies on standard culture medium (Li et al. 2014).

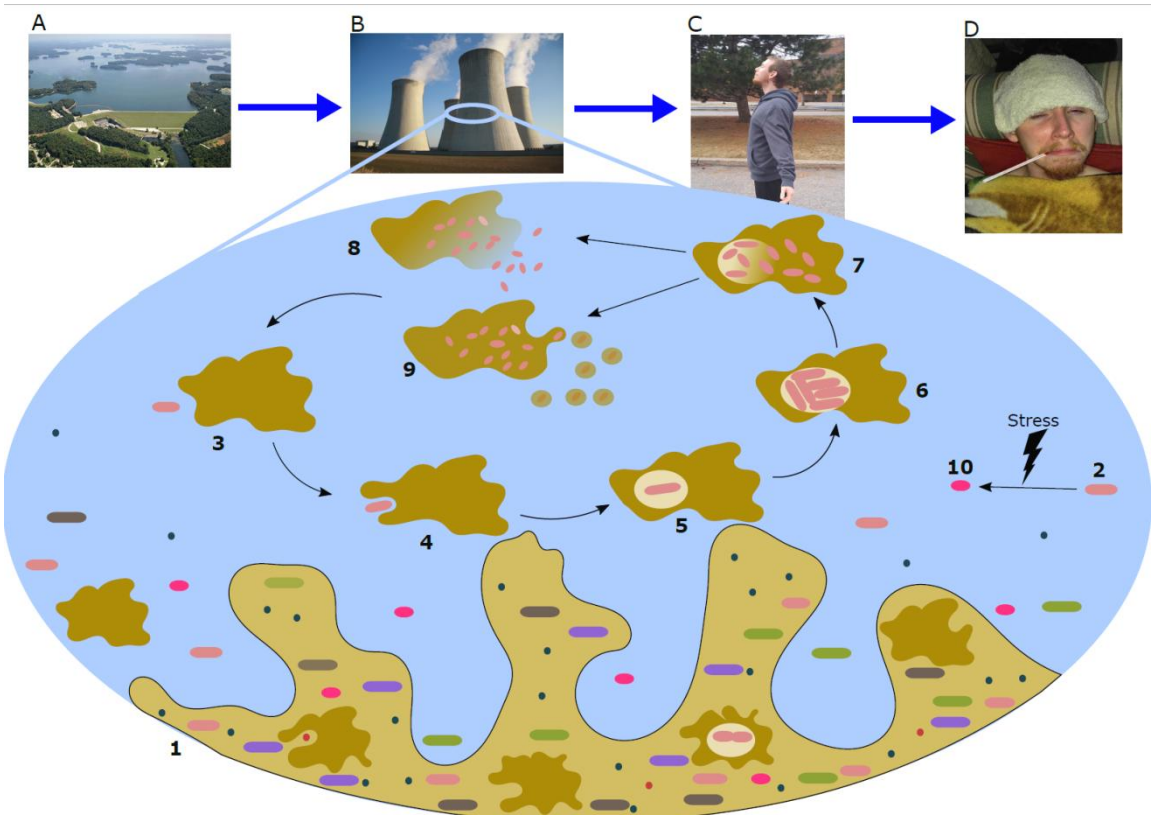

Figure 2.1: Overview of the *Legionella* life cycle in water. *Legionella* are found in many natural water sources (A) which are used to feed anthropogenic water distribution systems (ex: cooling towers) (B). When contaminated aerosols emitted by water distribution systems are inhaled by susceptible individuals (C), the bacterium can infect and replicate inside alveolar macrophages causing disease (D). In water, *Legionella* (pink rods) can exist within multispecies biofilm (1) or as planktonic cells (2). When the bacterium encounters a host amoeba (3), it gets internalized (4), after which the bacterium resides in a modified vacuole (5), termed the *Legionella* containing vacuole (LCV). Within this protected niche, *Legionella* replicates (termed the replicative phase) until host nutrient sources are depleted (6). Upon sensing nutrient deprivation, *Legionella* will enter the transmissive phase (7), where cells are virulent and stress resistant. They can further differentiate into mature infection forms (MIFs) before being released from the host. *Legionella* escapes from the host cell through host cell lysis (8) or is excreted inside host cell vesicles (9). *Legionella* can also enter the viable-but-non-culturable (VBNC) cells (bright pink rods) under some conditions (10).

2.6.1 Biofilm

Persistence of *Legionella* within biofilms is proposed as a major reason for the ubiquity of this bacterium in water distribution systems despite various disinfection measures (Abdel-Nour et al. 2013, Mérault et al. 2011, Rogers et al. 1994, Valster et al. 2010). As can be expected, biofilms pose a physical barrier for disinfectants within water distribution systems. There are also reports of bacteria gaining resistance to environmental stresses within biofilms, thereby contributing to *Legionella* persistence (Fux et al. 2005, Sanderson and Stewart 1997). Multiple heat shock and chemical disinfectant methods have been shown to be ineffective to kill biofilm-embedded *Legionella* (Falkinham et al. 2015, Farhat et al. 2012, Gião et al. 2009). According to some studies, repeated chlorination was found to be ineffective at eliminating biofilm embedded *Lp* strains when water systems were monitored between 3 months to 2.5 years (Cooper and Hanlon 2010, Cooper et al. 2008). Moreover, biofilm-derived bacteria were found to be more resistant to iodine than planktonic cells in water (Cargill et al. 1992).

Legionella are found within biofilms, and often at much higher concentrations than in the planktonic state (Borella et al. 2005a, Mercante and Winchell 2015, Rogers et al. 1994, Steinert et al. 2002). The substrata upon which the biofilm is formed greatly influences the degree of *Legionella* colonization; however conflicting reports suggest that multiple variables affect the establishment and shedding of *Legionella* from biofilm (Abdel-Nour et al. 2013, Buse et al. 2014, Rogers et al. 1994, Vervaeren et al. 2006). The bacterium has been shown to colonize pre-existing biofilm within 24 hours (Rogers et al. 1994). Freshwater amoeba feeding on biofilm bacteria also cohabit this environment, thereby serving as host cells in which *Legionella* can multiply (Mercante and Winchell 2015, Murga et al. 2001, Rogers et al. 1994, Valster et al. 2010). As such, one study found that increase in biofilm mass triggered *Legionella* growth within a model water system (van der Kooij et al. 2005). *Legionella* spp. have also been found in floating biofilm, commonly found in man-made water systems, where they can infect resident protozoa (Declerck et al. 2007, Hsu et al. 2011).

While this aquatic bacterium tends to colonize pre-formed multispecies biofilms (Abdel-Nour et al. 2013), the ability of *Legionella* to form biofilm itself has also been investigated (Hindré et al. 2008, Mampel et al. 2006, Pécastaings et al. 2010, Pécastaings and Roques 2013). Bacteria released from amoeba were found to have an enhanced capacity for polysaccharide production and self-aggregation, suggesting that environmental species may

contribute to biofilm production more significantly than previously reported (Bigot et al. 2013).

2.6.2 Viable-but-nonculturable (VBNC) status

A major concern for environmental bacteria detection is the existence of viable-but-non-culturable (VBNC) cells (Li et al. 2014). Culturing on bacteriological media still remains the gold-standard and most common method for detecting *Legionella* (Dunne Jr et al. 2017). Formation of VBNC *Legionella* has been reported in response to long-term exposure to nutrient-poor conditions (Hussong et al. 1987, Paszko-Kolva et al. 1992), disinfectants (Alleron et al. 2008, García et al. 2007) and various temperatures (Al-Bana et al. 2014, Hussong et al. 1987). One study found that culturability of *Legionella* decreased at water temperatures below 20^{o}C (Colbourne et al. 1988). In line with this finding, one group found that incubating water samples at 5^{o}C affected CFU counts (Wadowsky et al. 1985), while another group reported a similar effect at 4^{o}C (Hussong et al. 1987). In the latter study, cells were successfully resuscitated by infecting chick embryos (Hussong et al. 1987). VBNC cells produced by monochloramine treatment were shown to actively synthesize proteins, albeit at extremely low levels, and were metabolically active for over 4 months (Alleron et al. 2013, Alleron et al. 2008). When resuscitation was attempted by co-culturing with *Acanthamoeba castellanii*, only those VBNC cells produced with a treatment of less than 1mg/ml were recovered (Alleron et al. 2008). In another instance, *Legionella* was induced into the VBNC state by incubating the bacterium in water at 20°C for 125 days (Steinert et al. 1997). These VBNC cells were also recovered by *A. castellanii* infection (Steinert et al. 1997). Finally, chlorine treatment is also reported to cause entry into VBNC state, while infection of *A. polyphaga* recovered culturability (García et al. 2007). Only one report found that heat shock resuscitated VBNC cells naturally found in water (Colbourne et al. 1988).

2.6.3 *Legionella* growth *in vivo* and *in vitro*

Intracellular pathogens are inherently challenged with adaptation to often wildly different environments: the host cell and extracellular niche(s). As a result, many pathogens are known to undergo changes in gene expression and morphology that allow them to successfully adapt to a given environment.

Legionella exhibits a dimorphic lifestyle (Fig 2.1), switching between the replicative phase and the transmissive phase (Molofsky and Swanson 2004). The former is characterized by rapid growth during nutrient abundance, resistance to sodium, lack of motility and general sensitivity to stress (Byrne and Swanson 1998, Forsbach-Birk et al. 2004, Hammer et al. 2002). Cells enter the transmissive phase upon sensing nutritional stress, becoming salt sensitive, acquiring resistance to various stresses (heat, oxidative stress, acid stress), becoming motile as well as cytotoxic towards host cells, and expressing a variety of virulence regulators/genes that allow *Legionella* to become infectious and evade the host endosomal pathway (Byrne and Swanson 1998, Faucher et al. 2011, Forsbach-Birk et al. 2004, Hammer et al. 2002). In rich broth, the replicative state is mimicked by the exponential (E) phase, and the transmissive state by the post-exponential (PE)/stationary phase. Despite some similarities, gene expression is not identical between the *in vivo* and *in vitro* lifestyles (Bruggemann et al. 2006, Faucher et al. 2011). These transcriptomic changes manifest themselves to produce one of the best studied differences between *in vitro* and *in vivo* cells; the formation of the mature infectious form (MIF), which is exclusive to the intracellular transmissive phase (Garduño et al. 2002). Compared to stationary phase cells, MIFs are shorter, possess a distinct cell wall structure, are more resistant to alkaline pH, antibiotics and detergents, and are 10 times more infectious toward host cells (Faulkner and Garduño 2002, Garduño et al. 2002, Koubar et al. 2011). Intracellular growth has also been shown to enhance the bacterium's ability to resist acid and oxidative stress compared to stationary phase cells. However, stationary phase cells are equally heat resistant, despite increased membrane associated heat shock proteins (Garduño et al. 2002). Regulators that control transition to the stationary phase *in vitro* are important for the successful production of MIFs *in vivo* (Faulkner et al. 2008). Additionally, the *magA* gene, whose expression is highly upregulated in MIFs, is also upregulated in stationary phase, albeit to a lesser extent (Hiltz et al. 2004). The aforementioned evidence would suggest that stationary phase cells and MIFs are considered to be part of a continuum of transmissive phase bacteria; each one fine-tuning its morphology and characteristics in response to the *in vitro* or intracellular environment. Therefore, *Legionella* cultured to the E or PE phase are often used as a proxy for the study of the replicative or the transmissive phase respectively (Byrne and Swanson 1998, Hammer and Swanson 1999, Hammer et al. 2002, Lynch et al. 2003).

ppGpp and the stringent response (see section 2.8.1 The Stringent Response (SR)) has been conclusively linked to the transition between the replicative and transmissive phases (Bachman and Swanson 2001, Dalebroux et al. 2009, Hammer and Swanson 1999, Tiaden et al. 2007). Similarly to *E. coli*, *Legionella* accumulates ppGpp upon entry into the PE phase in response to amino acid depletion (Hammer and Swanson 1999) and fatty acid depletion (Dalebroux et al. 2009). The initiation of the SR by the ppGpp alarmone has been proven to be critical for differentiation in broth, as well as in macrophages (Dalebroux et al. 2009, Dalebroux et al. 2010, Hammer and Swanson 1999). Two other regulatory elements are involved in the bi-phasic lifestyle of *Legionella*: the alternative σ factor RpoS, which responds to ppGpp, and the LetA/S two-component system, which initiates the LetA/S-RsmYZ-CsrA cascade (see section 2.8.3) (Molofsky and Swanson 2004). Absence of RpoS or the LetA/S two-component system impairs the ability of cells to transition into MIFs and causes digestion by the host (Berk et al. 2008, Faulkner et al. 2008). *rpoS* and *letA/S* mutants also show impaired virulence and fail to exhibit other transmissive phase traits such as motility, stress resistance, pigment production and sodium sensitivity (Bachman and Swanson 2001, Bachman and Swanson 2004a, Hammer et al. 2002, Sahr et al. 2009, Zusman et al. 2002). Both the RpoS and LetA/S regulons have been linked to the SR, making ppGpp the master orchestrator of *Legionella*'s bi-phasic lifestyle.

2.6.3.1 Intracellular growth

2.6.3.1.1 Entry

Both human and protozoan host cells internalize *Legionella* through conventional or coiling phagocytosis (Bozue and Johnson 1996, Horwitz 1984). Internalization by phagocytes is facilitated by complement and antibodies (Horwitz and Silverstein 1981, Husmann and Johnson 1992, Payne and Horwitz 1987, Rechnitzer and Blom 1989); however, phagocytosis is also observed in the absence of serum (Newton et al. 2010, Weissgerber et al. 2003). Lectin-binding receptors, pili and the heat shock protein Hsp60 are also involved in non-opsonic entry into mammalian cells (Garduño et al. 1998a, Gibson III et al. 1994, Stone and Kwaik 1998, Weissgerber et al. 2003). In the case of protozoan hosts, phagocytosis is initiated by binding to receptors that vary from host to host (Abu Kwaik et al. 1998, Harb et al. 1998). A Gal/GalNAc

receptor was found to mediate entry in *H. vermiformis*; however, the host-cell binding moiety seems to vary in other amoeba and are yet to be determined (Venkataraman et al. 1997).

2.6.3.1.2 Establishing the *Legionella* Containing Vacuole (LCV)

Despite variations in the mechanisms of host cell attachment, intracellular replication of *Legionella* is remarkably similar between human cells and amoeba (Abu Kwaik 1996, Gao et al. 1997). After entry, *Legionella* resides and multiplies within a modified phagosome, namely the *Legionella* containing vacuole (LCV) (Horwitz 1983a). The LCV closely associates with smooth vesicles and mitochondria on its cytoplasmic surface starting at 15 minutes post-infection; after four hours of infection, these structures are less frequent and are replaced by rough vesicles and host ribosomes (Abu Kwaik 1996, Horwitz 1983a, b, Tilney et al. 2001). *Legionella* actively recruits host cell organelles, as formalin-killed bacteria did not associate with ribosomes, vesicles or mitochondria (Horwitz 1983a). In fact, within 15 minutes of infection, the lipid composition of the LCV membrane resembles that of the endoplasmic reticulum (ER) more closely than the plasma membrane (Tilney et al. 2001).

The typical endocytic pathway recruits host factors Rab5 and early endosome antigen 1 (EEA1) to the newly formed phagosome, followed by Rab7, lysosome-associated membrane glycoproteins (LAMPs), cathepsin D and acid hydrolases (Newton et al. 2010). The cumulative effect of this sequential host factor recruitment is the induction of phagosome-lysosome fusion resulting in degradation of the enclosed bacteria. Within the LCV niche, *Legionella* evades the conventional endocytic pathway and avoids phagolysosome formation (Bozue and Johnson 1996, Horwitz 1983b). As a result of these host cell manipulations, bacterial replication is initiated around 4-8 hours post-infection with a doubling time of 2 hours (Abu Kwaik 1996, Abu Kwaik et al. 1998, Horwitz and Silverstein 1980). Hundreds of *Legionella* accumulate within the LCV during this replication period (Molmeret et al. 2004a).

Paramount to the orchestration of host cell processes and subsequent replication by *Legionella* is a type IVB secretion system, Dot/Icm. The two **i**ntra**c**ellular **m**ultiplication (Icm) and **d**efective in **o**rganelle **t**ransport (Dot) loci encode the type IVB secretion system of *Legionella*, a major virulence factor that has been identified and studied to date (Marra et al. 1992, Sadosky et al. 1993, Segal et al. 1998). The Dot/Icm genes are related to the Tra genes of the Inc ColIb-P9 plasmid of *Shigella flexneri* (Christie and Vogel 2000, Vogel et al. 1998).

Interestingly, mutations within this system rendering it inactive and causing virulence defects are also accompanied by salt resistance (Vogel and Isberg 1999, Vogel et al. 1996). Having originated from plasmid transfer systems, Type IV secretion apparatus are capable of secreting plasmids, oncogenic DNA and proteins (Christie and Vogel 2000, Segal et al. 2005, Vogel and Isberg 1999). Indeed, the Dot/Icm machinery has also been proven to be used for conjugation (Segal et al. 1998, Vogel et al. 1998). It is also implicated in bacterial entry and early phagosome biogenesis (Hilbi et al. 2001, Roy et al. 1998, Wiater et al. 1998). At high multiplicities of infection (MOIs), *Legionella* initiates contact-dependent cytotoxicity towards mammalian cells, another virulence phenotype that has been linked to the Dot/Icm secretion system (Kirby et al. 1998).

More importantly, *Legionella* uses the type IVb secretion system to inject over 300 effector proteins into the host cell cytoplasm resulting in the manipulation of various host cell mechanisms (Gomez-Valero et al. 2011, Isaac and Isberg 2014). This results in the recruitment of the aforementioned host cell organelles to the LCV, arrest of the endocytic pathway and delayed acidification of the replication vacuole providing a safe niche for the bacterium to replicate (Sturgill-Koszycki and Swanson 2000). Effector proteins have similarity to eukaryotic genes, are thought to have been acquired through horizontal gene transfer, and play a variety of roles that allow successful replication of *Legionella* (Cazalet et al. 2004, Ge and Shao 2011, Ninio and Roy 2007). For example, VipA and VipD inhibit trafficking of lysosomal vacuoles (Shohdy et al. 2005); RalF recruits host cell GTPases that favour ER vesicle transport to the LCV (Nagai et al. 2002); SidM also recruits a host factor that hijacks the vesicular trafficking between the Golgi and ER (Machner and Isberg 2006); SidJ is also involved in recruiting ER vesicles (Liu and Luo 2007); SdhA and SidF prevent the premature apoptosis of the host cell allowing the bacterium to replicate (Banga et al. 2007, Laguna et al. 2006); and LepA/B are involved in the escape of *Legionella* from the host cell (Chen et al. 2004).

Multiple *Legionella* effectors target similar host pathways making Dot/Icm substrates highly redundant (Ge and Shao 2011, Ninio and Roy 2007). As such, mutations in a single effector rarely produce a significant intracellular multiplication defect (Nagai et al. 2002, Vanrheenen et al. 2006). This functional redundancy may be a result of gene duplication

followed by divergent evolution in response to the wide variety of host cells that *Legionella* is known to parasitize in its natural habitat (Ninio and Roy 2007).

In addition to the Icm/Dot system, *Legionella* species harbour a twin-arginine translocation (Tat) secretion system, type I, II and IVA secretion systems, all of which have been implicated in virulence (Lammertyn and Anné 2004). While a type I secretion system was found to be encoded by Lss genes in *Legionella* (Jacobi and Heuner 2003) and was hypothesized to transport an Rtx toxin (Lammertyn and Anné 2004), its involvement in virulence was only recently demonstrated (Fuche et al. 2015). Mutating structural genes within this secretion system produced an intracellular multiplication defect similar to a Dot/Icm mutation (Fuche et al. 2015). *Legionella* Tat genes were actively transcribed during growth inside amoebal host cells (De Buck et al. 2004). Mutations within the Tat secretion system have been shown to reduce virulence towards various mammalian and amoebal cell lines (De Buck et al. 2005, McCoy-Simandle et al. 2011, Rossier and Cianciotto 2005, Tyson et al. 2013). Notably, mutations within the Tat system were more pronounced during iron starvation conditions (Rossier and Cianciotto 2005). Working with the Sec/Tat systems, the type II secretion system is encoded by Lsp genes (*Legionella* secretion pathway) (Cianciotto 2005, Liles et al. 1998). The Lsp system, as well as the degradative enzymes that it transports, have been shown to aid in infection of macrophages and amoeba (Aragon et al. 2001, DebRoy et al. 2006, Hales and Shuman 1999a, Rossier et al. 2008, Rossier et al. 2004). A recent report found that the type II system aids in virulence by repressing cytokine production through the TLR-2 signaling pathway (Mallama et al. 2017, McCoy-Simandle et al. 2011).

A type IVA secretion system that resembled the VirB system of *Agrobacterium tumefaciens* is also encoded by *lvh* (*Legionella vir* homolog) genes (Segal et al. 1999). Given its relative similarity to the Type IVB system, some Lvh components are functionally redundant with the Dot/Icm genes for conjugation activity (Segal et al. 1999). While the Lvh secretion system was initially deemed dispensable for virulence in both mammalian and amoeba at 37^{o}C, deletion of *lvhB2* caused a drastic 100-fold reduction in the intracellular multiplication at 30^{o}C (Ridenour et al. 2003, Segal et al. 1999).

2.6.3.1.3 Metabolic Capacity of *Legionella*

Radio-labeling studies have established that the tricarboxylic acid (TCA) cycle is used by *Legionella* for energy production and for biosynthetic purposes (Eylert et al. 2010, Price et al. 2014, Tesh et al. 1983). *Legionella* uses amino acids as its primary source of carbon and energy. Indeed, a high-throughput substrate assay using 9 *Legionella* strains, including *Lp*, found that all strains metabolized amino acids and their derivatives most efficiently (Mauchline and Keevil 1991). Intracellular bacteria such as *F. tularensis* and *Listeria monocytogenes* acquire amino acids from the host cells (Alkhuder et al. 2009, Grubmüller et al. 2014). Similarly, when an amoeba is fed labeled amino acids, comparably labelled amino acid proportions are harvested from infecting *Legionella,* suggesting that the bacterium acquires the amino acids required for growth directly from the host cell (Schunder et al. 2014). More interestingly, the amino acid auxotrophies of *Legionella* overlap with that of its natural hosts; thereby, indicating that the bacterium may have evolved nutritional requirements with its host (Price et al. 2014).

The importance of amino acids for the growth of *Legionella* is evidenced in the numerous genes involved in amino acid acquisition; including 12 ABC transporters, permeases, peptidases and excreted proteases (Cazalet et al. 2004, DebRoy et al. 2006, Rossier et al. 2008). A transporter of threonine, an essential amino acid, was found to be crucial for *Legionella* growth in broth and inside host cells (Sauer et al. 2005). Interestingly, a knock-out strain of the threonine transporter induced premature post-exponential phase traits linking the acquisition of amino acids to cellular differentiation. Mutations of valine and threonine transporters also caused intracellular multiplication defects inside macrophages (Chen et al. 2008a, Fonseca et al. 2014). Finally, ArgR, a transcriptional repressor that responds to varying levels of arginine, is required for growth inside amoeba (Hovel-Miner et al. 2010). In addition to expression of bacterial proteins, *Lp* has been shown to recruit host cell transporters for the purpose of translocating host amino acids, significantly aiding in bacterial intracellular replication (Hoffmann et al. 2014, Wieland et al. 2005). Effector proteins are notoriously redundant, to the extent that deletion of one rarely results in an observable defect, which makes their characterization difficult. However, one well-studied effector is clearly indispensable for intracellular growth: AnkB (Al-khodor et al. 2008). This F-Box protein with eukaryotic domains recruits polyubiquitinated host proteins to the LCV which are subsequently degraded

to provide a pool of free amino acids for bacterial growth, further highlighting the importance of amino acid acquisition for growth *in vivo* (Price et al. 2009, Price et al. 2010, Price et al. 2011).

Despite carrying complete Embden-Meyerhoff and Entner-Doudoroff (ED) pathways, as well as an incomplete Pentose Phosphate Pathways (PPP) (Cazalet et al. 2004), early studies found conflicting evidence of the usage of sugars for energy by *Legionella* strains *in vitro*. Some reported that simple sugars were not utilized, but that *Legionella* did metabolize carboxylic acids including propionate, succinate and acetate (Mauchline and Keevil 1991). Others found that glucose was used slowly through the ED and the PPP, instead of the glycolytic pathway (Tesh et al. 1983). *In silico* analysis revealed that the *Legionella* genome coded for over 50 ABC-type transporters that may be involved in sugar uptake and several pathways involved in the degradation of complex sugars (Cazalet et al. 2004). More recent studies have established the importance of glucose metabolism for the intracellular growth of *Legionella* in human epithelial cells, mouse macrophages and in an amoebal host (Eylert et al. 2010, Harada et al. 2010). Glucose metabolism occurs during the non-replicative, transmissive state in host cells; under this condition, *Legionella* finds itself inside the cytosol after having exhausted the amino acid reserve of the host cell, and therefore, would rely on glucose for sustenance (Harada et al. 2010, Schunder et al. 2014). An *in vitro* study demonstrated the use of glucose in the post-exponential phase for the biosynthesis of poly-3-hydroxybutyrate (PHB) granules, a carbon and energy storage used under low nutrient conditions (Gillmaier et al. 2016, James et al. 1999).

Glycerol is used as a carbon source by *Listeria monocytogenes* and *Shigella flexneri* during macrophage infection (Eisenreich et al. 2010). *Legionella* was found to also use glycerol *in vitro* (Tesh et al. 1983) and express genes involved in its metabolism inside macrophages (Faucher et al. 2011). Recently, the *in vivo* use of host cell derived glycerol for anabolic processes was demonstrated using *A. castellanii* (Häuslein et al. 2016).

2.6.3.1.4 Bacterial Egress

Between 12-24 hours post-infection, *Legionella* exits the LCV and is found in the cytoplasm where it completes 1-2 rounds of proliferation (Molmeret et al. 2004a). In the human host, pore-formation is induced during the transmissive/post-exponential phase,

allowing for exit from the LCV and into the cytoplasm (Alli et al. 2000, Kirby et al. 1998). During replication, *Legionella* uses the Dot/Icm secretion system to both induce and control the host caspase pathway - the latter using anti-apoptotic factors (Abu-Zant et al. 2005, Molmeret et al. 2004b). Apoptosis allows *Legionella* to disperse (Amer 2010, Molmeret and Abu Kwaik 2002). Within the amoebal host, pore-formation leads to necrosis and subsequent cell lysis (Gao and Abu Kwaik 2000).

2.7. Factors Affecting *Legionella* Survival and Persistence in Water Distribution Systems

2.7.1 Temperature

Lp are most commonly associated with hot water distribution systems; however, their presence in cold water is also well established (Arvand et al. 2011, Borella et al. 2005a, Borella et al. 2005b, Lesnik et al. 2016, Principe et al. 2017, Yiallouros et al. 2013). Incidences of LD are highest during warmer seasons of the year (Cilloniz et al. 2017). In cooling towers, *Legionella* can survive under cooler, sub-optimal temperatures as low as 8^{o}C until warmer, summer temperatures allows for increase in bacterial numbers (Yamamoto et al. 1992). As such, *Legionella* is more frequently isolated from warm waters between 20-50^{o}C from natural and man-made sources (Fliermans et al. 1981, Lesnik et al. 2016). Similar to environmental sources, the bacterium has been isolated from water at temperatures above 60^{o}C, albeit at much lower levels (Darelid et al. 2002, Ezzeddine et al. 1989, Martinelli et al. 2000). Highest colonization levels are reported in waters between 30^{o}C and 45^{o}C, which reflects the optimal temperature range for *in vitro* growth (Borella et al. 2005a, Hruba 2009, Kruse et al. 2016). As a result of this penchant for mesophilic conditions, hot water systems are more prone to colonization by pathogenic *Legionella* and their subsequent growth within protozoa. Bacterial population diversity is lower in hot water compared to that of cold water systems (Henne et al. 2013, Lesnik et al. 2016); this may, in part, contribute to the enrichment of *Legionella* in hot water systems by eliminating competition with other bacteria. In support of this hypothesis, heat-treated tap-water was found to promote *Lp* growth within biofilms in an experimental model, whereas, untreated water had no impact on *Lp* counts (Vervaeren et al. 2006). A survey of drinking water distribution systems in one German town shows that *Legionella* species constituted a larger proportion of the hot water system bacterial population (2.4%) than the

cold water population (0.67%) (Lesnik et al. 2016). Indeed, *Lp* and *L. longbeachae* represented higher proportions of the total microbial community as the temperature increased from 47°C to 60°C, suggesting that these two epidemiologically important species of *Legionella* are well suited for survival in anthropogenic systems (Lesnik et al. 2016). Thermal treatment of water is also known to reduce the diversity of the eukaryotic community (Vervaeren et al. 2006). Whether this shift significantly contributes to enriching the water milieu with suitable hosts for *Legionella* is yet to be determined.

The global standard for hot water systems set forth by the World Health Organization for *Legionella* control is 60°C (WHO 2007). Maintenance of systems above this temperature has been consistently shown to correlate negatively with *Legionella* growth, while temperatures below 50°C are correlated with higher contamination rates (Borella et al. 2005b, Flannery et al. 2006, Plouffe et al. 1983). It is noteworthy that an elevated temperature of 60°C or higher severely reduces the rate of contamination, but does not eradicate the presence of *Legionella*, as evidenced in reports of bacterial isolation from sources surpassing this temperature standard (Fliermans et al. 1981, Hruba 2009). In fact, an early study isolating *Legionella* from hospitals in Columbus, Ohio, found that increasing the temperature of contaminated water distribution systems to 60°C did not successfully eliminate the pathogen (Plouffe et al. 1983). Reducing *Legionella* counts required flushing the plumbing system at a temperature of 70-75°C bimonthly. The researchers' finding that this treatment merely reduced the *Legionella* counts attests to the difficulty of eliminating *Legionella* from large water distribution systems once colonization has occurred. A study in Quebec City used a harsher thermal treatment, heating the water storage tank to 80°C for three days; nonetheless, similarly to the Ohio study, 20% of previously contaminated sites were *Legionella*-positive after 10 days and 50% of sites had reverted after 30 days (Bouchard et al. 1985). Yet another study reports finding high *Lp* counts in tap water two weeks after a 70°C heat shock treatment (van der Kooij et al. 2005). These results are echoed in subsequent publications that report entry into VBNC status or recurrence of *Legionella* contamination after thermal treatments at or exceeding 70°C (Allegra et al. 2011, Bédard et al. 2016a, Farhat et al. 2012), thereby, highlighting the current struggle to efficiently control this bacterium 40 years after its initial discovery.

Importantly, the conclusion of these reports brings to attention what most *Legionella* experts will agree with today; *Legionella* is a relatively thermotolerant bacterium and cannot be eradicated from water systems with current disinfection methods. A report published by the CDC sheds light on a structural component of the bacterium that may contribute to the observed temperature tolerance of *Lp*. The authors of the study found that branched-chain fatty acids, characteristic of Gram-positives, were highly abundant in the *Lp* strains that were tested, representing 81-90% of total fatty acids (Moss et al. 1977). Few Gram-negative bacteria are known to harbour similar amounts of branched-chain fatty acids (Verdon et al. 2011).

2.7.2 Chemical Disinfectants

Chlorination is a common method of disinfection used for water supplies, where water systems are treated with chlorines or chloramines (Lin et al. 2011, WHO 2014). *Lp* originating from rich laboratory media is highly susceptible to chlorine compared to water-exposed *Lp*, highlighting the increased resistance to disinfectants that can be expected from naturally-occurring *Legionella* strains (Cargill et al. 1992, Kuchta et al. 1985, States et al. 1989). Moreover, compared to *E. coli*, *Legionella* has been reported to be more resistant to chlorine (Borella et al. 2005a, Falkinham et al. 2015). Nonetheless, numerous reports attest to an effective decrease in *Legionella*-positive samples using chlorination, while low free chlorine concentrations are positively correlated with *Legionella* contamination (Flannery et al. 2006, Heffelfinger et al. 2003, Marchesi et al. 2013, Marrie et al. 1992, Schwake et al. 2016). It is also established that chlorination at higher temperatures increases the killing efficiency; in contrast, the rate of disinfectant decay was also higher at elevated temperatures (Dupuy et al. 2011, Marchesi et al. 2013). Increasingly, monochloramine is being reported as a better disinfectant for control of *Legionella* within water distribution systems (Flannery et al. 2006). This can be attributed to the longer half life of monochloramine allowing for prolonged persistence within water systems and its increased ability to penetrate biofilms compared to chlorines (Lin et al. 2011, WHO 2014). While studies report an initial decrease in the contamination level, like thermal treatments, chlorination has been proven unsuccessful for complete eradication (Marrie et al. 1992, Mérault et al. 2011, Mouchtouri et al. 2010). Two independent studies reported a positive correlation between chlorination and the abundance of *Lp* serogroup 1 (Borella et al. 2005b, Mérault et al. 2011). Lesnik et al. (2016) reported that chlorine treatment of municipal water negatively affected *Legionella* diversity. Continuous

chlorination may, therefore, select for *Legionella* species that are equipped to persist after disinfection, leading to significant populations of these organisms to reappear in water systems months after treatment. Treatment with chlorine or monochloramine induces *Lp* to enter a VBNC state (Alleron et al. 2008, Kirschner et al. 2012, Turetgen 2008), which may explain the observed increase in *Legionella* counts post-chlorination. Interestingly, environmental isolates were found to enter the VBNC status without cell death more readily than those cultured on laboratory media (Turetgen 2008).

Other common disinfectants used in water distribution systems include copper-silver ionization, silver stabilized hydrogen peroxide, ozone and hydrogen peroxide-peracetic acid (Ditommaso et al. 2005, Ditommaso et al. 2016, Farhat et al. 2012, Kim et al. 2002, Martin et al. 2015). Copper-silver ionization is also widely used and is reported to be more effective than thermal treatments (Miuetzner et al. 1997, Stout et al. 1998). Similarly to disinfection with chlorine, eradication of the bacterium is not possible and there are reports of resistance in systems that are treated using the metal ions (Chen et al. 2008b, Kusnetsov et al. 2001, Rohr et al. 1999).

2.7.3 Relationship with Eukaryotes

Aquatic amoeba and ciliates represent the host cells that are crucial for the persistence and growth of *Legionella* within water distribution systems (Buse and Ashbolt 2011, Fields et al. 1989, Wadowsky et al. 1988). The highest concentrations of *Legionella* are often correlated with the presence of amoeba (Barbaree et al. 1986, Barker et al. 1992, Paszko-Kolva et al. 1991, Winiecka-Krusnell and Linder 1999). In addition to providing an intracellular replicative niche, these unicellular eukaryotes are instrumental in protecting *Legionella*, as well as a variety of other pathogens such as *Acinetobacter baumanii*, *Pseudomonas aeruginosa* and *Mycobacterium avium*, from harmful environmental stresses (Falkinham et al. 2015, Farhat et al. 2012, Gast et al. 2011, Storey et al. 2004). Not only are protozoa more resistant to disinfectants (Thomas et al. 2004), amoeba encyst in response to harmful environmental stimuli such as chlorine, thereby trapping intracellular *Legionella* and protecting the latter from the effects of biocides (Kilvington and Price 1990, King et al. 1988). Various pathogenic bacteria are reported to be 50 times more resistant to residual chlorine than their free-living counterparts when they experience the biocide stress inside encysted protozoa (King et al.

1988). Growth within *Hartmanella vermiformis* rendered *Legionella* 2.5-1000 times more resistant to oxidative stress, heat shock, acid stress and osmotic stress (Kwaik et al. 1997). A similar resistance to disinfection and antibiotics has been reported for *Legionella* exiting *A. polyphaga* and *A. castellanii* (Barker et al. 1992, Barker et al. 1995, Dupuy et al. 2011, García et al. 2007, Garduño et al. 2002).

In terms of human infection, growth within protozoa contributes to enhanced virulence towards protozoan and human cells compared to agar grown *Legionella* (Cirillo et al. 1999, Cirillo et al. 1994). Moreover, amoeba infection influenced the entry of *Lp* into mammalian cells, whereby it increased entry through coiling phagocytosis which has been reported to be fairly infrequent (Cirillo et al. 1999, Newton et al. 2010). Co-inoculation with *H. vermiformis* into the lungs of mice caused a 10-100 fold increase in the *Legionella* counts compared to mice infected solely with the bacterium (Brieland et al. 1997). LD patients are reported to have antibodies against amoeba from the infection source in addition to *Legionella* antibodies (Winiecka-Krusnell and Linder 1999). Taken together, close association with amoeba enhances *Legionella*'s virulence, seemingly independently of intracellular infection.

Alternatively to exiting the host cell through lysis, *Tetrahymena* and *Acanthamoeba* spp. have been shown to expel *Legionella* into the surrounding environment inside vacuoles (Berk et al. 2008, Berk et al. 1998, Faulkner et al. 2008, Greub and Raoult 2003). These vacuoles provide protection from biocides and other environmental stresses (Berk et al. 1998, Bouyer et al. 2007), and have been proposed as the infectious particle that cause LD (Rowbotham 1980). *Legionella* expelled within vesicles were shown to survive for at least 6 months (Bouyer et al. 2007).

While the parasitic relationship between *Legionella* and amoeba is well documented, the former cannot infiltrate all protozoa. One study identified three groups of amoeba that resisted *Legionella* infection: one group did not ingest *Legionella*, a second released the bacterium in a pellet after uptake, and the third group was able to digest *Legionella* (Amaro et al. 2015). The authors propose these predatory protists present a selective pressure for the acquisition of virulence traits by *Legionella* spp.

2.7.4 Other Abiotic Factors

Copper is often used to disinfect water, attesting to its microbicidal activity (Kim et al. 2002). The presence of copper ions in water has been associated with a lower rate of *Legionella* (Leoni et al. 2005, Marrie et al. 1992). Moreover, the use of copper piping has been shown to slow down biofilm accumulation, reduce *Legionella* colonization of biofilm and lead to cultivability loss faster than stainless steel or elastomeric materials (Buse et al. 2016, Moritz et al. 2010, Rogers et al. 1994, van der Kooij et al. 2005). Nonetheless, plumbing systems containing copper are not immune to *Legionella* colonization and does not completely inhibit its growth once established (Buse et al. 2014, van der Kooij et al. 2005). Older water distribution systems using copper lose their antimicrobial activity with the accumulation of corrosion products on the surface of the plumbing material (van der Kooij et al. 2005).

Iron and zinc are metals that have also been linked to the presence of *Legionella* in water systems. Iron is essential for the growth of *Legionella in vitro* and *in vivo* (Cianciotto 2015). The adherence of *Legionella* to host cells is zinc-dependent and a zinc mettaloprotease has been shown to contribute to pathogenesis (Moffat et al. 1994, Yaradou et al. 2007). In water, iron and zinc levels (<1mg/L) are positively correlated with the presence of this bacterium (Bargellini et al. 2011, Marrie et al. 1994, Rakic et al. 2012, States et al. 1985). On the other hand, excessive zinc and iron appear to be protective against colonization of water systems by *Legionella* (Borella et al. 2004, States et al. 1985). While the importance of manganese for the survival and virulence of *Legionella* has not been studied, several studies point to a correlation between high manganese levels and *Lp* contamination of water systems (Bargellini et al. 2011, Rakic et al. 2012). It is noteworthy, that these general correlations have not been observed in some studies, while others have reported the opposite (Edagawa et al. 2008, Mathys et al. 2008, Rakic et al. 2012). Other parameter ,s associated with a higher presence of *Legionella* include calcium and magnesium, which contribute to water hardness, potassium, phosphate, sodium, nitrate, dissolved organic content and scale/sediment (Bargellini et al. 2011, Borella et al. 2004, Marrie et al. 1994, Ng et al. 2008, Ortiz-Roque and Hazen 1987, Vickers et al. 1987, Wullings and van der Kooij 2006).

The relationship between the pH of water and the presence of *Legionella* is unclear. Survival and intracellular growth within amoeba have been observed between pH 5.5 to 9.2 (Ohno et al. 2003, Wadowsky et al. 1985). While some reports found that high pH correlates

negatively with some *Legionella* spp. (Mouchtouri et al. 2007), others have found a positive correlation (Ji et al. 2015, Kusnetsov et al. 2003, Leoni et al. 2005, Marrie et al. 1994). Yet another study testing over 60 cooling towers found no correlation between pH and *Legionella* contamination (Ikedo and Yabuuchi 1986). It is noteworthy that pH can affect the efficacy of some disinfection protocols. For example, in one case study, silver-copper ionization worked less efficiently in a hospital where the pH of the water was between 8.5-9.0, whereas decreasing the pH to a neutral 7.0 resulted in 100,000-fold increase in killing efficiency (Lin et al. 2002). Copper is less soluble and, therefore, less available at higher pH (Lin et al. 2002). In the case of chlorination, alkaline conditions are known to prolong chlorine persistence in cooling towers (Mead et al. 1988, States et al. 1987); however, it can also favour the formation of scale which can impede heat transfer and water flow creating a niche for *Legionella* to flourish (States et al. 1989). Conflicting reports of the relationship between *Legionella* and water pH may, therefore, be due to overlooking the disinfection methods employed in each case. In general, it is clear that the survival or persistence of *Legionella* in water distributions systems is multifactorial, and that any one parameter cannot contribute to its absolute success.

2.7.5 Interaction with Other Microbial Agents

It is generally accepted that high heterotrophic plate counts (HPC) correlate positively with the presence of *Legionella* in potable water systems (Bargellini et al. 2011, Duda et al. 2015, Goutziana et al. 2008, Solimini et al. 2014). In sediments that accumulate inside water heaters, one study found that *Legionella* was more frequently present with higher counts of non-*Legionella* bacteria (Dewailly and Joly 1991, Kusnetsov et al. 2003). Sediments provide nutrients for other microorganisms, which in turn, favour the survival of *Legionella* directly or indirectly (Stout et al. 1985, Vickers et al. 1987). It is possible that these microorganisms represent a food source of amoeba that act as host cells for *Legionella*. Alternatively, it is possible that products excreted by these co-habitants promote the survival of *Legionella* in a yet undefined fashion. In reality, it is likely an amalgamation of these factors contributing to promoting survival of *Legionella* within a given microbial community. Only a few direct interactions between *Legionella* and other bacteria species have been studied to-date. Some of the earliest reports found that *Legionella* was capable of growth and oxygen uptake using products excreted by *Fischerella*, a cyanobacterium (Bohach and Snyder 1983, Tison et al. 1980); however, the growth observed in these studies were modest and the biological relevance

of the observed effect is questionable. A more substantial increase in the number of *Legionella* was observed in the presence of a *Synechocystis* sp., a *Scenedesmus* sp. and a *Chlamydomonas* sp. (Pope et al. 1982).

Bacteria belonging to the genus *Flavobacterium* are often isolated with *Legionella* from water (Grimes 1991, States et al. 1985, Stojek and Dutkiewicz 2011, Surman et al. 1994). *Flavobacterium breve* was found to support the formation of *Legionella* satellite colonies on standard CYE agar without cysteine (an essential amino acid), presumably by providing L-cysteine or a related product (Wadowsky and Yee 1983). Several bacteria, including *Pseudomonas* spp. and *Flavobacterium* were also found to enhance the viability of *Lp* on R2A medium (Surman et al. 1994). Co-habitation of *Legionella* with *Mycobacterium chelonae* in biofilm was also found to slightly increase the culturability of *Lp*, while association with *Acidovorax* sp. and *Sphingomonas* sp. decreased culturability on agar (Gião et al. 2011). The exact mechanism with which each organism exerts their positive or negative effects is unknown. Two common bacterial members of the respiratory microflora, *Neisseria meningitides* and *Haemophilus influenza*, were found to promote the growth of *Lp* on cysteine deficient CYE agar (Stout et al. 1986).

In contrast, other studies report the inhibition of *Legionella* growth by other oropharyngeal and respiratory tract species including *Staphylococcus aureus, Klebsiella pneumonia, Pseudomonas aeruginosa,* and *Streptococcus* viridans group (Carrington 1979, Flesher et al. 1980). Toze et al. (1990) tested the ability of 100 HPC isolates for their ability to affect the growth of various *Legionella* strains on CYE agar. *Aeromonas* spp. and *Pseudomonas vesicularis* were identified as inhibitors of *Legionella* by one study, while *Vibrio fluvialis* inhibited the growth of *Legionella* with the exception of *Lp* (Toze et al. 1990). The authors also reported the isolation of two unidentified microorganisms that stimulated *Legionella* (Toze et al. 1990).

It is important to note that the associations between various microbial species and *Legionella* described above have been reported in the context of growth on rich medium, except for *P. aeruginosa*. Indeed, the presence of this bacterium has been shown to reduce the survival of *Lp* in water (Solimini et al. 2014). Therefore, while the presence of the respective microorganism in environmental samples may definitely impact the detection of *Legionella*,

whether or not these microorganism impact the survival of *Legionella* in water is still unclear. Moreover, given the highly diverse array of organisms present in environmental samples, it is likely that the presence of one organism does not significantly contribute to the survival or detriment of *Legionella*. It is the balance of organisms that act both positively and negatively within the microbial community which ultimately has the potential to impact *Legionella* survival in any significant manner.

2.8 Regulators of virulence and survival in water

The major regulators encoded by *Legionella* were discovered and have been studied for their role in virulence and pathogenesis. These regulators are involved in the transition from the replicative to the transmissive phase *in vitro* and *in vivo*, and control the expression of a variety of virulence genes. Major regulators of virulence include RelA, SpoT and RpoS which are part of the stringent response (SR), the LetA/S, CpxR/A and PmrA/B two-component systems (TCSs), the FliA sigma factor, as well as the quorum sensing systems encoded by Lqs genes.

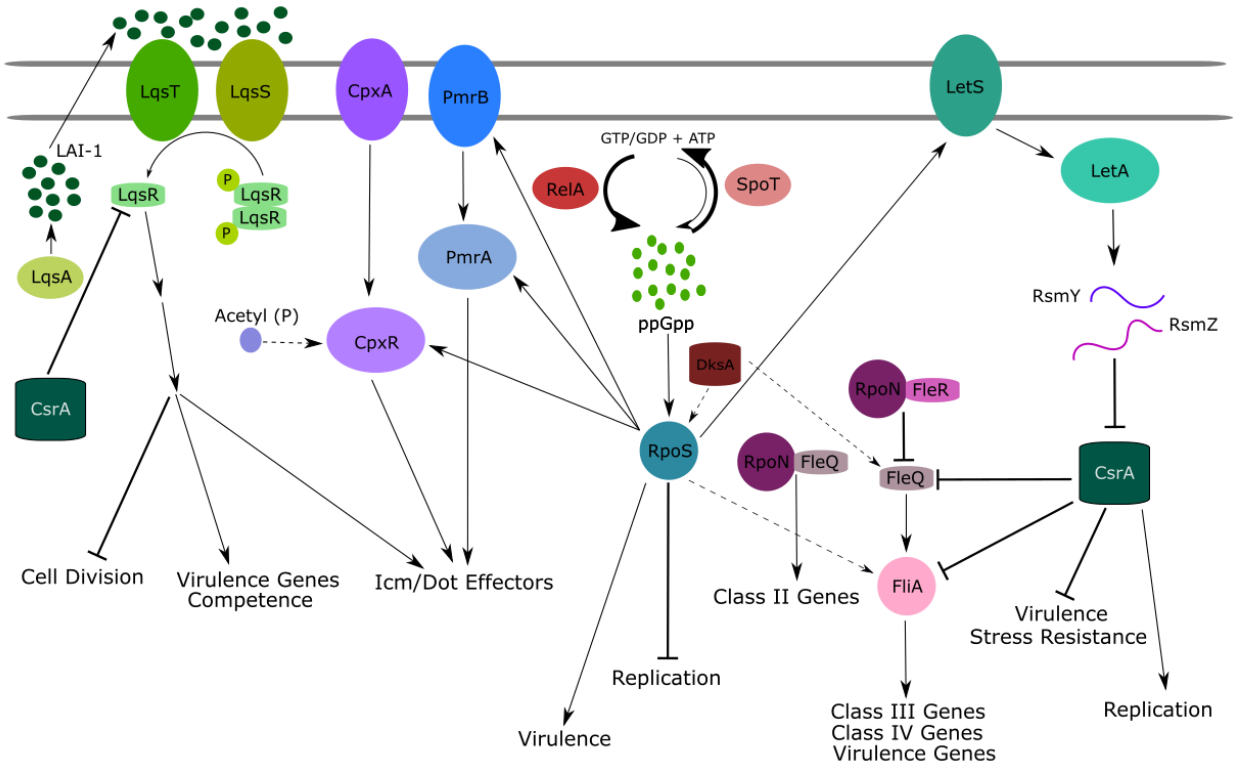

Figure 2.2 Overview of major *Legionella* regulators. RelA and SpoT produce the cellular alarmone ppGpp in response to nutrient deprivation. ppGpp along with the DksA protein increase levels of RpoS. The alternative sigma factor RpoS halts replication and induces virulence during the transmissive phase. RpoS affects the LetA/S, CpxR/A and PmrA/B two-component systems (TCSs). LetA/S initates the transcription of the sRNAs RsmY/Z, which antagonize the RNA-binding protein CsrA. The LetA/S cascade promotes virulence, motility and stress resistance, while inhibiting replication. FleQ and RpoN induce the timely expression of flagella proteins. CpxR/A and PmrA/B TCSs induce type IV secretion system substrates. The CpxR response regulator can be phosphorylated through CpxA and likely also by acetyl phosphate. The quorum sensing (QS) Lqs system produces the autoinducer molecule LAI-1 through the LqsA synthetase. Two sensor kinases, LqsS and LqsT, respond to LAI-1. At high concentrations, LqsS and LqsT dephosphorylate LqsR which likely acts with yet unidentified factors to initate downstream regulatory events leading to the expression of Icm/Dot effectors and other virulence genes, increased competence and repression of cell division.

2.8.1 The Stringent Response (SR)

The addition of a pyrophosphate moiety, from ATP to GDP or GTP, produces (p)ppGpp or ppGpp respectively, herein referred to collectively as ppGpp (Hauryliuk et al. 2015). ppGpp is a cellular alarmone that accumulates when cells sense nutrient deprivation (Potrykus and Cashel 2008). This accumulation inside the cytoplasm triggers the stringent response (SR) which is a global stress response to starvation (Potrykus and Cashel 2008, Srivatsan and Wang 2008).

In β- and ɣ-proteobacteria, RelA, a monofunctional synthase, and SpoT, a bi-functional synthase/hydrolase, are the two enzymes that maintain the intracellular pool of ppGpp (Battesti and Bouveret 2009). Production of ppGpp by the ribosome-associated RelA is triggered upon sensing uncharged tRNA (Haseltine and Block 1973). As a result, RelA initiates the SR in response to amino acid starvation (Potrykus and Cashel 2008). On the other hand, SpoT reportedly responds to various other stresses including fatty acid (Seyfzadeh et al. 1993), phosphate (Spira et al. 1995, Spira and Yagil 1998), carbon (Xiao et al. 1991) and iron starvation (Vinella et al. 2005). ppGpp production upon fatty acid deficiency is mediated by the direct interaction of SpoT with a co-factor involved in fatty acid metabolism, the acyl carrier protein (ACP) (Battesti and Bouveret 2006, Gong et al. 2002, Seyfzadeh et al. 1993). When activated, SR halts cellular growth through: a massive transcriptional remodeling that results in the downregulation of stable RNA molecules such as rRNA and tRNA synthesis and upregulates genes that favour survival, shut down of protein synthesis, and blocking DNA replication (Potrykus and Cashel 2008, Svitil et al. 1993). ppGpp can bind directly to the β-subunit of the RNA polymerase (RNAP) and change transcription in response to starvation (Chatterji et al. 1998, Toulokhonov et al. 2001, Zuo et al. 2013). Transcriptional regulation by ppGpp is enhanced in the presence of DksA, another regulatory protein that can bind RNAP (Paul et al. 2004, Paul et al. 2005). The cellular levels of the alternative sigma factor RpoS is positively regulated by ppGpp (Brown et al. 2002, Dalebroux and Swanson 2012, Lange et al. 1995). RpoS is the general stress response sigma factor and its regulon is crucial for the expression of genes that promote survival under various stresses, including starvation, oxidative stress, heat and pH (Battesti et al. 2011, Landini et al. 2014).

2.8.2 Stringent response regulators: RelA, SpoT and RpoS

The production of ppGpp in the PE phase is immediately followed by motility, cytotoxicity and sodium sensitivity in *Legionella* (Dalebroux et al. 2010, Hammer and Swanson 1999). *Legionella* becomes sensitive to sodium in the PE phase, a trait that correlates with the presence of a functional Icm/Dot secretion system (Sadosky et al. 1993, Vogel et al. 1996). It is, therefore, commonly used as an indicator of virulence. Ectopic expression of the ppGpp synthetase, *relA* in E phase cultures, induces the expression of flagellin, the major subunit of the *Legionella* flagellum, as well as sodium sensitivity, osmotic stress and heat shock resistance, contact-dependent cytotoxicity and infectivity of macrophages (Hammer and Swanson 1999). ppGpp also halts the growth of E phase cells (Hammer and Swanson 1999). Much like in *E. coli*, the *Legionella* SpoT responds to fatty acid synthesis, and RelA, to amino acid availability (Dalebroux et al. 2009), thereby allowing differentiation of the bacterium in response to nutrient conditions. DksA, the regulatory protein that potentiates ppGpp affects in *E. coli* was also found to contribute to virulence traits, growth arrest and pigmentation in the transmissive phase of *Lp* (Dalebroux et al. 2010)

The alternative sigma factor RpoS, which is activated by and lies directly beneath ppGpp in the SR regulatory cascade, affects approximately 25% of the *Legionella* genome in the PE phase, directly and indirectly (Dalebroux and Swanson 2012, Dalebroux et al. 2010, Hovel-Miner et al. 2009). In fact, 50% of its regulon is induced while the remaining 50% is repressed. One study reported that over 300 genes in the RpoS regulon contained an RpoS-dependent promoter (Sahr et al. 2012). While *rpoS* mRNA is expressed maximally during E phase, the RpoS protein level is highest in the PE phase (Bachman and Swanson 2004a, Hales and Shuman 1999b, Lynch et al. 2003). This transcriptional pattern led to the finding that RpoS contributed to E phase gene regulation as well (Bachman and Swanson 2004a). Accordingly, transcriptomic data revealed a set of over 200 genes affected by RpoS during the E phase, approximately half of which were negatively affected and the other half positively influenced by RpoS (Hovel-Miner et al. 2009). In general, the transcriptomic analysis also revealed that RpoS negatively affected genes associated with translation and metabolism, while positively affecting genes related to virulence, motility, signal transduction, transcription factors and Icm/Dot effector proteins (but not the Icm/Dot structural proteins) (Hovel-Miner et al. 2009). Interestingly, most effector proteins were negatively affected by RpoS in the E phase, consistent with a model where RpoS

acts during the E phase to dampen virulence genes and promotes their transcription during PE phase (Hovel-Miner et al. 2009). In contrast to other avirulent strains that promptly get digested inside the host, the *rpoS* mutant is able to evade the early endosomal pathway, but fails to replicate (Bachman and Swanson 2001). This further explains the important role of RpoS in the E phase, wherein it promotes growth. The repression mechanism employed by RpoS during the E phase is yet to be elucidated. On the other hand, it is likely that the observed effect of RpoS on the Icm/Dot effector proteins is indirect, as 25 putative transcription factors were found to be under its control, including CpxR, PmrA, RpoE, FleQ and FliA (Hovel-Miner et al. 2009). Interestingly, CpxR/A and PmrA/B, two two-component systems (TCSs) that directly regulate multiple effectors, were highly upregulated by RpoS in the PE phase (Hovel-Miner et al. 2009). The same study also found that RpoS was imperative for the expression of LetA/S, a TCS that works with RpoS to express transmissive phase phenotypes (Hovel-Miner et al. 2009).

The transcriptomic pattern of the *rpoS* mutant is reflected in the *in vitro* and *in vivo* phenotypes reported (Hovel-Miner et al. 2009). RpoS contributes to the expression of flagellin, virulence and sodium sensitivity, as an *rpoS* mutant was unable to fully induce these traits even in the presence of ppGpp (Bachman and Swanson 2001, Hammer and Swanson 1999). RpoS was found to strongly affect FliA, another alternative sigma factor that is related to the expression of the flagellum (See section 2.8.5). While growth and cytotoxicity in cultures with macrophages (THP-1 and HL-60) were not affected by RpoS (Hales and Shuman 1999b), the use of primary murine macrophages revealed some virulence defects. The *rpoS* mutant is partially deficient in intracellular multiplication (ICM), recruitment of ER vesicles to the nascent LCV, and evading the early endosomal pathway (Bachman and Swanson 2001, Swanson and Isberg 1996). Nonetheless, blocking lysosomal fusion to the LCV was found to be RpoS-independent, a process that is seemingly Icm/Dot-independent (Bachman and Swanson 2001). While virulence in mammalian macrophages is not fully RpoS-dependent, an *rpoS* mutant exhibited a significant virulence defect in the amoebae *A. castellanii* and *A. polyphaga* (Abu-Zant et al. 2006, Hales and Shuman 1999b, Hovel-Miner et al. 2009). Defects associated with infections can be explained by subsequent reports which found that RpoS controlled the expression of *mip*, a protein that is required for full infection potential of both amoeba and macrophages (Cianciotto and Fields 1992), as well as Icm/Dot effectors and regulators of these proteins, i.e. LetA/S, CpxR/A, PmrA/B and LqsR (Bachman and Swanson 2004a, Hovel-Miner et al. 2009).

Unlike its counterparts in *E. coli* (Hengge-Aronis 1993), *V. cholera* (Yildiz and Schoolnik 1998) and *P. aeruginosa* (Suh et al. 1999), RpoS in *Legionella* does not contribute to growth phase-dependent stress resistance such as heat shock, acid stress or oxidative stress (Bachman and Swanson 2001, Hales and Shuman 1999b, Hammer and Swanson 1999). It is noteworthy that over-expression of the sigma factor has lead to unsuccessful complementation and negative effects on wild-type (WT) strains, demonstrating the importance of RpoS levels to induce the appropriate response (Bachman and Swanson 2001, Hales and Shuman 1999b, Trigui et al. 2014). Over-expression of *rpoS* led to the depletion of *csrA*, a major repressor of transmission phase genes, which in turn can lock the cell in E phase (Bachman and Swanson 2004a). As a result of the need for tightly controlled levels of this alternative sigma factor, RpoS levels in the cell are intricately controlled at the transcriptional, post-transcriptional and translational level (Lowewen et al. 1998, Schellhorn 2014).

In addition to regulating the transition between the replicative and transmissive phase, RpoS and its downstream regulon was shown to be an important genetic determinant for the survival of *Legionella* in water (Trigui et al. 2014). While the number of genes affected by RpoS in water (Trigui et al. 2014) was similar to that in the PE phase (Hovel-Miner et al. 2009), the regulatory pattern was different. In water, over 75% of the differentially regulated genes were repressed by RpoS (Trigui et al. 2014). Indeed, RpoS was required to shut down translation, transcription and replication in response to water. Interestingly, RpoS was found to initiate a positive feedback loop, wherein it reduced *spoT* levels in order to maintain high ppGpp levels during starvation (Trigui et al. 2014).

2.8.3 LetA/S-RsmY/Z-CsrA Cascade

Since the *rpoS* mutant only contributed partially to transmissive phase traits (such as virulence, growth arrest, and stress resistance), at least one other factor is responsible for growth arrest and stress resistance in the PE phase of *Legionella*. Given the contribution of the ***Le****gionella* **t**ransmission **a**ctivator and **s**ensor (LetA/S) system to the aforementioned phenotypes, the two-component system (TCS) was proposed as an important determinant of differentiation (Bachman and Swanson 2001, Bachman and Swanson 2004a, Gal-Mor and Segal 2003b, Hales and Shuman 1999b, Hammer and Swanson 1999). The LetA/S TCS was first identified as another important regulator of the the transmissive phase by Hammer et al. (2002). LetS is the

sensor kinase and, LetA, its cognate response regulator (Hammer et al. 2002). Upon being activated by a yet-unknown signal, LetS, phosphorylates LetA; which in turn activates the transcription of the sRNAs RsmX/Y/Z (Edwards et al. 2010, Sahr et al. 2009, Sahr et al. 2012). The last member of this cascade is CsrA, a global post-trascriptional regulator (Fettes et al. 2001, Forsbach-Birk et al. 2004, Molofsky and Swanson 2003). In *Legionella*, CsrA represses transmissive traits and promotes replication in the presence of sufficient nutrients by binding to target mRNA (Molofsky and Swanson 2003). As such, reducing *csrA* levels in E phase cells causes the premature expression of transmission traits and growth arrest (Fettes et al. 2001, Molofsky and Swanson 2003, Sahr et al. 2017). In the replicative phase, CsrA binding can: 1) repress translation of transmissive phase genes by blocking the ribosomal binding site and/or promoting mRNA degradation, or 2) enhance the translation of replicative phase genes by affecting the stability and translation, but the exact mechanisms are yet to be determined (Romeo et al. 2013). However, when the cell senses nutritional deprivation, LetA/S induced RsmX/Y/Z production. The sRNAs are able to competitively bind CsrA, releasing the previously bound mRNA which can now act to repress replication and promote virulence traits (Romeo et al. 2013, Sahr et al. 2009, Sahr et al. 2012). To date, Rsm sRNAs are considered to be the only targets of LetA, therefore the regulatory changes initiated by the cascade are seemingly mediated by the direct and indirect effects of CsrA binding.

The LetA/S system influences a variety of transmissive traits including motility, virulence, cytotoxicity, oxidative stress, heat shock and acid stress (Hammer et al. 2002, Lynch et al. 2003, Molofsky and Swanson 2003, Sahr et al. 2009). While mutants within the LetA/S cascade exhibit virulence defects related to entry and LCV establishment, ICM within host cells are not impacted by loss of function of the TCS (Sahr et al. 2009, Sahr et al. 2017). This further indicates that the cascade is dispensable for growth while highlighting its vital role in the transmissive phase. In contrast, mutations of *csrA* did not impact the entry of *Legionella*, but severely diminished intracellular growth (Molofsky and Swanson 2003, Sahr et al. 2017). In regards to virulence, LetA/S affects the expression of Mip, Icm/Dot structural proteins as well as effectors which explains the virulence deficits reported by multiple groups (Gal-Mor and Segal 2003b, Hammer et al. 2002, Nevo et al. 2014, Rasis and Segal 2009, Sahr et al. 2009, Shi et al. 2006). LetA/S also influences the hydrolytic capacity of *Legionella*; however, it is still unclear whether this is mediated through the direct regulation of hydrolytic enzymes, the type II

secretion systems that transport the enzymes or by controlling the expression of a chaperone (Broich et al. 2006). Elements under positive control of the LetA/S regulatory network include the heat shock sigma factor RpoH and the global stress response protein, GspA, which likely contributes to stress resistance conferred by the cascade (Kwaik et al. 1997, Sahr et al. 2017). Genes that are negatively regulated by activation of LetA/S include those involved in iron acquisition systems, energy production, transcription, cell division and translation (Molofsky and Swanson 2003, Sahr et al. 2009, Sahr et al. 2017).

Much like in other bacteria, the RpoS and LetA/S regulatory systems are intrinsically linked to each other. RpoS was found to be essential for the transcription of *letS* in the PE phase, and therefore, crucial for the downstream cascade (Hovel-Miner et al. 2009). Moreover, the *rpoS* transcript seems to be repressed by CsrA, while RsmY/Z naturally increases its expression (Sahr et al. 2009, Sahr et al. 2017). In contrast, RelA levels were positively affected by CsrA (Sahr et al. 2017). While this regulatory pattern seems counterintuitive to the SR being activated in the transmissive phase, it is noteworthy that the ppGpp contributes to bacterial growth rate control (Potrykus et al. 2011). It is, therefore, likely that the aforementioned observation reflects a role for RelA during the E phase. Nonetheless, there is significant overlap between the RpoS and LetA/S regulons and phenotypes (Broich et al. 2006, McNealy et al. 2005); however, it is curious that the *rpoS* mutation exhibits less severe phenotypes in relation to virulence, motility and stress resistance than mutations within the LetA/S system given the dependence of the latter on the former.

Unlike canonical sensor kinases, tripartite sensor kinases such as LetS can induce varied regulons in response to varying environmental stimuli (Cotter and Miller 1997, Deora et al. 2001). As such, mutational analysis found that the *Legionella* LetS is able to induce a subset of its regulon when its phosphorylation sites were manipulated (Edwards et al. 2010). Moreover, while it has been long hypothesized that ppGpp acts as the activating signal for LetS sensor kinase (Molofsky and Swanson 2003, Rasis and Segal 2009, Sahr et al. 2009), the TCS was also found to be activated by nicotinic, acetic and propionic acid to induce flagellin (Edwards et al. 2013, Edwards et al. 2009). Nonetheless, direct stimulation of LetS by any stimuli has not yet been proven.

While the importance of LetA/S for the survival of *Legionella* has never been tested, the CsrA-repressor, CsrR, was found contribute to the bacterium's survival in tap water (Abbott et al. 2015). CsrR is a paralog of CsrA and was found to repress *csrA* translation, forming a positive feedback loop enhancing persistence during nutrient deprivation (Abbott et al. 2015).

Given the contribution of the LetA/S cascade to cellular differentiation, it is likely that the TCS also contributes to surviving the starvation conditions in water in a CsrR-dependent and -independent manner.

2.8.4 CpxR/A Two-Component System

In *E. coli*, the CpxR/S TCS senses and responds to misfolded proteins within the periplasm and is positively regulated by RpoS in the stationary phase (De Wulf et al. 1999). The response regulator CpxR and the sensor kinase CpxA were found to be highly induced during infection, regardless of growth phase (Bruggemann et al. 2006, Faucher et al. 2011). In *Legionella*, CpxR regulates Icm/Dot structural genes and effectors both positively and negatively (Altman and Segal 2008, Feldheim et al. 2016, Gal-Mor and Segal 2003a, Tanner et al. 2016a, Vincent et al. 2006). The expression of *icmR* is controlled directly by CpxR in *Lp* (Gal-Mor and Segal 2003a). *icmR* is a chaperone for the pore-forming IcmQ protein (Raychaudhury et al. 2009). In other *Legionella* spp., the functional homologs of IcmR are influenced by CpxR and/or PmrA, the other response regulator that is a direct inducer of Icm/Dot genes (Feldman and Segal 2007). A transcriptomic study also identified 14 type II secretion system substrates that were downregulated in the absence of CpxR (Tanner et al. 2016a). This secretion system is also known to contribute to the virulence of *Legionella* (Cianciotto 2014). The CpxA sensor kinase is partially dispensable for the phosphorylation that activates CpxR, as the latter can be phosphorylated by the intracellular phosphate donor molecule, acetyl-phosphate (Feldheim et al. 2016, Gal-Mor and Segal 2003a, Vincent et al. 2006). As such, *cpxA* mutations show less severe regulation defects than *cpxR* mutations (Gal-Mor and Segal 2003a). As a consequence of virulence gene regulation, the *cpxR* and *cpxA* mutants are associated with defective ICM in the amoebal host *A. castellanii* (Feldheim et al. 2016, Tanner et al. 2016a). Corroborating its positive regulation of the Icm/Dot machinery, *cpx* mutants are resistant to sodium and are deficient for translocation of effector proteins (Altman and Segal 2008, Tanner et al. 2016a).

In addition to Icm/Dot genes, CpxR was also found to control the transcription of two regulatory genes; repressing *oxyR* and activating *letE* (Feldheim et al. 2016). LetE was initially identified with the LetA/S system, and found to contribute to the transmission phase phenotypes under LetA/S control such as motility, pigment production, heat sensitivity and cell size reduction (Bachman and Swanson 2004b, Hammer et al. 2002). However, LetE is also induced by CpxR, and was found to repress RsmY/Z and at least 10 effector genes that are positively regulated by the LetA/S system (Feldheim et al. 2016). Therefore, CpxR, whose expression is controlled by RpoS (Hovel-Miner et al. 2009), may be inducing negative feedback on the LetA/S system via LetE. OxyR is a putative oxidative stress response regulator of *Lp* (Tanner et al. 2016b). While it is highly expressed during infection, OxyR is reported to be dispensable for ICM in amoeba and in macrophages (Tanner et al. 2016b). In contrast to what is reported by Feldheim et al. (2016), Tanner et al. (2016b) found that OxyR directly repressed both the *cpx* operon and *icmR*. It is noteworthy that the former study used the Lp02 strain of *Lp*, while the latter study used the JR32 strain.

2.8.5 PmrA/B Two-Component System

PmrA/B is one of the TCSs that directly controls the expression of Icm/Dot effectors (Al-Khodor et al. 2009, Al-khodor et al. 2008, Zusman et al. 2007). PmrA is the response regulator and PmrB is the sensor kinase (Zusman et al. 2007). A *pmrA* mutant strain was unable to replicate within *A. castellanii* and *A. polyphaga*, but was partially defective for ICM within a cultured macrophage cell line (Al-Khodor et al. 2009, Zusman et al. 2007). This virulence defect is likely partially conferred by the positive regulation of effector proteins by PmrA, but also by a lack of *csrA* (Zusman et al. 2007). Indeed, *csrA* levels were severely diminished in a Δ*pmrA* strain regardless of growth phase (Rasis and Segal 2009). Given that a lack of CsrA halts growth (Molofsky and Swanson 2003), the ICM defects observed are probably due to the inability of cells to enter the replicative phase. PmrA/B was also found to affect some flagella genes, general stress resistance genes, the *pilE* pilin gene involved in host cell adherence, type II secretion system substrates and Icm/Dot structural genes in addition to effectors, which may contribute to the virulence defect (Al-Khodor et al. 2009).

The PmrA/B system of *S. enterica* is activated in response to infection, acid stress and Fe^{3+} (Merighi et al. 2005, Perez and Groisman 2007, Wösten and Groisman 1999). The *pmrB*

mutant exhibited an increased sensitivity to a pH 3 acid stress in E and PE phase (Al-Khodor et al. 2009), suggesting that the TCS system in *Legionella* also likely responds to acid stress, which was never investigated.

PmrA/B was found to be positively controlled by RpoS in the PE phase (Hovel-Miner et al. 2009). The *pmrA/B* transcripts were also found to be directly bound by CsrA (Sahr et al. 2017). Taken together, this TCS is part of the complicated regulatory network that controls the differentiation and the acid stress response of *Legionella*.

2.8.6 Flagella Regulation

The expression of the monotrichous *Legionella* flagellum was found to be influenced by temperature, growth phase and osmolarity (Byrne and Swanson 1998, Hammer and Swanson 1999, Heuner et al. 1995, Heuner et al. 1999, Ott et al. 1991). *Legionella* is motile only during the transmissive phase (*in vivo* and *in vitro*), and amino acid supplementation is able to curtail the expression of flagellin in the post-exponential phase, which suggests that flagella expression may be linked to the starvation response (Byrne and Swanson 1998, Hammer and Swanson 1999, Heuner et al. 1999, Heuner and Steinert 2003). As a result, motility and the expression of flagella coincide with the virulent stages of *Legionella* both *in vitro* and *in vivo* (Bruggemann et al. 2006, Byrne and Swanson 1998, Heuner and Steinert 2003). It is, therefore, not surprising that global regulators involved in the SR and virulence are also involved in the regulation of flagella, including RpoS, LetA/S, CsrA and LqsR (Heuner and Steinert 2003, Tiaden et al. 2007).

Flagella expression is highly ordered and hierarchical, where genes can be categorized into classes depending on their temporal expression (McCarter 2006). In *Legionella*, four classes of genes have been identified (Bruggemann et al. 2006, Schulz et al. 2012). Class I is comprised of FleQ and RpoN, which induce the expression of class II genes coding for the basal body (Albert-Weissenberger et al. 2010). RpoN (σ^{54}) is an alternative sigma factor that requires an activator protein to initiate transcription (Wigneshweraraj et al. 2008). The activator binds both RpoN and an enhancer sequence in the promoter region of the target gene (Wigneshweraraj et al. 2008). *Legionella* codes for three such activators; FleQ, FleR and PilR (Jacobi et al. 2004). Of these, FleQ is the main activator protein that positively affects over half of the flagella genes, thereby making it the master regulator of flagellar expression in

Legionella (Albert-Weissenberger et al. 2010, Jacobi et al. 2004). Surprisingly, FleQ was also found to alter the expression of class III and class IV genes independently of RpoN, a highly unusual characteristic for a σ^{54} activator protein (Albert-Weissenberger et al. 2010). This is mediated by positively regulating the transcription of *fliA* by a yet-unknown mechanism (Albert-Weissenberger et al. 2010). That FleQ acts in concert with RpoD, the house keeping sigma factor, or by binding to cyclic di-GMP (c-di-GMP) has been proposed, but is yet to be verified (Albert-Weissenberger et al. 2010). According to one transcriptomic study, RpoN transcript levels are relatively constant throughout growth in broth and *in vivo*, while FleQ mRNA increased approximately 8-fold in PE phase and 4-fold in the transmissive phase *in vivo* (Faucher et al. 2011). This is corroborated in an independent report that found an initial increase of *fleQ* mRNA and FleQ protein in E phase, followed by a decrease in PE phase (Schulz et al. 2012). The aforementioned data supports FleQ levels as the major determinant for flagella biosynthesis and also coincides with FleQ as the regulator of flagella genes that are required earlier in the assembly process.

Similar to *E. coli* (Arnosti and Chamberlin 1989), *Salmonella typhimurium* (Ohnishi et al. 1990) and *P. aeruginosa* (Starnbach and Lory 1992), the alternative sigma factor FliA (σ^{28}) controls the expression of the gene coding for major subunit flagellin (FlaA/FliC), as well as fellow class III and class IV genes (Bruggemann et al. 2006, Heuner et al. 1997). While FleQ impacts the transcription of *fliA* in the later phases of growth, basal *fliA* expression in E phase seems to be under RpoD control and is possibly mediated by DksA (Schulz et al. 2012). In the PE phase, *fliA* transcription may be increased by the concerted efforts of ppGpp, DksA, RpoS and FleQ (Hovel-Miner et al. 2009, Schulz et al. 2012). To this end, the putative negative regulator of FleQ, FleR was found to be upregulated in an *rsmYZ* mutant (Sahr et al. 2009). Moreover, it appears that the translation of *fliA* and *fleQ* are repressed by the CsrA protein (Fettes et al. 2001, Molofsky and Swanson 2003, Sahr et al. 2017). Therefore, both transcriptional and post-transcriptional mechanisms contribute to the flagella expression process.

The production of flagella is implicated in enhanced invasion of both amoebal and human hosts, and contact-dependent cytotoxicity, but does not affect adhesion or ICM (Albert-Weissenberger et al. 2010, Bosshardt et al. 1997, Dietrich et al. 2001, Pruckler et al. 1995, Schulz et al. 2012). Using a variety of flagella structural mutants, one study determined that

motility, not the presence of the flagella, increased host cell uptake of *Legionella* (Molofsky et al. 2005). On the other hand, a *fliA* mutant exhibits more significant virulence defects than flagella mutants, including the inability to avoid phagosomal degradation (Hammer et al. 2002, Molofsky et al. 2005). The absence of FliA is also reported to cause a more significant virulence defect in *D. discoideum*, but not in *A. castellanii* (Heuner et al. 2002). This suggests that, in addition to structural flagella genes, the FliA regulon includes virulence genes, which may support host-specific invasion. Indeed, the non-flagella genes of the FliA regulon include two *enhA* genes which are involved in invasion of host cells, which may partially explain the defects observed during the infection process (Bruggemann et al. 2006, Sahr et al. 2012, Schulz et al. 2012). Moreover, both RpoN and FleQ regulons are known to contain Icm/Dot effectors and other virulence genes (*gspA, mip, lvgA*) respectively (Albert-Weissenberger et al. 2010).

Finally, FleR is thought to act with RpoN to form a negative feedback loop inhibiting flagella expression when the appendage is no longer required (Albert-Weissenberger et al. 2010). Indeed, a *rpoN* mutant was found to have higher levels of *fleQ* mRNA and FleQ protein, suggesting a negative regulatory role for RpoN (Schulz et al. 2012). It is also noteworthy that the aforementioned studies used *Lp* strain Paris and/or Corby, and while the regulatory pattern observed was similar between the two strains, the positive effect of FleQ on *fliA* was stronger in Paris (Schulz et al. 2012). This may suggest that some variation with regards to regulatory strength may exist between *Legionella* species. In general, it is clear that flagella regulation is multi-layered and complex, integrating major regulators such as RpoS and LetA/S. The finer details of this intricate regulatory pathway still need to be elucidated.

2.8.7 The *Legionella* Quorum Sensing (Lqs) System

The quorum sensing (QS) system of *Lp* is similar to the CqsA/S system of *V. cholerae* and is coded by *lqs* genes (Ng and Bassler 2009). In *V. cholerae,* two quorum sensing systems exist. CqsA produces the autoinducer CAI-I, which is sensed by CqsS, while a second QS molecule, AI-2, is sensed by the LuxP protein that is complexed with the LuxQ sensor kinase (Ng and Bassler 2009). When activated by their respective autoinducer molecules, CqsS and LuxPQ transfer a phosphate to a small phosphotransfer protein called LuxU, which in turn, will phosphorylate the response regulator LuxO (Ng and Bassler 2009). LuxO initiates the

transcription of four sRNAs that are able to reduce the transcripts of another transcriptional regulator, HapR (Ng and Bassler 2009). HapR directly activates and represses genes belonging to the quorum sensing regulon.

Of the three classes of autoinducer molecules present in *Vibrio* spp. (AI-2, AHL and AHK), *Lp* seems to solely employ α-hydroxyketone (AHK) signaling (Tiaden et al. 2010a). This quorum sensing system, while present in all *Lp* genomes investigated thus far, does not seem to be present in *L. longbeachae*, suggesting that it is not a conserved regulatory system within members of the *Legionella* genus (Cazalet et al. 2010). To date, four members of the *Lp* Lqs system have been identified; LqsA, LqsS, LqsT and LqsR. LqsA synthesizes a diffusible AHK autoinducer called the *Legionella* autoinducer-1 (LAI-1) (Spirig et al. 2008). The *lqsA* gene is transcribed during the E phase in broth and during replication within amoeba, indicating a role for Lqs in virulence (Spirig et al. 2008). LqsS and LqsT are two sensor kinases that respond to LAI-1 (Kessler et al. 2013, Tiaden et al. 2010b).

LqsR is proposed to be the response regulator (Tiaden et al. 2007), despite the lack of a DNA-binding domain (Segal 2014). It is possible that LqsR is in fact the homolog of the *V. cholerae* LuxU, a phosphotransfer protein (Ng and Bassler 2009). In further support of this hypothesis, Tiaden et al. (2007) found a "*lux* box" upstream of *lqsR* which could indicate that *lqsR* is regulated by a yet-unidentified HapR homolog. The transcription of *lqsR* was completely inhibited in a Δ*rpoS* strain, and somewhat reduced in a Δ*letA* strain (Tiaden et al. 2007). Recently, the *lqsR* mRNA was found to be directly bound by CsrA (Sahr et al. 2017), corroborating a previous finding that RsmY/Z influences its transcript levels (Sahr et al. 2009), and that treating WT *Lp* cells induces the expression of RsmY/Z (Schell et al. 2016). Moreover, transcriptomic analysis revealed that deletions of the Lqs system affected gene expression primarily in the stationary phase (Tiaden et al. 2008, Tiaden et al. 2007). The aforementioned evidence establishes the Lqs system as a contributor to cellular differentiation in *Lp*.

Disrupting the quorum sensing cascade led to salt resistance, which is indicative of a virulence defect. Indeed, *lqs* mutations demonstrated decreased host cell uptake, cytotoxicity, and the inability to successfully hijack host cell processes in both amoebal and human host cells leading to inefficient ICM (Kessler et al. 2013, Tiaden et al. 2008, Tiaden et al. 2010b, Tiaden et al. 2007). In the stationary phase, 386 genes were affected by the deletion of *lqsA/S/R*, wherein

190 of them were repressed and 196 were activated (Tiaden et al. 2008). In line with the observed virulence deficiencies, some of the genes which were repressed in the absence of *lqs* were virulence-related, such as pili, flagella, enhanced entry (Enh) and Icm/Dot effector coding genes (Tiaden et al. 2008). It is also noteworthy that despite a significant decrease in *flaA* (flagellin), the *lqsR* mutant produced a flagellum, indicating that while the Lqs contributes to flagella expression, it is not the main regulator of flagellar genes (Tiaden et al. 2008, Tiaden et al. 2007). Nonetheless, LAI-1 was found to contribute to the motility of *Lp* (Schell et al. 2016).

It was also noted that Δ*lqsR* entered the replicative phase faster or slower than the WT at 37^{o}C and 30^{o}C, suggesting that the QS system plays a role in switching between E to PE and also PE to E phase (Tiaden et al. 2008, Tiaden et al. 2007). Disruptions to the Lqs system (*lqsR* over-expression or deletion of all four Lqs genes) also resulted in elongated cells in the stationary phase compared to short WT cocci (Tiaden et al. 2008, Tiaden et al. 2007), pointing to a role of QS in cell division and/or cell size control in response to growth conditions. As such, cell division genes such as *ftsL* and *ftsZ,* and the replicative phase transcriptional regulator Fis were affected by LqsR (Tiaden et al. 2007).

Yet another phenotype linked to the Lqs system is competence. Mutations of Lqs components were found to increase competence of WT cells, presenting Lqs as a negative regulator of natural competence (Kessler et al. 2013). Lack of *lqs* genes increased the transcripts of ribosomal proteins, similarly to *letA/S* mutants (Sahr et al. 2009, Tiaden et al. 2008). This could illustrate the link between the *Lp* QS system and other transmissive state regulators which is used to achieve balanced and timely growth phase-driven gene expression, as was proposed by Tiaden et al. (2007).

Deletion of *lqsA* or *lqsS* resulted in defects that were less severe than those observed in an *lqsR* mutant (Tiaden et al. 2010b). This finding makes sense given that deletion *lqsR* affects a larger number of genes than deletions of *lqsA*, *lqsT, lqsS* alone (Kessler et al. 2013, Tiaden et al. 2008, Tiaden et al. 2010b, Tiaden et al. 2007). Moreover, deleting multiple *lqs* genes caused more severe defects than a single *lqsR* mutation, which suggests missing links in the Lqs regulatory network (Tiaden et al. 2008). Finally, over-expressing *lqsA* was able to rescue *lqsS* and *lqsT* single mutants, but not a *lqsS/T* double mutant (Kessler et al. 2013, Tiaden et al. 2010b), suggesting that *Lp* uses LqsS and LqsT as the sole sensors of LAI-1. Taken together, this

data suggests that LqsR lies beneath LqsA and LqsS in the regulatory cascade. It has been proposed that the two sensor kinases respond to varying levels of LAI-1 (Kessler et al. 2013). The transcription of *lqsS* coincides with an increase in *lqsR* transcripts in late E phase preceding *lqsA* increase, while *lqsT* transcripts maintain low levels throughout growth in broth (Kessler et al. 2013). In addition, while some Icm/Dot substrates are inversely regulated by LqsS and LqsT, the authors conclude that the two sensors may still act synergistically given that both respond to LAI-1 and double mutants exhibit more severe phenotypes than single mutants (Kessler et al. 2013).

Given the known hierarchy of Lqs components and phosphorylation studies, the current model of the *Lp* QS system is as follows. The presence of LAI-1 inhibits autophosphorylation of LqsS and LqsT (Schell et al. 2016). Therefore, under low LAI-1 concentrations, such as those encountered during the E phase, the sensor kinases are phosphorylated. LqsR is known to interact with both LqsS and LqsT, and likely gets phosporylated by the latter (Schell et al. 2014). Phosphorylated LqsR dimerizes and the downstream regulatory cascade is inactivated (Schell et al. 2016). Conversely, the high LAI-1 levels during PE phase leads to dephosphorylation of LqsS/T and LqsR (Schell et al. 2016). In its unphosphorylated, monomeric state, LqsR induces expression of genes contributing to virulence and motility (Schell et al. 2016).

2.8.8 Genes Involved in Survival in Water

A massive transcriptional remodeling occurs within *Lp* when exposed to water (Li et al. 2015). This change increases the resistance of *Lp* to antibiotics (Li et al. 2015), and likely increases the bacterium's persistence in the face of many other stresses. Indeed, one study exposed stationary phase *Lp* cultures, which are naturally more stress resistant than E phase bacteria, to a nutrient-poor buffer that is similar to a water system (Bandyopadhyay et al. 2004). The authors found that resistance to acid, hydrogren peroxide and antibiotic stress were further enhanced by the treatment, termed Ers treatment (Bandyopadhyay et al. 2004). Together, the data suggests that *Legionella* initiates unique transcriptomic and proteomic changes to adapt to and survive in water.

As described above, the SR and CsrR contribute to *Legionella'*s adaptation to an aquatic environment. Nonetheless, few other genetic elements have been identified as contributors to the survival of *Legionella* within the water systems it inhabits. One study found

that the survival of *Lp* in tap water was enhanced by the type II secretion system at 17^{o}C; however at higher temperatures, the effect was detrimental (Söderberg et al. 2008). The type II effectors that would promote survial in this low-nutrient environment are unknown.

Poly-3-hydroxybutyrate (PHB) is an intracellular energy reserve that is produced by *Legionella* during the PE phase (Gillmaier et al. 2016), and is used by the bacterium during prolonged exposure to water (James et al. 1999). Indeed, a mutation of the RpoS-regulated *bdhA* gene that disrupts the PHB degradation pathway was shown to affect long-term survival in water (Li et al. 2015). LasM is a membrane protein that is also positively regulated by RpoS, the alternative sigma factor essential for survival in water (Li and Faucher 2016, Trigui et al. 2014). This newly identified putative metal transporter was found to promote the culturability of *Lp* in water, and has been proposed to contribute to long-term survival (Li and Faucher 2016). Finally, a putative LuxR family protein, Lpg2524, was also found to confer a survival advantage to *Lp* in water (Li and Faucher 2017). Interestingly, over-expression of this gene in a WT strain decreased survival, and the initial survival defect was abrogated if *Lp* was exposed to water at a high cell density (Li and Faucher 2017). As such, Lpg2524 seems to require careful titration in order to manifest its positive effect on survival at low density, which may suggest that it is tightly regulated by or is part of a major regulatory pathway. The cell density dependency could also point to an unidentified product excreted by *Legionella* that enhances survival. This may be Lpg2524-dependent or merely bypasses the deficiency created by the mutant (Li and Faucher 2017).

2.9 Hypothesis

It is evident that a major research gap exists with regards to our understanding of the molecular mechanisms employed by *Legionella* to successfully survive in the water environment. In *Legionella,* the major regulators involved in virulence are expressed in the post-exponential/transmissive phase that is characterized by starvation, thereby contributing to the bi-phasic lifestyle of the bacterium. Therefore, virulence and the starvation responses are intrinsically linked during intracellular and *in vitro* growth. Consequently, we hypothesized that these same virulence regulators will contribute to the adaptation and survival of *Lp* when faced with the nutrient-deprived water environment.

2.10 Objectives

I. Establish a reproducible freshwater model for studying *Lp* in water. This new medium will be used to test one or several environmental factors in order to corroborate general trends observed in the literature, thereby validating its use for future investigations.

II. Screen *Lp* regulatory mutants for their potential contribution to: 1) surviving nutrient-deprivation in water or 2) overcoming additional environmental stresses such as heat shock, exposure chlorine, or treatment with oxidants during water survival.

III. Elucidate the regulon of each regulator that contributes to survival in water using transcriptomic analysis. When possible, study the molecular mechanisms by which select regulatory elements enhance survival.

CONNECTING TEXT

The systematic study of *Lp* in water has not been undertaken until recently. In order to accurately study the bacterium in its natural setting, we found it imperative to identify a suitable freshwater medium for the following reasons;

- Gene expression can be influenced by medium composition; therefore, a stable water source is needed in order to evaluate the survival of the bacterium in any given condition
- A minimal medium allows precise control over the environmental factors, and hence, the genes that are involved in adapting to each condition
- A reproducible medium would serve as a useful tool when comparing data originating from different research groups
- Differences between *Lp* strains can be accurately evaluated

Therefore, I evaluated Fraquil, an artificial freshwater medium, as a suitable water model for the study of *Legionella* by testing the survival of a laboratory strain of *Lp* exposed to various temperatures, pH and trace metal concentrations.

Contributions of authors: Nilmini Mendis contributed to the experimental design, conducted the temperature and pH experiments, and wrote the manuscript. Peter McBride conducted the trace metal experiments. Sebastien Faucher contributed to the experimental design, writing and editing the manuscript.

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CHAPTER 3: Short-Term and Long-Term Survival and Virulence of *Legionella pneumophila* in the Defined Freshwater Medium Fraquil

3.1 Abstract

Legionella pneumophila (*Lp*) is the etiological agent responsible for Legionnaires' disease, a potentially fatal pulmonary infection. *Lp* lives and multiplies inside protozoa in a variety of natural and man-made water systems prior to human infection. Fraquil, a defined freshwater medium, was used as a highly reproducible medium to study the behaviour of *Lp* in water. Adopting a reductionist approach, Fraquil was used to study the impact of temperature, pH and trace metal levels on the survival and subsequent intracellular multiplication of *Lp* in *Acanthamoeba castellanii*, a freshwater protozoan and a natural host of *Legionella*. We show that temperature has a significant impact on the short- and long-term survival of *Lp*, but that the bacterium retains intracellular multiplication potential for over six months in Fraquil. Moreover, incubation in Fraquil at pH 4.0 resulted in a rapid decline in colony forming units, but was not detrimental to intracellular multiplication. In contrast, variations in trace metal concentrations had no impact on either survival or intracellular multiplication in amoeba. Our data show that *Lp* is a resilient bacterium in the water environment, remaining infectious to host cells after six months under the nutrient-deprived conditions of Fraquil.

3.2 Introduction

Identified as the etiological agent of Legionnaires' disease in the late 1970's, *Legionella pneumophila* (*Lp*) is a Gram-negative, water-borne bacterium (Fields et al. 2002). Inhalation of *Legionella*-contaminated aerosols can lead to Legionellosis, which is comprised of the mild, flu-like Pontiac fever and the more serious pneumonia, Legionnaires' disease (Diederen 2008, Glick et al. 1978). *Legionella* species are often found in freshwater bodies as well as an assortment of man-made water distribution systems (Kuroki et al. 2009, Tachibana et al. 2013, Walczak et al. 2013a, Walczak et al. 2013b) with the exception of *Legionella longbeachae* whose presence in potting soils is linked to the incidence of Legionnaires' disease in Australia and New Zealand (Whiley and Bentham 2011). In Europe and North America, *Lp* is responsible for over 90% of reported Legionellosis cases (Yu et al. 2002). Public health concerns related to *Lp* are mainly associated with its contamination of cooling towers and other man-made water distribution systems (WHO 2007).

In the natural or man-made water environment, *Lp* can be found in a motile planktonic state, a sessile state within mixed species biofilms, growing intracellularly in amoeba and in a persistent state (viable but non-culturable, VBNC) (Lau and Ashbolt 2009). In the water niche, *Lp* is exposed to a changing environment, such as variations in temperature, concentration of dissolved oxygen, minerals, anthropogenic chemicals and organic matter (Lau and Ashbolt 2009). The most important parameter associated with the presence of *Lp* in a given water system is the heterotrophic plate count (HPC), which measures the general contamination of a system. The higher the HPC of a system, the higher the odds are of finding *Lp* within it (Bargellini et al. 2011, Edagawa et al. 2008, Mouchtouri et al. 2010, Serrano-Suárez et al. 2013). Elevated total organic content (TOC) in water distribution systems has also been correlated with an increase in the incidence of *Legionella* (Leoni et al. 2005). Presumably, amoeba are attracted to contaminated sites in water systems to feed on susceptible bacteria and multiply, providing a source of prey for *Lp* (Lau and Ashbolt 2009). In addition, *Lp* can persist in mixed species biofilms in natural and human-made water systems (Stewart et al. 2012). Nevertheless, growth of *Lp* associated with such biofilms requires the presence of amoeba, such as *Hartmannella vermiformis* (Murga et al. 2001). Symbiotic and competitive interactions with other bacterial species seem to have a major impact on the net number of *Lp* in the water environment (Taylor et al. 2009). As an example, the persistence of *Lp* in water is impaired by the presence of

Pseudomonas aeruginosa (Guerrieri et al. 2008, Messi et al. 2011). Physico-chemical factors, such as temperature, pH, and the concentration of dissolved metals also seem to have an impact on the presence of *Lp* in water systems (Bargellini et al. 2011, Dufresne et al. 2012, Serrano-Suárez et al. 2013, WHO 2007); however, there have been conflicting results regarding the extent of their effects on *Lp* persistence in water (Edelstein et al. 1987, Fliermans et al. 1981, Joly 1985, Mauchline et al. 1994, Moritz et al. 2010).

To date, no standardized system has been presented to study the effect of individual environmental factors on the survival of *Lp*. Knowledge pertaining to environmental conditions that affect the presence of *Lp* in such systems has been gained mainly through prospective studies in the field (Bargellini et al. 2011, Borella et al. 2005a, Edagawa et al. 2008, Fliermans et al. 1981, Mouchtouri et al. 2010, Serrano-Suárez et al. 2013) or by using tap water models (Bargellini et al. 2011, Paszko-Kolva et al. 1992, Serrano-Suárez et al. 2013, Wadowsky et al. 1985). The impact of different environmental factors such as water temperature, pH, and the presence of trace metals has been recorded mainly as a result of on-site and environmental samplings, or using sterilized tap or distilled water (Borella et al. 2004, Fliermans et al. 1981, Loret et al. 2005, Messi et al. 2011, Wadowsky et al. 1988, Yee and Wadowsky 1982). As a result, the interpretation of this data does not take into account variations in water composition depending on the locality or the seasonality. Thus, there is lack of a standardized artificial freshwater medium to study *Legionella* and environmental factors affecting survival, and their subsequent effect on the virulence potential of the bacterium. A defined water medium will allow elimination of variations in water composition that are dependent on seasonality and geography. It will also provide a foundation to build water microcosm, whose complexity can be increased gradually, thus allowing the evaluation of each additional parameter separately.

We have previously shown that the sigma factor RpoS, and the stringent response is required for *Lp* to survive in water (Trigui et al. 2014). Consequently, the RpoS mutant was unable to survive in tap water, and in two defined freshwater media, DFM and Fraquil. DFM composition was derived from the salt and buffer content of the chemically defined media used for *Legionella* growth (Reeves et al. 1981), and contains 50mg/L NaCl, 20 mg/L KH_2PO_4 and 50 mg/L KCl. Fraquil is an approximation of freshwater found in North America (Morel et al. 1975) (see Table 1 for the composition of Fraquil). Our previous results show that *Lp* survive in Fraquil

as well as in tap water for 30 days, but show a slight defect in DFM (Trigui et al. 2014). Therefore, we decided to further investigate the behaviour of *Lp* in Fraquil. Here, we use Fraquil to study the impact of individual environmental factors on *Lp* in a controlled water environment. To further this goal, the consequences of changing temperature, acidity and trace metal content over a short time period were investigated by tracking the capacity of *Lp* to survive in Fraquil under these conditions, and its subsequent capacity for intracellular multiplication (ICM) in amoeba. Furthermore, we investigated the long-term influence of temperature on the survival of *Lp* and on its ICM potential.

3.3 Materials & Methods

3.3.1 Bacterial Strains and Media

All experiments were conducted using JR32 or its derivatives. JR32 is a salt-sensitive, streptomycin-resistant, restriction negative mutant of *Legionella pneumophila* (*Lp*) strain Philadelphia 1 (Sadosky et al. 1993). The *dotA*$^-$ strain, used as a negative control in intracellular multiplication (ICM) assays, is a transposon mutant carrying a mutation in the type IVb secretion system that is essential for intracellular multiplication in *Lp* (Sadosky et al. 1993). Strains stored at -80^{o}C in 10% glycerol were grown on BCYE (buffered charcoal yeast extract) agar supplemented with 0.25mg/ml L-cysteine and 0.4mg/ml ferric pyrophosphate. AYE broth (BCYE without agar and charcoal) was used as the liquid medium. The defined water medium used for water exposure experiments, Fraquil was prepared as described by Morel et al. (1975) with a final iron concentration of 10nM and was filter-sterilized using a 0.2µm filter (Sarstedt). Ultrapure Type 1 water (18.2 MΩ·cm at 25 °C), produced with a Synergy Ultrapure Water System (EMD Millipore), was used to prepare Fraquil. The complete composition of Fraquil is presented in Table 1.

Table 3.1 Composition of Fraquil

Components	Concentration
$CaCl_2 \cdot 2H_2O$	0.25µM
$MgSO_4 \cdot 7H_2O$	0.15µM
$NaHCO_3$	0.15µM
K_2HPO_4	10nM

$NaNO_3$	0.1μM
$FeCl_3 \cdot 6H_2O$	10nM
$CuSO_4 \cdot 5\ H_2O$	1nM
$(NH_4)_6Mo_7O_{24} \cdot 4\ H_2O$	0.22nM
$CoCl_2 \cdot 6\ H_2O$	2.5nM
$MnCl_2 \cdot 4\ H_2O$	23nM
$ZnSO_4 \cdot 7\ H_2O$	4nM

3.3.2 Water Exposure Experiments

JR32 cultured on BCYE agar at 37°C for 3 days was washed three times with Fraquil and suspended in fresh Fraquil at an OD_{600nm} of 0.1. Then, 1ml of the bacterial suspension was mixed with 4ml of fresh Fraquil in a $25cm^2$ cell culture flasks. Three biological replicates were used for each experiment. To test the impact of temperature, the flasks were incubated at six temperatures: 4°C, 10°C, 25°C, 30°C, 37°C, 42°C. For water experiments with varying pH, aliquots of Fraquil were adjusted to pH 4, 5, or 6 with 0.01M HCl and *Lp* was suspended at each pH as described above. To test the effect of varying trace metal concentrations, Fraquil was prepared without the addition of trace metal (0X), by adding twice the volume of trace metals than standard Fraquil (2X) or by adding 10 times the volume of trace metals (10X). Standard Fraquil (1X metals, please refer to Table 3.1 for composition) was used as a control. *Lp* was suspended in each trace metal concentration as described above. For each environmental parameter tested, CFU counts on BCYE agar were used at defined time points to track survival over time.

3.3.3 Intracellular Multiplication Assays

Intracellular multiplication was measured in the amoeba, *Acanthamoeba castellanii* and THP-1-derived human macrophages with a multiplicity of infection (MOI) of 0.1. *A. castellanii* was cultured in peptone yeast glucose (PYG) broth (Moffat and Tompkins 1992). PYG contained 20g/L proteose peptone, 1g/L yeast extract, 0.1M glucose, 0.4mM $MgSO_4$, 0.05mM $CaCl_2$, 0.1mM sodium citrate, 0.005mM $Fe(NH_4)_2(SO_4)_2$, 0.25mM Na_2HPO_4 and 0.25mM KH_2PO_4, and the pH was adjusted to 6.5 using 1M HCl. Each infection well in a 24-well plate was seeded with $5x10^5$ *A. castellanii* cells in 1ml of PYG. One hour prior to infecting *A. castellanii* with *Lp*, the

media in each well was replaced with 1ml of Ac buffer (PYG without proteose peptone, yeast extract and glucose). The plate was incubated for an additional hour at 30^{o}C before introducing approximately 5 x 10^4 *Lp* to each well. THP-1 monocytes were cultured in RPMI (GIBCO) supplemented with L-glutamine and 5% FBS. For the THP-1 infection, 5x10^5 cells treated with 10^{-7} nmol phorbol myristate acetate (PMA) were seeded into a 24-well plaque in 1ml of RPMI 3 days prior to infection and left to incubate at 37^{o}C in 5% CO_2. One hour prior to infecting the macrophages with *L. pneumophila*, the media in each well was replaced with fresh RPMI. THP-1 cells were infected with approximately 5 x 10^4 *Lp* to each well.

The infection wells were sampled daily to detect the extracellular increase of CFUs in Ac buffer relative to time zero. The laboratory wild type JR32 was used as a positive control, while the *dotA*$^-$ mutant, deficient in intracellular replication, served as a negative control. Both control strains were grown on BCYE agar and suspended in AYE broth at an OD_{600} of 0.1, and were then further diluted 10 fold to obtain an approximate OD_{600} of 0.01. 2µl of this final solution was used to infect the amoeba. CFU counts at 24-hour intervals tracked the intracellular growth of the bacteria inside host cells and subsequent release in the medium.

For bacterial samples originating from water experiments testing temperature, pH or trace metal content, a CFU count was done 3 days prior to the infection. On the day of the infection assay, this count was used to determine the volume representing 1 x 10^4 bacterial cells, thus resulting in an MOI of 0.1. When necessary, bacteria were diluted in Fraquil.

3.3.4 Statistical Analysis

The graphs show the average of at least three biological replicates and the standard deviation. We used unpaired the one tail Student's T-test to access statistical significance.

3.4 Results

3.4.1 Short-term effect of temperature on the survival of *Lp* and subsequent ICM potential

We tested the survival of *Lp* in Fraquil, hereafter called water, exposed to six different temperatures ranging from refrigeration to the high end of the temperature spectrum recorded as supporting *Legionella* growth (Rowbotham 1980): 4^{o}C, 10^{o}C, 25^{o}C, 30^{o}C, 37^{o}C and 42^{o}C. Using CFU counts, the survival of the JR32 strain was monitored for 49 days (Fig 3.1A). It was clear that changes in temperature had a definite impact on the survivability of *Lp* in this water system.

The 42^{o}C experimental condition resulted in the fastest CFU decline starting at 15 days, reaching the detection limit of 100 CFU/ml in all three replicates after 28 days. At 37^{o}C, which is in the range of optimal growth temperatures for *Legionella*, bacterial numbers were stable for approximately 1 month after which the CFU counts decreased dramatically reaching the detection limit at the end of 42 days. A moderate dip in the CFU counts was observed at 30^{o}C at day 42, while *Lp* incubated at 25^{o}C showed no decrease in bacterial counts during the 49-day tracking period testing survivability in water. At 10^{o}C, *Lp* seems to maintain relatively stable bacterial counts. The set of samples at 4^{o}C showed a small decrease in CFU counts after approximately two weeks and then stabilized for the remainder of the time tested (49 days). Our results show that even moderate temperatures between 30^{o}C to 42^{o}C significantly impact the survivability of *Lp* in a minimal water system (Fig 3.1A).

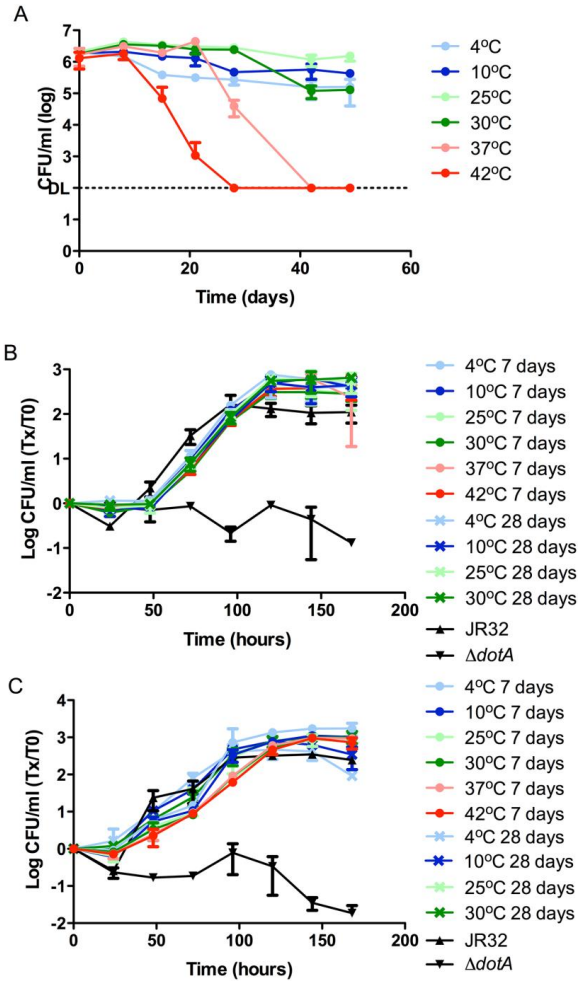

Figure 3.1: Impact of temperature on the short-term survival of *Lp* in Fraquil and intracellular multiplication (ICM) after exposure to different temperatures. A) The JR32 strain was exposed to Fraquil at six different temperatures. Weekly CFU counts were performed to track survival. DL, detection limit. At 4^{o}C, 10^{o}C, 37 ^{o}C and 42 ^{o}C, CFU counts from 8 days were statistically different ($P \leq 0.05$) than CFU counts at 25°C. At 30^{o}C, CFU counts from day 42 were statistically different that CFU counts at 25°C. **B)** *A. castellanii* was infected with JR32 that had been exposed to the respective temperatures tested for 7 days or 28 days at an MOI of 0.1. Daily CFU counts monitored the ICM inside amoeba and are presented as the ratio over CFU counts at day 0. JR32 from BCYE was used as the positive control and *dotA*$^{-}$ was used as the negative control. **C)** Cultured human macrophages were infected with JR32 that had been exposed to the respective temperatures tested for 7 days or 28 days at an MOI of 0.1. Daily CFU counts monitored the ICM and are presented as the ratio over CFU counts at day 0. JR32 from BCYE was used as the positive control and *dotA*$^{-}$ was used as the negative control.

Following water exposure, we tested the infectivity of *Lp* incubated at different temperatures towards *Acanthamoeba castellanii*, a natural host of *Legionella* found in natural and man-made water systems (Rowbotham 1980). The *dotA*$^{-}$ mutant strain was used as a negative control in the infection assays due to its deficiency in intracellular multiplication (ICM) in *A. castellanii* as a result of a Type IVb secretion system defect (Sadosky et al. 1993). To detect any changes of virulence potential in response to different incubation times in water, we used bacteria that had been exposed to the respective temperatures for both 7 and 28 days (Fig 3.1B). *Lp* exposed to 37^{o}C and 42^{o}C was not used in the 28 day infections since the CFU counts were either too low (37^{o}C) or non-existent (42^{o}C). The volume of the 37^{o}C samples required to infect the cells at an MOI of 0.1 was too large and would have compromised the dynamics of the infection assay, thus making the results unreliable. At the end of the infection cycle (168 hours), there was no significant difference in the CFU increase during infection between the different temperatures. Moreover, no significant differences were observed between 7-day and 28-day exposure times. As for the infection of amoeba, there were no significant differences between the positive control and the bacterial cultures originating from water. Since no significant difference was observed between temperature-treated samples in macrophages, subsequent ICM experiments were conducted exclusively in *A. castellanii*, which is more relevant in the context of the water environment.

These infection assay results show that *Lp* is able to maintain its ICM potential for at least 28 days post-incubation in the nutrient-deficient water environment at temperatures ranging from 4^{o}C to 30^{o}C, and retains ICM at least 7 days after exposure to water at 37^{o}C and 42^{o}C, temperatures that are environmentally relevant (Fig 3.1B and 3.1C).

3.4.2 Effect of trace metal concentrations on the survival and subsequent ICM of *Lp*

To test whether a water environment with a reduced level of trace metals impairs the ability of *Lp* to survive, or if an increase in these metal concentrations may help it survive better, we tested four different trace metal concentrations in Fraquil; namely without the addition of trace metals (0X), standard Fraquil (1X), double the concentration of trace metals (2X) and 10 times the concentration of trace metals (10X) used in standard Fraquil (Fig 3.2A). Over a period of 56 days, there were no observable significant differences in the CFU counts between *Lp* exposed to different trace metal concentrations.

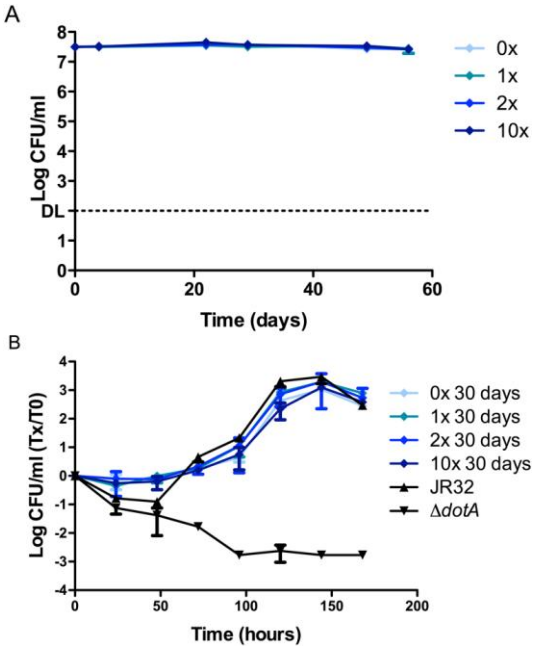

Figure 3.2: Survival of *Lp* at different trace metal concentrations and subsequent intracellular multiplication (ICM). A) The JR32 strain of *Lp* was exposed to Fraquil at four different metal concentrations: no addition of trace metals (0X), standard Fraquil (1X), double the quantity of trace metals (2X) and 10 times the quantity of trace metals (10X) than in standard Fraquil. Weekly CFU counts were performed to track survival. DL, detection limit. **B)** *A. castellanii* was infected with JR32 that had been exposed to the respective levels of trace metals tested for 30 days at an MOI of 0.1. Daily CFU counts monitored the ICM inside amoeba and are presented as the ratio over CFU counts at day 0. JR32 from BCYE was used as the positive control and *dotA*$^{-}$ was used as the negative control.

The ICM capacity of *Lp* exposed for 30 days to different trace metal concentrations in Fraquil was tested (Fig 3.2B). The ICM rate of the water exposed *Lp* samples were comparable to that of *Lp* cultured on BCYE agar (positive control). Furthermore, no difference was observed in ICM between *Lp* exposed to different trace metal concentrations. Therefore, it would seem that exposure to water containing a higher amount of trace metals, or a lack thereof, does not impact the ICM of *Lp* in the amoebal host.

3.4.3 Effect of pH on the survival and subsequent ICM of *Lp*

The survival of *Lp* in Fraquil at three different pH levels (4, 5 & 6) was tested in addition to the standard Fraquil whose pH hovers around 7.3. The pH experiment was conducted at 25^{o}C since it is a permissive temperature for the survival of *Lp* in water (Fig 3.1A). Changing the pH had a much more drastic effect on the survivability of *Lp* compared to temperature variations. When the water medium was adjusted to a pH value of 4, CFU counts steadily declined to the detection limit within 33 days (Fig 3.3A). At a pH value of 5, the CFU counts started to visibly decrease after 24 days of incubation in the defined water medium. The higher pH values of 6 and 7.3 of normal Fraquil resulted in no loss of culturability over the course of the experiment (52 days).

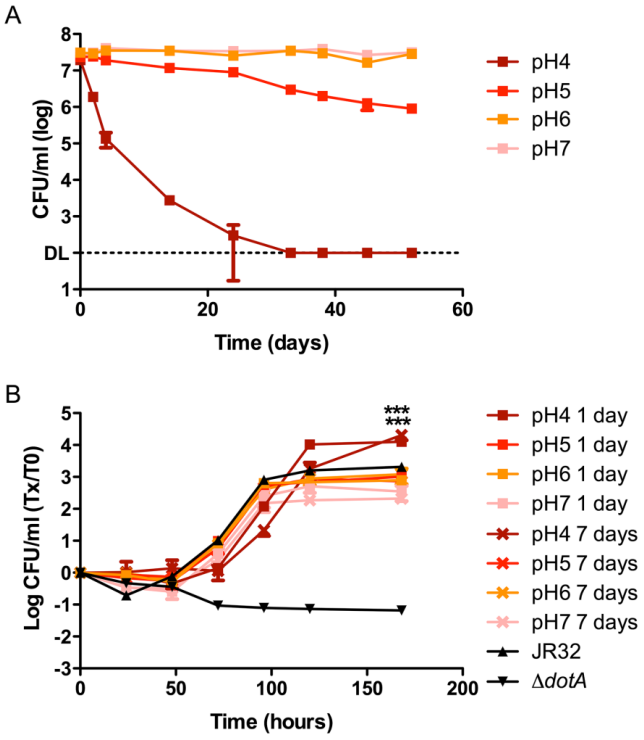

Figure 3.3: Impact of pH on the short-term survival of *Lp* in Fraquil and intracellular multiplication (ICM) after exposure to different pH. A) The JR32 strain of *Lp* was exposed to Fraquil at four different pH. Weekly CFU counts were performed to track survival. DL, detection limit. At pH 4 and pH 5, CFU counts from 2 days are statistically different ($P \leq 0.05$) than CFU counts at pH 7. At pH 6, CFU counts from 38 days are statistically different than CFU counts at pH 7. **B)** *A. castellanii* was infected with JR32 that had been exposed to the respective pH tested for either 1 day or 7 days at an MOI of 0.1. Daily CFU counts monitored the ICM inside amoeba and are presented as the ratio over CFU counts at day 0. JR32 from BCYE was used as the positive control and *dotA*$^-$ was used as the negative control. *** $P \leq 0.005$ versus control.

The virulence potential of *Lp* incubated in acidified Fraquil was tested by following the intracellular multiplication (ICM) in the same manner used for the temperature and trace metal variation experiments in *A. castellanii*. Since the CFU counts decreased dramatically in the first week of exposure to pH 4, we used *Lp* that had been exposed to water for 24 hours and for 7 days to test the ICM in amoeba. Interestingly, compared to samples at pH 7 and the positive control, incubation in Fraquil at pH 4 at both exposure times resulted in a statistically significant, approximate 1 log increase of CFU counts at the end of a 168 hour infection cycle (Fig 3.3B).

The ICM of *Lp* exposed to pH 5, pH 6 and standard Fraquil produced a similar increase in bacterial load upon infection of *A. castellanii* compared to *Lp* originating from rich media.

3.4.4 Long-term survival of *Lp* in water and subsequent effect on ICM

To test whether *Lp* was able to survive in Fraquil over a longer period of time, inoculums were tested at four temperatures (4^{o}C, 15^{o}C, 25^{o}C and 37^{o}C). The lower end of the temperature spectrum was used, since higher temperatures were found to be detrimental to *Lp* in the short-term (Fig 3.1A). We used 37^{o}C as a short-term survival temperature control. CFU counts were tracked over a period of 211 days in total (Fig 3.4A). As was expected (Fig 3.1A), the fastest decline in the bacterial population was observed at 37^{o}C, where no CFUs could be detected on agar plates after 70 days. At 25^{o}C, inoculums were stable for 98 days after which the CFU counts started to decrease, reaching the detection limit by 176 days. At 4^{o}C, the CFU counts reached the detection limit by 149 days. We tested long-term survival at 15^{o}C instead of 10^{o}C. *Lp* was initially exposed to 15^{o}C for 176 days, but the flasks were incubated at 10^{o}C for the following 32 days prior to testing ICM. On the long term, *Lp* survived at 15^{o}C even better than at 25^{o}C showing no significant decrease in CFU counts until 183 days.

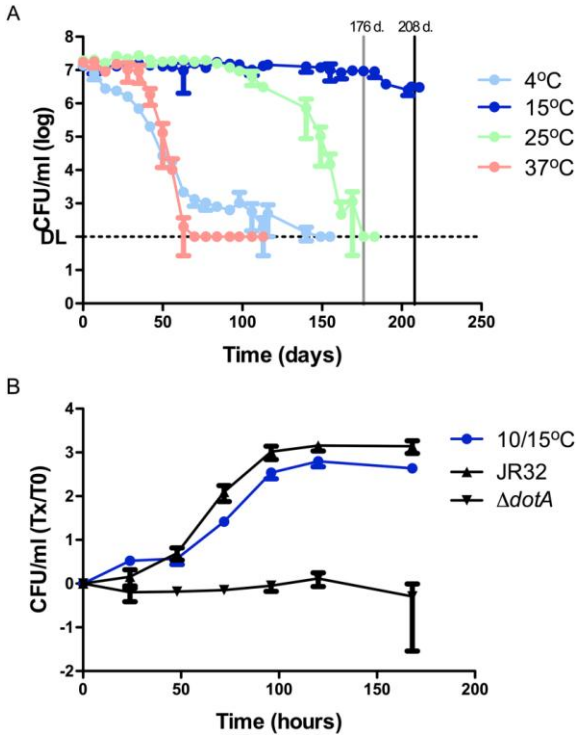

Figure 3.4: Long-term survival of *Lp* in Fraquil and intracellular multiplication (ICM) after long-term exposure to a moderate temperature. A) The JR32 strain was exposed to Fraquil and CFU counts were used to track survival over a 211-day time period. Weekly CFU counts were performed to track survival. DL, detection limit. The vertical lines indicate the time points at which the temperature of incubator was reduced from 15°C to 10°C (grey – 176 days) and when samples were harvested to infect amoeba (black – 208 days). At 4°C, CFU counts from 14 days are statistically different ($P \leq 0.05$) than CFU counts at 15°C. At 25°C, CFU counts from 113 days are statistically different than CFU counts at 15°C. At 37°C, CFU counts from 42 days are statistically different than CFU counts at 15°C. **B)** *A. castellanii* was infected with JR32 that had been exposed to 15°C for 176 days and 10°C for an additional 32 days at an MOI of 0.1. Daily CFU counts monitored the ICM inside amoeba and are presented as the ratio over CFU counts at day 0. JR32 from BCYE was used as the positive control and *dotA*$^-$ was used as the negative control.

To test whether the ICM capacity of *Lp* had been affected negatively or positively after a long incubation period in Fraquil, *A. castellanii* was infected with bacteria that had been incubated in water for a total of 208 days, 176 days at 15°C and another 32 days at 10°C. While

the increase in bacterial counts during the infection is statistically lower than that of *Lp* originating from rich media, *Lp* seems to retain most of its capacity for ICM even after an extended time in water and in the absence of any additional nutrients (Fig 3.4B).

3.5 Discussion

A variety of water distribution systems can harbour *Lp* including shower heads, hot water tubs and hot water tanks (Azuma et al. 2013, Dufresne et al. 2012, Roig et al. 2003). In fact, the design of some systems can further favour *Legionella* contamination and persistence in water, either by having cooler bodies of water within hot water systems or dead legs creating areas of stagnant water (Joly 1985, Thomas et al. 2004). For example, domestic water heating units in Quebec City, Canada, powered by electricity were shown to contain an area of water that was at a significantly lower temperature than the rest of the tank, thus, allowing the survival and growth of *Legionella* species in contaminated units (Dufresne et al. 2012, Joly 1985).

In this study, we have used Fraquil to explore the effect of temperature, pH and trace metal concentration on the survival of *Lp* and on its subsequent capacity to grow inside amoeba. It is noteworthy that the JR32 laboratory strain was used for this study and that environmental isolates of *Lp* may behave differently in Fraquil. We are currently screening multiple clinical and environmental isolates.

Several studies report finding the bacterium at temperatures exceeding 50^{o}C in the environment while others have shown that *Lp* is metabolically active above 45^{o}C (Fliermans et al. 1981, Kusnetsov et al. 1996, Moritz et al. 2010, Qin et al. 2013, Tison et al. 1980, Wadowsky et al. 1982). In rich media, *Lp* grows optimally under laboratory conditions between 25^{o}C and 37^{o}C. Early studies also showed that *Lp* is able to multiply in unsterilized tap water containing amoeba between 25^{o}C and 42^{o}C over a period of 21 days, but that it could not replicate in the absence of amoeba at temperatures above 37^{o}C (Wadowsky et al. 1988, Wadowsky et al. 1985, Yee and Wadowsky 1982). Wadowsky et al. (1985) demonstrated that an environmental isolate survived for 28 days in distilled water at 35^{o}C. An early study by Dennis et al. (1984) showed that *Lp* is more temperature tolerant on the short term than *Pseudomonas* and *Micrococcus* species, both of which are found in water distribution systems with *Lp*. This characteristic has been taken advantage of when isolating *Legionella* spp. from environmental sources, using a

mild heat treatment to increase isolation (AFNOR. 2010, Guillemet et al. 2010). Therefore, we expected to find that *Lp* would be relatively resistant to heat in Fraquil, the artificial freshwater medium used in this study. To our surprise, we found that *Lp* survived for only 6 and 3 weeks at 37^{o}C and 42^{o}C, respectively. Our study suggests that the reported persistence of *Lp* in water systems at high temperatures is positively affected by variables other than temperature; these variables may include protection inside thermophilic amoebal hosts, amoeba cysts or inside biofilm (Kilvington and Price 1990, Pryor et al. 2004, Wadowsky et al. 1988).

The steady decrease in CFUs observed at 4^{o}C (Fig 3.4A) is consistent with recorded loss of culturability at 4^{o}C, but the survival or culturability periods vary according to different groups (Borella et al. 2005a). For example, Wadowsky et al. (1985) observed that storing environmental samples at 5^{o}C resulted in a decrease of CFUs. In addition, the earliest reports of viable-but-nonculturable (VBNC) *Lp* cells elude to a 4^{o}C incubation temperature inducing this state (Hussong et al. 1987). We, therefore, suspect that *Lp* enters a VBNC state in water at 4^{o}C; however, this will require further experimentation.

A moderate water temperature of 25^{o}C allowed *Lp* to survive approximately three months in Fraquil (Fig 3.4A). At 15°C, *Lp* survived for at least 208 days. Similarly, Paszko-Kolva et al. (1992) show that in drinking water samples, a clinical *Lp* strain has no significant difference in the CFU counts after incubation at 15^{o}C for approximately 200 days, but that CFU counts decreased more significantly when using creek or estuarian waters. This ability to survive over a period of several months would allow *Lp* to persist in a water system and replicate in amoebal hosts when the latter arrive into the system. Most community outbreaks of Legionnaires' disease occur in the late summer or early fall seasons (Carratalà and Garcia-Vidal 2010, Marston et al. 1994). A gradual increase of *Lp* in a water system over this time frame will eventually allow the bacterium to attain a concentration sufficient for disease transmission in humans during the spring and summer, and survive over the winter at lower temperature.

We demonstrate that *Lp* incubated at a temperature ranging from 4°C to 42°C were able to replicate in *A. castellanii* and in cultured human macrophages after exposure to water for at least one week, while *Lp* at lower temperatures maintained intracellular multiplication (ICM) capacity for at least 28 days (Fig 3.1B). Temperature is known to regulate the expression of virulence factors in other pathogenic bacteria like *Shigella*, *Streptococcus* and *P. aeruginosa*

(Grosso-Becerra et al. 2014, Maurelli et al. 1984, Smoot et al. 2001); however, the interplay between *Lp* virulence and temperature is yet to be clearly defined. A growth temperature of 37^{o}C was shown to increase virulence of *Lp* while growth at 24^{o}C results in an avirulent strain in a guinea pig model, establishing a first link between temperature and virulence of *Lp* (Mauchline et al. 1994). More importantly, the avirulence observed at 24^{o}C was corrected when the temperature was shifted to 37^{o}C (Mauchline et al. 1994). In contrast, another study showed that *Lp* grown at 25^{o}C is more lethal to guinea pig macrophages *in vitro* (Edelstein et al. 1987). While contradictory, both studies show a definite effect of temperature on the virulence of *Lp*. In addition, pili and flagella are expressed in *Lp* in a temperature-dependent manner, and the expression of both structures have been linked to the bacterium's virulence in host cells (Heuner et al. 1999, Heuner and Steinert 2003, Liles et al. 1998, Molofsky et al. 2005, Stone and Kwaik 1998). The type II secretion system is involved in the production of the type IV pili in *Lp* (Liles et al. 1998). Soderberg et al. (2008) reported that the type II secretion system of *Lp* allowed survival in water at 4^{o}C and 17^{o}C and also played a role in intracellular multiplication (ICM) in amoeba at 22^{o}C-25^{o}C. Moreover, pili were shown to play a role in adherence to both amoeba and human cells (Stone and Kwaik 1998). Flagella expression has also been demonstrated to be temperature regulated and is implicated in virulence (Heuner et al. 1999, Heuner and Steinert 2003).

Moreover, we demonstrate that *Lp* incubated at 15^{o}C were able to infect and kill amoeba after resting in Fraquil for approximately six months, albeit at a slightly lower rate than the positive control (Fig 3.4B). Our results suggest that, even in the absence of its natural hosts and lack of sufficient nutrients for growth (i.e. without the contamination of water systems by organic material), *Lp* may still pose a significant threat to public safety for a long period of time, as it remains virulent and competent for ICM. Therefore, *Lp* is able to easily linger in a clean water system for many months before coming into contact with its amoeba prey.

The presence of some metals has been linked to a decrease of *Legionella* in water systems while other metals are considered contributors to its survival (Manske and Hilbi 2014). Copper and silver ions are used to disinfect water distribution systems and have been studied for their negative effects on *Legionella* species, and they are known to decrease contamination levels in conjunction with other treatments (Cachafeiro et al. 2007, Landeen et al. 1989). Indeed, a

negative correlation is observed between the incidence of *Lp* and elevated trace levels of copper ions (Bargellini et al. 2011, Serrano-Suárez et al. 2013). In addition, as observed in the cases of many other bacteria, *Lp* requires iron for optimal growth on artificial media and in host cells as evidenced by its multiple iron acquisition systems (Allard et al. 2006, Cianciotto 2007, Ristroph et al. 1981, Robey and Cianciotto 2002, Viswanathan et al. 2000, Zheng et al. 2013). In accordance, *Lp* contamination is positively correlated with higher concentrations of iron in water systems (Bargellini et al. 2011, Edagawa et al. 2008, Serrano-Suárez et al. 2013). Furthermore, higher concentrations of manganese, zinc and cobalt have also been shown to correlate positively with *Lp* contamination of water systems (Bargellini et al. 2011, Reeves et al. 1981).

No survival defect (Fig 3.2A) was observed in any of the trace metal variations that were tested. While a positive relationship has been shown between the concentrations of manganese, iron and zinc and the presence of *Lp* in hot water systems in some studies (Bargellini et al. 2011), no such correlation was found in others (Edagawa et al. 2008, Serrano-Suárez et al. 2013). There was no difference in ICM after 30 days of exposure to the respective trace metal concentrations (Fig 3.2B). The infection itself was performed in Ac buffer, which contains 5nM of iron. Therefore, any negative effects on ICM caused by the lack of iron in Fraquil without trace metals may have been rescued upon exposure of *Lp* to Ac buffer. The Ac buffer does not contain the other trace metals found in Fraquil; therefore, it is possible to conclude that the trace metals in Fraquil other than iron do not affect the ICM of *Lp* in the concentrations tested. Our results suggest that the impact of the concentration of trace metals on the survival and growth of *Lp* in water systems is linked to other conditions, potentially the presence of susceptible amoeba hosts.

Once inside a host cell, *Lp* grows within the *Legionella* containing vacuole (LCV), evading the normal endocytic pathway of its host by hijacking the host cell machinery (Horwitz 1983b). While virulent *Lp* disturbs the acidification of the LCV, the recruitment of V-ATPases that carry out the acidification is not fully blocked in a small number of cases (Sturgill-Koszycki and Swanson 2000, Xu et al. 2010). Therefore, there is a biological need for *Lp* to have and to use mechanisms to survive pH stress. This may explain the relative hardiness of *Lp* toward varying pH levels that has been observed in nature and in man-made water systems. In fact, environmental sampling shows that *Lp* can be recovered at a wide range of pH (5.5 to 8.1) (Fliermans et al. 1981). Wadowsky et al. (1985) also found that an environmental isolate of *Lp*

was able to replicate in filter-sterilized tap water from pH 5.5-9.2. Furthermore, isolating environmental strains of *Legionella* is known to be greatly enhanced by an acid treatment, suggesting that it is relatively more tolerant to acid than other bacteria found in water (Bopp et al. 1981, Buesching et al. 1983). *Lp* has been shown to tolerate a pH 2 treatment for at least 30 minutes (Wang et al. 1979). More recently, the *Lp* genome was shown to encode carbonic anhydrases (Nishimori et al. 2014). These enzymes are involved in pH regulation and may have a role in the bacterium's survival inside the LCV, further supporting a relatively high level of tolerance to pH in *Legionella* species (Nishimori et al. 2014).

A low pH of 4 significantly affected the survival of *Lp* in the water environment, resulting in no CFU counts at the end of one month. It is conceivable that *Lp*, already under the stress of adapting to the nutritionally poor water environment, is unable to cope with the additional stress of an acidic pH. Variations in pH are known to cause the precipitation of some trace metals and have been used to evaluate trace metal contents in environmental samples (Lee et al. 2002, Tessier et al. 1979). A more acute deprivation of metal cofactors caused by precipitation reactions may be responsible for the sensitivity of *Lp* at low pH, but this hypothesis is negated by Fig 3.3A showing no survival defect when no trace metals are added to Fraquil. Moreover, exposure to pH 4 for 24 hours or seven days resulted in higher ICM (Fig 3.3B). Another study demonstrated that a pH 6.5 acid treatment over a 24 hour time period provided increased resistance to a second pH stress, to oxidative stress, and induced virulence in previously non-virulent strains (Bandyopadhyay et al. 2004). It is possible that prior exposure to pH 4 primes *Lp* for the intracellular environment that is reported to attain a pH value of 5.6 (Allombert et al. 2014, Sturgill-Koszycki and Swanson 2000). This possibility will require further study.

3.6 Conclusions

We have successfully used Fraquil to investigate the survival of *Lp* in water. Temperature and pH were found to have a determinant effect on the survival, but the trace metal concentration does not impact survival of *Lp*. The ICM of *Lp* seems to increase in response to low pH, but the concentration of trace metals and temperature seem to have little effect on its ICM capacity. Importantly, our results show that *Lp* retains its ability to infect host cells after long-term survival in Fraquil. Our results support the use of Fraquil as a defined freshwater medium to

study the biology of *Lp* in water systems, such as the transcriptomic response of *Lp* to Fraquil (Li et al. 2015), and to facilitate the interpretation of datasets originating from different research groups.

3.7 Acknowledgements

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CONNECTING TEXT

Based on Chapter 3, Fraquil was determined to be a suitable proxy for water found within anthropogenic systems. In the next step, we assayed the contribution of several regulatory genes to survival in water. Given that nutrient deprivation is a key signal in the differentiation of *Lp in vitro* and *in vivo*, genes that are involved in the transition from the replicative to the transmissive phase were first tested for their role in adapting to and surviving the starvation condition of water. One gene in particular, *letS*, caused a significant defect that is less severe, but similar to a stringent response mutant, making it a viable candidate for further study. The regulatory pathway under LetS control is currently known as a major virulence determinant in *Lp*. This is the first study demonstrating its important role in water survival. I also look at the transcriptomic changes under LetS control in water. Finally, I investigate whether the cellular alarmone ppGpp that initiates the stringent response also activates the LetS sensor kinase.

Contributions of authors: Nilmini Mendis contributed to experimental design, conducted the survival experiments at 4^{o}C and 25^{o}C, constructed SPF291, performed and analyzed the microarray, analyzed qPCR results, conducted other phenotypic characterizations (microscopic observation, heat shock and pigment production), Northern Blots and wrote the manuscript. Peter McBride conducted the survival experiments, assisted in constructing SPF291, performed the microarray, performed qPCR, and analyzed the transcriptomic and microscopic data. Sebastien Faucher constructed SPF41, contributed to the experimental design, writing and editing the manuscript.

This chapter will be submitted for publication.

Chapter 4: The LetA/S two-component system is essential for the survival of *Legionella pneumophila* in water

4.1 Abstract

Surviving the nutrient-poor aquatic environment for extended periods of time is important for transmission of various water-borne pathogens to the host, including *Legionella pneumophila* (*Lp*). *Lp* is a leading cause of community-acquired and nosocomial pneumonia called Legionnaires' disease. The remarkable ability of the bacterium to survive in water for periods ranging from several months to years under starvation conditions alludes to regulatory pathways that mediate adaptation to the water environment. In the present study, we investigated a potential role for the LetA/LetS signal transduction system in the successful survival of *Lp* in water. During infection of host cells, the LetA/LetS two-component system controls the transition from the replicative phase to the transmissive phase in response to nutrient deprivation. In accordance with previous work, the *letS* mutant used in the present study is defective for pigment production and contributed to cell size reduction in the post-exponential phase. LetS also contributed to cell size reduction when *Lp* was exposed to water. Importantly, absence of the sensor kinase resulted in a significantly lower survival rate in water at 4^{o}C, 25^{o}C and 42^{o}C. Transcriptomic analysis indicated that a general transcriptomic downshift of major pathways is orchestrated by LetA/LetS upon water exposure leading to better survival, suggesting a potential link with the stringent response. However, the expression of the LetA/S regulated small regulatory RNAs RsmY and RsmZ was not changed in a *relAspoT* mutant, which indicates that the stringent response and the LetA/S response are two distinct regulatory systems important for the survival of *Lp* in water.

4.2 Introduction

Legionella pneumophila (*Lp*) is a bacterium ubiquitous in many freshwater environments (Fliermans et al. 1981). *Lp* is an intracellular parasite of amoeba, and mainly targets alveolar macrophages in the context of human infection (Buse and Ashbolt 2011, Prashar and Terebiznik 2015, Tyson et al. 2013). During intracellular replication, *Lp* undergoes morphological and underlying transcriptional changes that allow transition from a replicative state, where the bacterium undergoes rapid replication when host nutrients are abundant, to a transmissive phase state when the nutrient sources are exhausted (Byrne and Swanson 1998, Garduño 2008). Its transmission to and infection of the human host is incumbent upon successful survival in relatively harsh, man-made water systems. The inhalation of *Lp* contaminated aerosols that are generated from these systems transmits the bacterium to the human lungs, where it proliferates (Borella et al. 2005a, Fields et al. 2002, Zimmerman 1979). Therefore, identifying the molecular mechanisms that *Lp* uses to survive in water is crucial, not only to better understand the operating system of the organism, but also for better water systems management.

Bacteria employ two-component systems (TCSs) to sense and respond to a variety of environmental stresses, ranging from temperature, antibiotics, quorum sensing autoinducer molecules and intermediates of the TCA cycle (Krell et al. 2010, Mascher et al. 2006). TCSs consist of a sensor kinase (SK), that can detect an environmental stimulus, and a response regulator (RR), that can bind and control the transcription of target genes (Capra and Laub 2012, Stock et al. 2000). Upon sensing an activating signal, the SK autophosphorylates a conserved histidine residue. This phosphoryl group can then be shuttled to an aspartate residue on the cognate RR, which will initiate the downstream transcriptional changes that allow the bacterium's response to the environment (Capra and Laub 2012, Stock et al. 2000).

The LetA/LetS TCS of *Lp* (Hammer et al. 2002) is the ortholog of BarA/UvrY in *Escherichia coli* (Pernestig et al. 2001), GacS/GacA in *Pseudomonas* spp. and *Erwinia* spp. (Cui et al. 2001, Marutani et al. 2007), VarS/VarA in *Vibrio* spp. (Lenz et al. 2005), BarA/SirA in *Salmonella* spp. (Teplitski et al. 2003) and ExpS/ExpA in *Pectobacterium* spp. (Broberg et al. 2014). Prototypical sensor kinases have a histidine phosophotransferase domain that autophosphorylates a conserved histidine residue with the aid of a catalytic ATPase domain (Capra & Laub 2014). LetS belongs to a family of tripartite sensor kinases which deviate from

this traditional model. Similar to its well-studied counterpart BvgS in *Bordetella* spp., LetS architecture includes 3 major domains (transmitter, receiver and histidine phosphotransfer domains) that are involved in a phosphorelay which activates its cognate response regulator LetA (Dupré et al. 2015, Edwards et al. 2010). Upon activation by an environmental signal, the transmitter domain (T) is phosphorylated first by accepting a phosphate group from an ATP molecule. This, in turn, phosphorylates the receiver domain (R) and finally, the histidine phosphotransfer domain (HPT) receives the phosphate from R and relays it to the response regulator LetA (Edwards et al. 2010). The modular nature of this SK has allowed it to act as a rheostat; the TCS can be activated by multiple stimuli, where each stimulus leads to the transcription of a different set of genes (Cotter and Miller 1997, Deora et al. 2001). Following the rheostat model of BvgS in *Bordetella*, LetS was shown to regulate only a subset of post-exponential phase genes in response to nicotinic acid (Edwards et al. 2010).

The RRs of orthologous TCSs activate transcription of small regulatory RNAs (sRNAs): CsrB/C in *E. coli* and in *Salmonella* (Teplitski et al. 2003, Zere et al. 2015), RsmW/X/Y/Z in *Pseudomonas* (Kay et al. 2005, Miller et al. 2016), and CsrB/C/D in *Vibrio cholera* (Lenz et al. 2005). Those sRNAs sequester a messenger RNA (mRNA) binding protein: CsrA in *E. coli, V. cholera, S. enterica* (Altier et al. 2000, Lenz et al. 2005, Suzuki et al. 2002) or RsmA in *Pseudomonas* (Miller et al. 2016). Since the CsrA/RsmA protein binds to target mRNA and inhibits their translation, sequestration of these proteins by sRNAs allows the translation of the target mRNAs (Altier et al. 2000, Lenz et al. 2005, Pessi et al. 2001, Suzuki et al. 2002, Zere et al. 2015). These TCSs and their accompanying regulatory cascade are involved in the virulence phenotypes of a number of pathogens, including *Salmonella*, where it is involved in the regulation of pathogenicity islands (Ahmer et al. 1999, Marcus et al. 2000, Martínez et al. 2011), *E. coli* through regulation of fimbriae and exopolysaccharide production (Herren et al. 2006, Mitra et al. 2013, Palaniyandi et al. 2012), *Pseudomonas* spp. (Coggan and Wolfgang 2012, Heurlier et al. 2004, Kay et al. 2005, Marutani et al. 2007, Pessi et al. 2001) and *Vibrio cholerae* (Mey et al. 2015). Members of this TCS family are also involved in quorum sensing, stress resistance, biofilm formation, switching between glycolytic and gluconeogenic carbon sources, and iron acquisition in various bacterial species (Frangipani et al. 2014, Herren et al. 2006, Lenz et al. 2005, Mitra et al. 2013, Pernestig et al. 2003, Pessi et al. 2001). In *Lp*, LetA binds to the promoters and positively affects transcription of the two sRNAs, RsmX, RsmY and RsmZ (Sahr

et al. 2009, Sahr et al. 2012). RsmX is, however absent in the *Lp* Philadelphia strain and *L. longbeachae*, where only RsmY/Z expression is induced by LetA (Sahr et al. 2012). RsmY/Z antagonizes the mRNA-binding protein CsrA, which represses PE phase traits (Molofsky and Swanson 2003). Competitive binding of CsrA by RsmY/Z activates the post-exponential (PE) phase and transmissive phase traits in *Legionella* (Forsbach-Birk et al. 2004, Hammer et al. 2002, Molofsky and Swanson 2003, Sahr et al. 2009). As a result, mutations within this cascade have been linked to attenuated virulence within host cells, motility, as well as sensitivity to oxidative and acidic stress (Hammer et al. 2002, Lynch et al. 2003, Molofsky and Swanson 2003, Sahr et al. 2009).

The present study establishes the role of the LetA/S regulatory cascade in a relevant water model. Similar to what is reported in rich media and in host cells, evidence presented in this report shows that LetS is responsible for initiating morphological changes, as well as a genome wide repression of major pathways in response to water exposure. Northern blotting showed that the cellular alarmone, ppGpp, is not an activating signal for the sensor kinase, advocating other environmental stimuli to be investigated.

4.3 Materials and Methods

4.3.1 Bacterial Strains and Media

The wild-type strain KS79 is a Δ*comR* mutant of the JR32 strain rendering it constitutively competent. JR32 is a salt-sensitive, streptomycin-resistant, restriction negative mutant of *Lp* strain Philadelphia 1 (Sadosky et al. 1993). A complete list of strains used in this study can be found in Table 4.1. Bacterial strains stored at -80^{o}C in 10% glycerol were grown on CYE (charcoal yeast extract) agar supplemented with 0.1 mg/ml α-ketoglutarate, 0.25 mg/ml L-cysteine and 0.4 mg/ml ferric pyrophosphate (Feeley et al. 1979). AYE broth (CYE without agar and charcoal) was used as the liquid medium (Feeley et al. 1979). When necessary, media were supplemented with 5μg/ml chloramphenicol, 2.5 μg/ml kanamycin and/or 0.1mM isopropyl β-D-1-thiogalactopyranoside (IPTG).

4.3.2 Deletion of *rsmY* and *rsmZ*

Construction of the Δ*rsmYZ* strain was performed by allelic exchange as previously described (Hovel-Miner et al. 2009), replacing first *rsmY* with a kanamycin resistance cassette

and then *rsmZ* with a gentamicin resistance cassette. Two 1-kb fragments corresponding to the upstream and downstream of *rsmY* were amplified using primers rsmY-BF/rsmY-BR and rsmY-EF/rsmY-ER, respectively. A kanamycin cassette was amplified from pSF6 using primers rsmY-BRKN/rsmY-EFKN. PCR fragments were purified on gel using a gel extraction kit (Qiagen). The three fragments were ligated together by PCR using primers rsmY-BF/RsmY-ER. The resulting 3 kb fragment was purified on gel, as described above, and introduced into KS79 by natural transformation (Sexton and Vogel 2004). The recombinants were selected for kanamycin resistance and the allelic exchange confirmed by PCR. Deletion of *rsmZ* was performed similarly by using primers rsmZ-BF/rsmZ-BR and rsmZ-EF/rsmZ-ER to amplify the upstream and downstream fragments, and rsmZ-BRGT and rsmZ-EFGT to amplify and gentamycin cassette from pBBR1MCS-5. The resulting 3 kb fragment was introduced into the Δ*rsmY* strain, recombinants were selected for kanamycin and gentamicin resistance and the deletion of *rsmZ* was confirmed by PCR. The resulting strain was named SPF41. Northern blot was used to confirm absence of expression of both sRNA. All PCR amplifications were carried with Phusion polymerase (NEB) according to the manufacturer's protocol.

4.3.2 Cloning of *rsmY*

The *rsmY* gene was amplified with its own promoter from the KS79 wild-type strain using primers rsmY-F and rsmY-R (Table 4.2). The pXDC39 vector and the amplicon were then digested with BamHI and HindIII (New England Biolabs) according to the manufacturer's protocol, and purified using a QIAGEN MiniElute Purification kit. The digested vector and insert were ligated overnight using T4 DNA Ligase (New England Biolabs) at 16^{o}C. The recombinant plasmid was then transformed into *E. coli* DH5α (pSF86). The transformed population was incubated at 37^{o}C shaking for 90 minutes before plating on 25ug/ml chloramphenical plates. Colonies that grew on antibiotic plates were patched and tested by PCR for insertion of the *rsmY* gene into the vector. The recombinant plasmid was extracted using a QIAGEN Plasmid Extraction kit and introduced into the Δ*rsmYZ* mutant to produce the Δ*rsmYZ* + p*rsmY* strain (SPF291).

4.3.3 Survival in Water

Survival in water was tested in the artificial freshwater medium, Fraquil, as described previously (Li et al. 2015, Mendis et al. 2015). Briefly, *Lp* strains cultured on CYE agar at 37^{o}C

for 3 days were suspended in Fraquil at an OD_{600nm} of 0.1 and washed three times with Fraquil. One millilitre of this bacterial suspension was mixed with 4ml of fresh Fraquil in a $25cm^2$ cell culture flasks (Sarstedt) and incubated at 4^oC, 25^oC or 42^oC. To test survival in water at high cell densities, strains were suspended in Fraquil at an OD_{600nm} of 1 and washed three times with Fraquil. Five millilitres of this bacterial suspension was placed in $25cm^2$ cell culture flasks (Sarstedt) and incubated at 42^oC. Survival of the strains in water was monitored using CFU counts.

4.3.4 Microscopic Analysis

Lp strains were grown on CYE agar for 3 days at 37^oC. AYE broth was inoculated with the respective strains and grown to exponential phase (OD_{600} 0.4-0.7) or post-exponential phase (OD_{600} >3) at 37^oC shaking (200 rpm). To test the effect of water, strains were suspended in Fraquil at an OD_{600} of 1 and incubated at 42^oC for 2 or 24 hours. 20µl of each sample was placed on a clean microscope slide, covered with a cover slip. Samples were observed under 1000X magnification under oil immersion using digital phase contrast microscopy with a Nikon Eclipse 80i microscope (Nikon Coporations, Tokyo, Japan). The objective lens was a Nikon Plan Fluor 100X/1.30 oil lens with the following specifications: OFN25 ∞/0.17 WD 0.16. The illumination source was a 12V/100W long-life halogen lamp. A Nikon Digital Sight DS-Ri1 camera attachment was used to capture microscopic images with a 1920 X 1536 resolution. For each strain, at least 10 images of random microscopic fields were captured using the NIS Element Software (Nikon Instruments, Inc.). ImageJ 1.51f software (Schindelin et al. 2015) was used for quantitative analysis of cell length. 10 cells from 3 different fields of view were randomly chosen and analyzed for a total of 30 cells per strain per treatment. Multiplying cells (presence of a septum) were excluded and only individual, non-filamentous cells were used for analysis.

4.3.5 Pigment Production

Lp strains grown on CYE for 3 days at 37^oC were inoculated into AYE broth. Strains were grown to stationary phase at 37^oC shaking at 200 rpm. 10ml of each strain was pelleted at 4500 rpm for 10 minutes. The supernatant was then removed and filtered using a 0.2µm pore sized syringe filter. The optical density of the supernatant was measured at 550nm.

4.3.6 Heat Shock

Lp strains cultured on CYE agar at 37^{o}C for 3 days were suspended in Fraquil at an OD_{600nm} of 0.1. One milliliter aliquots of each strain were transferred to 13ml tubes (Sarstedt) and were allowed to acclimate to the water environment for 2 hours at room temperature. At the end of the incubation period, tubes were submerged in a 55^{o}C water bath. At each time point tested, three biological replicates from each strain were removed from the water bath and the CFU counts enumerated on CYE agar.

4.3.7 DNA Microarray

The WT, Δ*letS,* and the complemented strain induced (ON) or not (OFF) with IPTG were grown on CYE agar for 3 days at 37^{o}C. Each culture was suspended Fraquil in triplicate at an OD_{600} of 1. 20ml of each strain was placed in 75cm^2 cell culture flasks and incubated at 42^{o}C for 2 hours. After incubation, 10ml aliquots were pelleted for 5 minutes at 5000g. After centrifugation, the supernatant was removed, and the cell pellets were re-suspended in 1ml of TRIzol reagent (Invitrogen). Three biological replicates of each strain were used for the transcriptomic analysis. The three remaining replicates of each strain were preserved at -20^{o}C for further experimentation. RNA extractions were done according to the manufacturer's protocol. To remove DNA contamination, extracted RNA was subsequently treated with Turbo DNase (Ambion) as per the manufacturer's protocol. The purity and concentration of RNA were determined by UV spectrophotometry. Fifteen micrograms of RNA was labeled with aminoallyl-dUTP (Sigma) during reverse transcription (Supercript II, Life Technologies) using random hexamers (Life Technologies) as previously described (Faucher and Shuman 2013, Li et al. 2015). Genomic DNA was used as a reference channel and was labeled by random priming using Klenow fragments, aminoallyl-dUTP and random primers as described previously (Faucher and Shuman 2013). DNA was subsequently coupled to the succinimidyl ester fluorescent dye (Life Technologies) AlexaFluor 546 (for cDNA) or AlexaFluor 647 (for gDNA) following the manufacturer's protocols. The microarray used (GPL19458) and the protocol for hybridization, data acquisition and data analysis have been published previously (Li et al. 2015). Statistical analyses were performed using an unpaired one-tailed Student's *t*-test. Genes were considered differentially expressed if they demonstrated a ratio-to-control value of ±2-fold with a $P<0.05$.

4.3.8 qPCR

One replicate of the WT and Δ*letS* strains exposed to water as described above were used to validate the microarray results using qPCR. RNA was extracted as described above. 1µg of total RNA was transcribed to cDNA (Supercript II, Life Technologies) using random hexamers. qPCR was performed as described previously using the primers described in Table 4.2 (Li et al. 2015). Ct values were normalized to the 16S rRNA.

4.3.9 Northern Blot

Lp strains grown on CYE agar for 3 days at 37^{o}C were used to inoculate AYE broth. All strains were grown to exponential phase (OD_{600} 0.4-0.7) at 37^{o}C shaking (200 rpm). For each strain, 10ml of exponential phase bacterial culture was centrifuged for 10 minutes at 4500rpm, the supernatant removed and the pellet re-suspended in 10ml of Fraquil. Water-exposed bacteria were incubated at 42^{o}C for 2 hours, after which cells were pelleted and suspended in 1ml of TRIzol reagent (Invitrogen). RNA was extracted according to the manufacturer's protocol. RNA from the WT was also extracted from 10ml of exponential phase culture and 5ml of post-exponential phase culture (OD_{600} >3). 1µg of RNA was loaded and migrated on a 6% Tris-borate-EDTA-urea polyacrylamide gel (Ambion) at 180mV. The RNA was transferred onto a positively charged nylon membrane (Thermo Scientific) using a semi-dry gel blotting system (BioRad) for 20 minutes at 200mA. The membranes were pre-hybridized in ULTRAhyb-Oligo Hybridization Buffer (Ambion AM8663) for 1 hour at 37^{o}C before hybridization with 5' biotinylated RsmY and RsmZ probes (Integrated DNA Technologies). Hybridization was performed overnight in a rotating chamber at 37^{o}C. Blots were washed twice with 2X SSC (0.15M NaCl and0.015M sodium citrate) and 0.5% SDS for 30 minutes. The biotinylated probed were detected using the Chemiluminescent Nucleic Acid Detection Module (Thermo Scientific) as per the manufacturer's instructions.

4.3.10 Bioinformatic Analyses

NCBI Conserved Domain Database (CDD https://www.ncbi.nlm.nih.gov/cdd/) was used to search for conserved protein domains that may be implicated in signal sensing within LetS. The Accession number YP_095929.1 representing Lpg1912 of *Legionella pneumophila* Philadelphia-1 strain was queried. The TMHMM server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM/) and TOPCONS (http://topcons.net/) were used to

predict the transmembrane helices on the N-terminal of LetS (Krogh et al. 2001, Tsirigos et al. 2015).

4.4 Results

4.4.1 LetS is important for the survival of *Lp* in water

The survival of the Δ*letS* mutant was compared to that of the wild-type (WT) at 42^{o}C (Fig 4.1A). An inducible plasmid carrying the *letS* gene (p*letS*) was introduced into Δ*letS* to complement the mutant strain. This strain was either artificially induced with 0.1mM IPTG to express *letS*, herein referred to as ON, or was left uninduced, herein referred to as OFF, in water. Starting at early time points during survival in water, a significant defect was observed in the survival of Δ*letS* compared to the WT. While partial complementation was observed during the early stages, inducing the expression of *letS* in ON only upon entry into water did not provide a benefit when compared to OFF, as evidenced by similar survival curves (Fig 4.1A). Both ON and OFF demonstrated a survival defect relative to the WT, albeit to a lesser extent to that of the Δ*letS* mutant. Leaky transcription from the pMMB207c vector used for complementation likely explains partial complementation observed in the first month of water exposure in the non-induced strain. To test whether the failure to complement was caused by a requirement for LetS expression prior to water exposure, the survival of *Lp* was re-tested, but, in contrast to Figure 4.1A, ON was induced on both agar and in water using 0.1mM IPTG. Consequently, inducing the expression of LetS on agar prior to water exposure significantly improved the survival of ON, resulting in a survival rate which is similar to the WT (Fig 4.1B). Δ*letS* and OFF have similar survival curves. This implicates the LetA/LetS two-component system as an important tool for the survival of *Lp* in water at 42^{o}C.

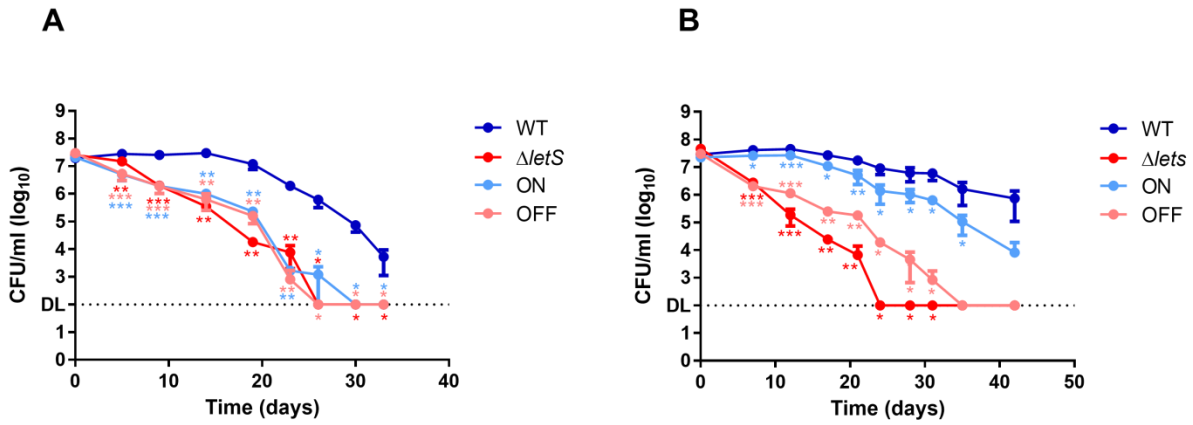

Figure 4.1: LetS increases the survival of *Lp* in water. The survival of the WT, Δ*lets* and the induced (ON) or uninduced (OFF) Δ*letS* + p*letS* in water at 42^{o}C was monitored using CFU counts. The strains were suspended in water at an OD_{600} of 0.02. In panel A, the Δ*letS* + p*letS* strain was induced (ON) in water using 0.1mM IPTG. In panel B, the ON strain was induced using 0.1mM IPTG on agar prior to water exposure and with 0.1 mM IPTG during water exposure. DL, detection limit. Student's t-test was used to assess statistical significance *versus* the WT. * $P < 0.05$; ** $P < 0.005$; *** $P < 0.0005$.

The survival of the WT, Δ*letS*, ON and OFF was also tested at 4^{o}C and 25^{o}C to ensure that the survival defect observed at 42^{o}C was not temperature specific. As a general trend, strains reached the detection limit slower as the incubation temperature decreased (Fig 4.1 and S4.1). Similar to Fig 4.1B, a loss of *letS* caused a survival defect when *Lp* was exposed to water at 4^{o}C and 25^{o}C (Fig S4.1A and 4.1B). The defect was well complemented when *letS* had been induced with 0.1mM IPTG on agar prior to water exposure and throughout the course of the experiment. OFF has a survival curve similar to that of Δ*letS*. When incubated at 25^{o}C, the induced complement demonstrated only partial complementation, mirroring the WT strain better during the early stages of water exposure (Fig S4.1B). IPTG is reported to be a stable inducer of gene expression with minimal decay in broth (Politi et al. 2014); however, its long-term stability in water over the course of several months has never been investigated. Given the complementing pattern observed at 4^{o}C and 25^{o}C, it would seem that IPTG degradation is slower at colder temperatures. Therefore, we postulate that incomplete complementation observed in the 25^{o}C

survival experiments may be due to reduced *letS* expression in ON at later time points, and the resulting regulatory changes affecting the cells capacity to survive continued nutritional stress. Taken together, the survival experiments suggest that expression of LetS ensures the survival of *Lp* in water; however, its continued expression, likely at low levels, ensures an appropriate transcriptomic response over the long-term. As a whole, the aforementioned water survival experiments establish the LetA/LetS TCS as an important regulator that allows *Lp* to successfully survive the nutrient poor aquatic environment.

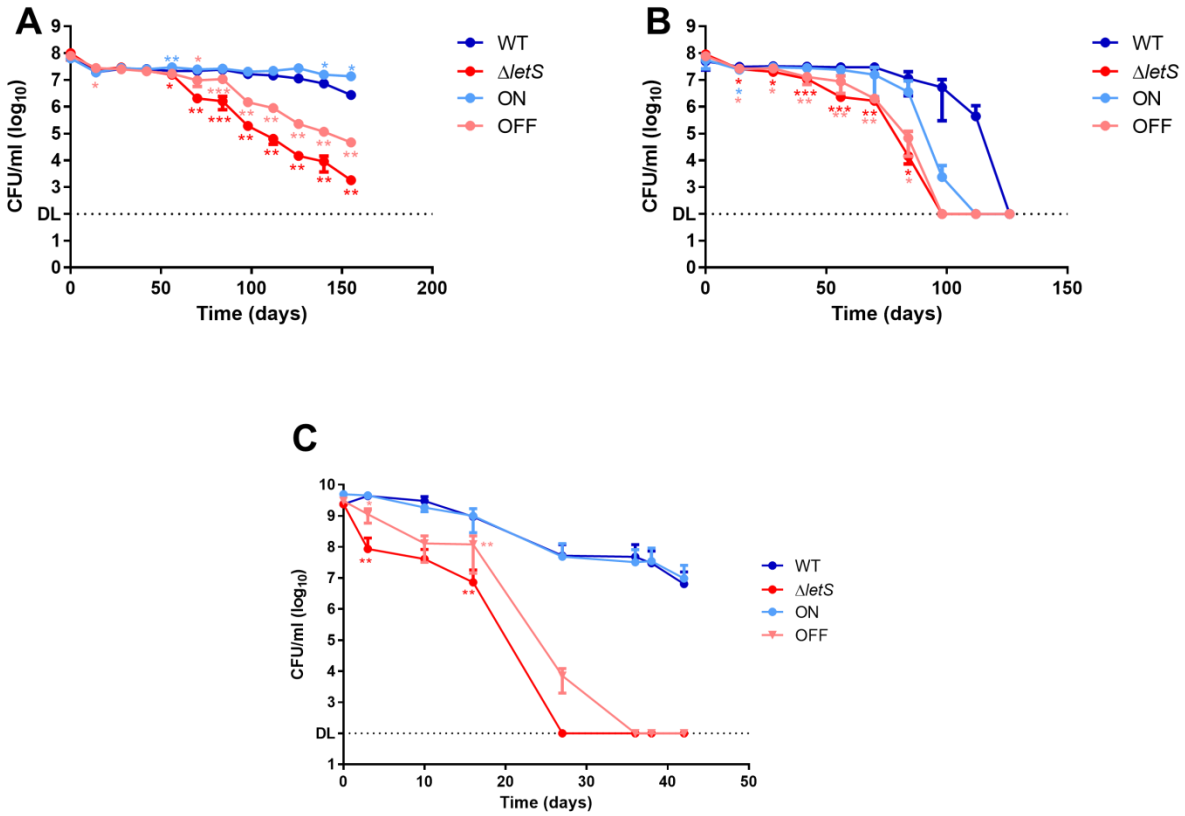

Figure S4.1: The survival of Lp in water at various temperatures and at OD_{600} of 1 at 42°C. The WT, Δ*lets* and the induced (ON) or uninduced (OFF) Δ*letS* + p*letS* were exposed to water at 4°C (A) and 25°C (B) at an OD_{600} of 0.02. Alternatively, *Lp* strains were exposed to 42°C at OD_{600} of 1. ON was induced using 0.1mM IPTG on agar prior to water exposure and with 0.1 mM IPTG during water exposure. DL, detection limit. Student's t-test was used to assess statistical significance *versus* the WT. * $P < 0.05$; ** $P < 0.005$; *** $P < 0.0005$.

4.4.2 LetS influences morphological changes in water, pigment production and resistance to heat shock

WT, Δ*letS*, ON and OFF strains were grown to exponential phase, post-exponential phase or exposed to water for 2 hours at 42^{o}C. Using phase contrast microscopy, each strain was examined for any differences in morphology under these conditions. In the exponential phase, there were no significant differences in the cell lengths between the four strains (Fig 4.2E, S4.2). In contrast, strains exhibited significant differences upon entry into the post-exponential phase (Fig 4.2E, S4.2, S4.3). Compared to the exponential phase, the WT significantly shortened its average cell length upon entry into the post-exponential phase (Fig 4.2E, S4.3) (Forsbach-Birk et al. 2004). While a size downshift was apparent for all strains, the Δ*letS* mutant and OFF strain cells were significantly longer in the post-exponential phase compared to the WT. A similar morphology has been reported in *Lp* over-expressing CsrA (Fettes et al. 2001). A small cell size reduction also occurred when WT and ON cells were exposed to water (Fig 4.2A and C); however, this size reduction was not statistically significant compared to exponential phase, except in the case of ON. Nonetheless, Δ*letS* cells were significantly longer than WT cells in water. The ON strain produced *Lp* cells that were shorter than the WT (Fig 4.2A, C and E), which could be due to a higher level of *letS* expression. Notably, we observed that filamentous cells were more commonly found in the *letS* mutant and OFF strains than in the WT and ON strains when *Lp* was exposed to water. Microscopic analysis highlights the contribution of LetS to cell size reduction in both the post-exponential phase and under the starvation condition of water, but not during the exponential phase of growth.

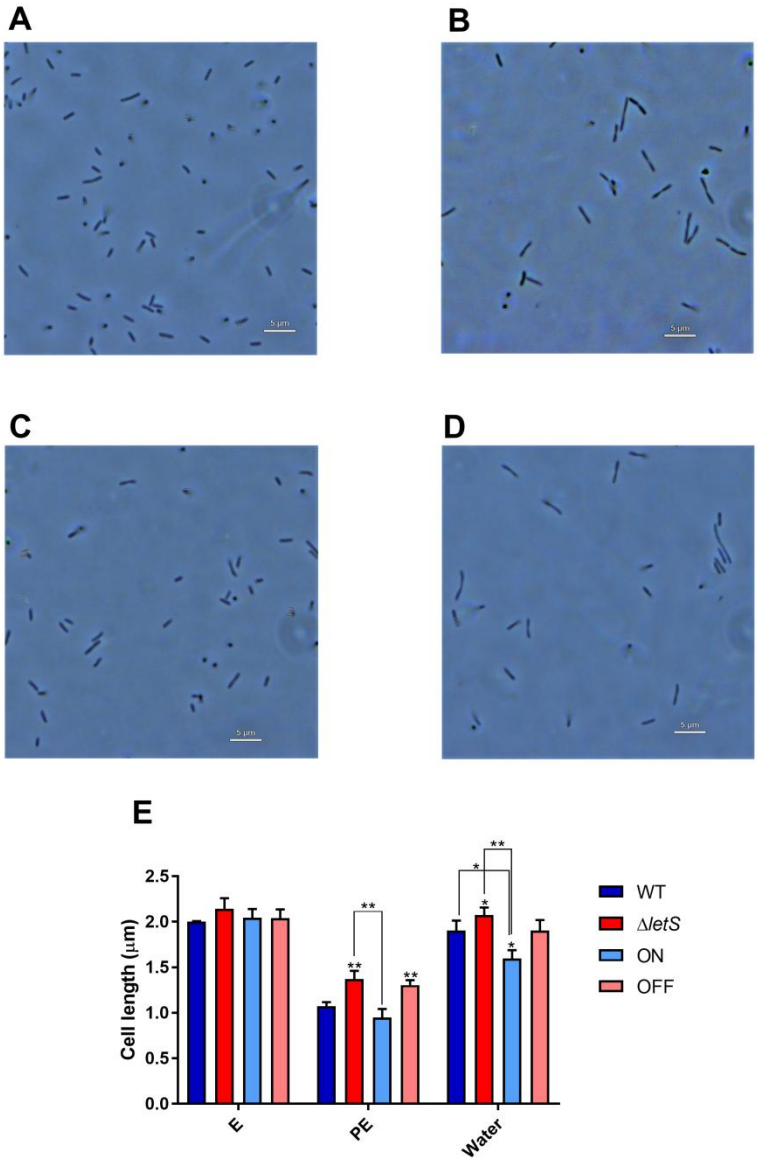

Figure 4.2: Deletion of *letS* affects the cell morphology of *Lp* in water. A) Phase contrast microscopy was used to visualize morphological changes at 1000X magnification. A representative image of the WT (A), Δ*letS* (B), ON (C) and OFF (D) exposed to water for 2 hours are shown. The induced complemented strain (ON) was exposed to 0.1mM IPTG. (E) Image J 1.51f software was used to quantify the length of the WT, Δ*letS*, ON and OFF grown to the exponential phase (E), in the post-exponential phase (PE) and after 2 h in water. The scale bar on the microscopic images is equivalent to 5µm. An unpaired Student's t-test was used to assess statistical significance *versus* the WT, unless identified otherwise. * $P < 0.05$; ** $P < 0.005$.

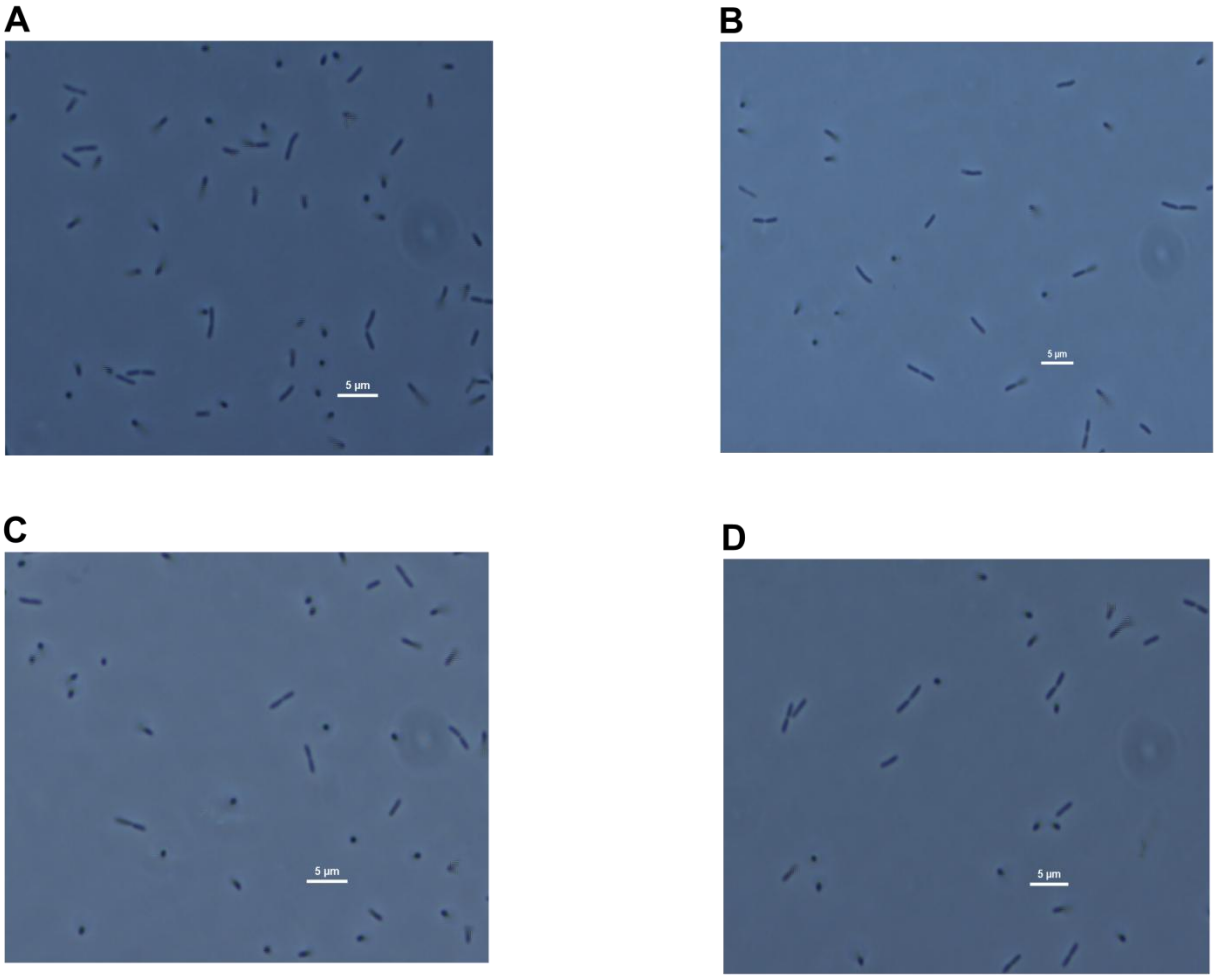

Figure S4.2 Microscopic images of exponential phase cells. A representative image of the WT (A), Δ*letS* (B), ON (C) and OFF (D) in the exponential phase. The induced complemented strain (ON) was grown with 0.1mM IPTG. Phase contrast microscopy was used to visualize morphological changes at 1000X magnification under oil immersion. The scale bar is equivalent to 5µm.

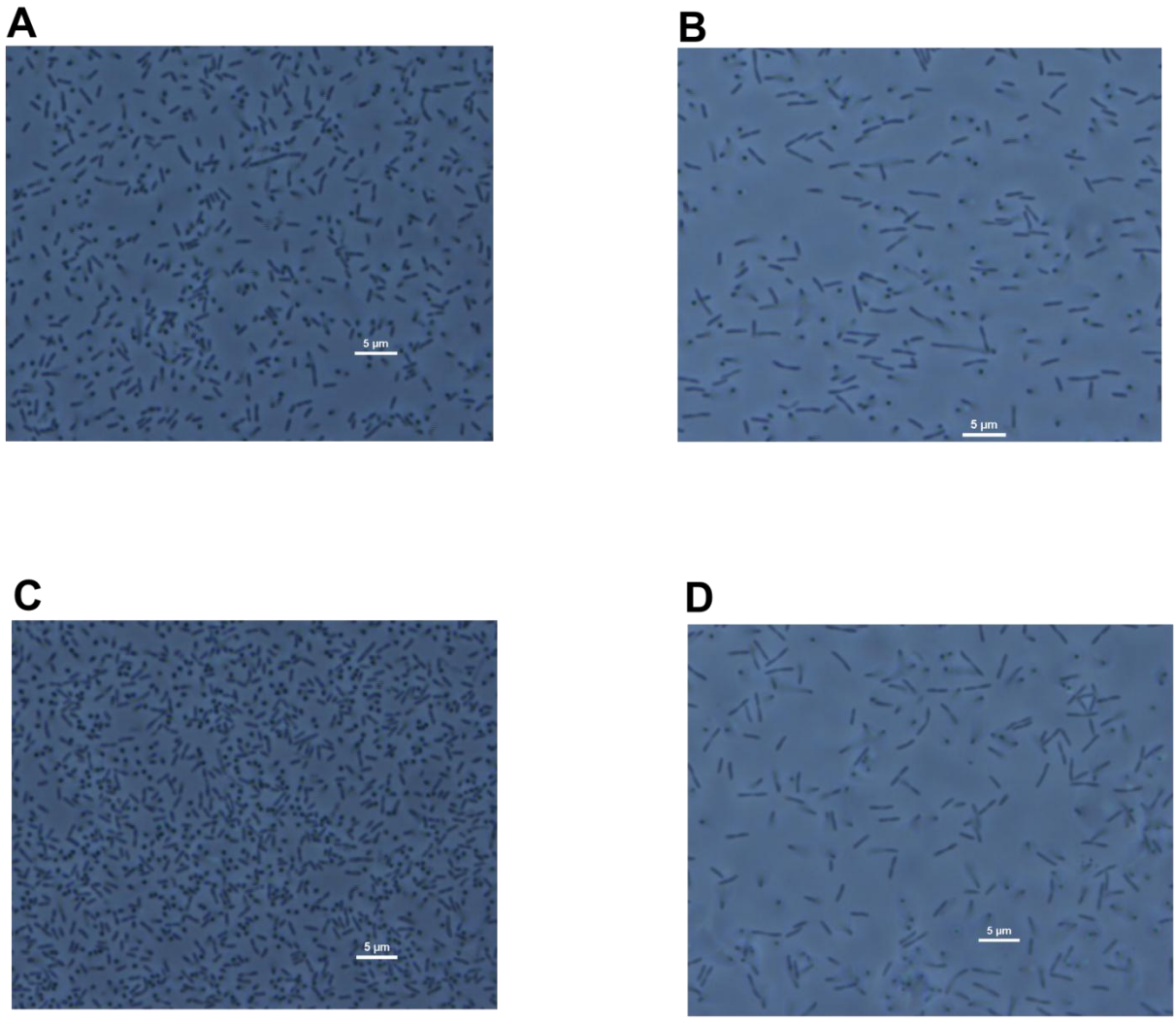

Figure S4.3 Microscopic images of post-exponential phase cells. A representative image of the WT (A), Δ*letS* (B), ON (C) and OFF (D) in the post-exponential phase. The induced complemented strain (ON) was grown with 0.1mM IPTG. Phase contrast microscopy was used to visualize morphological changes at 1000X magnification under oil immersion. The scale bar is equivalent to 5µm.

In agreement with previously published work studying the LetA/LetS cascade (Molofsky and Swanson 2003, Sahr et al. 2009), the Δ*letS* mutant showed a defect in pigment production during the stationary phase (Fig 4.3A). It is noteworthy that pigment production was not completely inhibited in the Δ*letS* mutant, which produced visible pigmentation once cultures

reached stationary phase. Nonetheless, the amount of pigment produced by the mutant was significantly lower than the WT (Fig 4.3A). The reduced pigment production observed in the Δ*letS* mutant is corrected in ON strain, while OFF pigment production resembles Δ*letS* (Fig 4.3A).

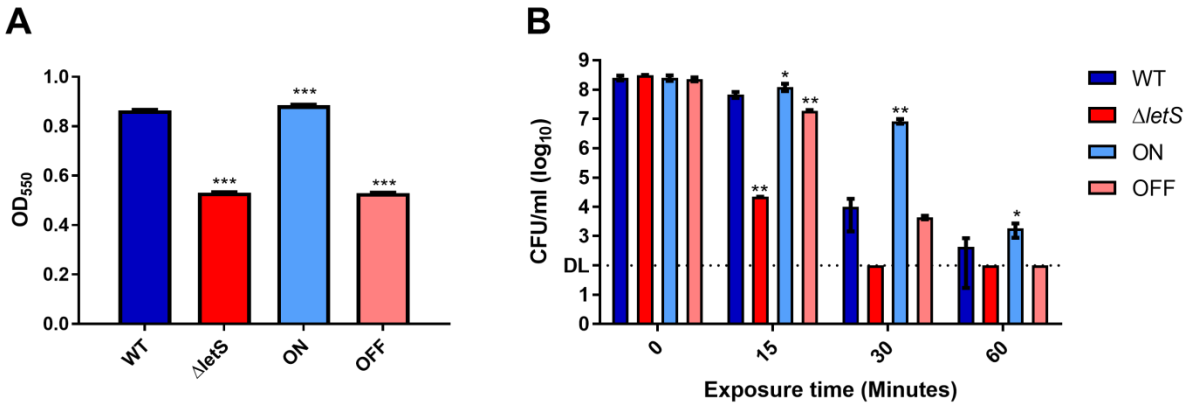

Figure 4.3: Deletion of *letS* affects pigment production and sensitivity to heat shock. A) The WT, Δ*letS* and complemented strain (ON and OFF) were grown to stationary phase in AYE broth at 37°C. The optical density (OD_{550}) of 1ml of cell-free supernatant was measured in triplicate. B) The WT, Δ*letS*, ON and OFF were suspended in water for 2 hours and incubated in a 55°C water bath for 15, 30 or 60 minutes. CFU counts were enumerated on CYE agar. DL, detection limit. An unpaired Student's t-test was used to assess statistical significance *versus* WT. * $P < 0.05$; ** $P < 0.005$; *** $P < 0.0005$.

Lp frequently encounters high temperatures within man-made water distribution systems and is known to persist despite continuous heat treatments (Bédard et al. 2016a, Bouchard et al. 1985, Farhat et al. 2012, Plouffe et al. 1983). Therefore, we tested the ability of the *letS* mutant to withstand heat shock at 55°C in water. WT CFU counts dropped by 5 orders of magnitude after 30 minutes at 55°C, but remained above the detection limit after 60 minutes. In contrast, the *letS* mutant was more sensitive, as CFU counts dropped below the detection limit after a 30-minute heat shock treatment (Fig 4.3B). Expression of *letS* in ON afforded *Lp* an increased resistance to heat compared to the WT. The OFF strain resulted in lower heat tolerance compared

to the WT and ON; however, its survival was markedly better than that of the *letS* mutant, presumably because of leaky *letS* expression from the vector.

4.4.3 Transcriptomic analysis of the LetS regulon in water

In order to identify genes under the control of the LetA/S regulatory cascade in response to water exposure, DNA microarrays were used to probe the transcriptomic differences between the WT, Δ*letS*, ON and OFF strains. Bacterial cultures at an OD_{600} of 1 were exposed to water for 2 hours at 42^{o}C, after which their transcriptomes were compared to identify differential gene expression. In order to extract sufficient amounts of RNA for transcriptomic analysis, a higher cell density was required during water exposure. The survival defect observed at low cell density (Fig 4.1 – OD_{600} 0.02) was confirmed at high cell density (Fig S4.1C - OD_{600} 1). Justification for the use of an early time point were two-fold: 1) *Lp* is known to reduce transcription dramatically after 6h in Fraquil (Li et al. 2015), and 2) we expect that the regulatory changes initiated by the LetA/S TCS are important for early adaptation in *Lp* as evidenced by the need for induction of *letS* on agar prior to water exposure to achieve proper complementation (Fig 4.1).

In total, 753 genes were upregulated and 153 genes were down-regulated in the Δ*letS* mutant compared to the WT strain upon water exposure (Fig 4.4). Differential gene expression between ON and OFF was also compared. Similar to the *letS* mutant, the OFF strain over-expressed more genes (1221) than it under-expressed (68) compared to ON (Fig 4.4B). Among the significantly up-regulated open reading frames (ORFs), 569 genes and 49 annotated sRNAs (a total of 618) were shared in both the mutant and OFF strain compared to WT and ON strain respectively (Fig 4.4B). 16 genes, including the two sRNAs, RsmY and RsmZ were significantly downregulated in both the *letS* mutant and OFF strain compared to the WT and ON strain respectively (Fig 4.4B, Table 4.4). qPCR was performed on six randomly chosen genes (four protein-coding genes and two small RNAs); three of which were upregulated and three of which were downregulated in the microarray dataset (Fig 4.5). The fold change pattern observed in the microarray analysis was mirrored in the qPCR results.

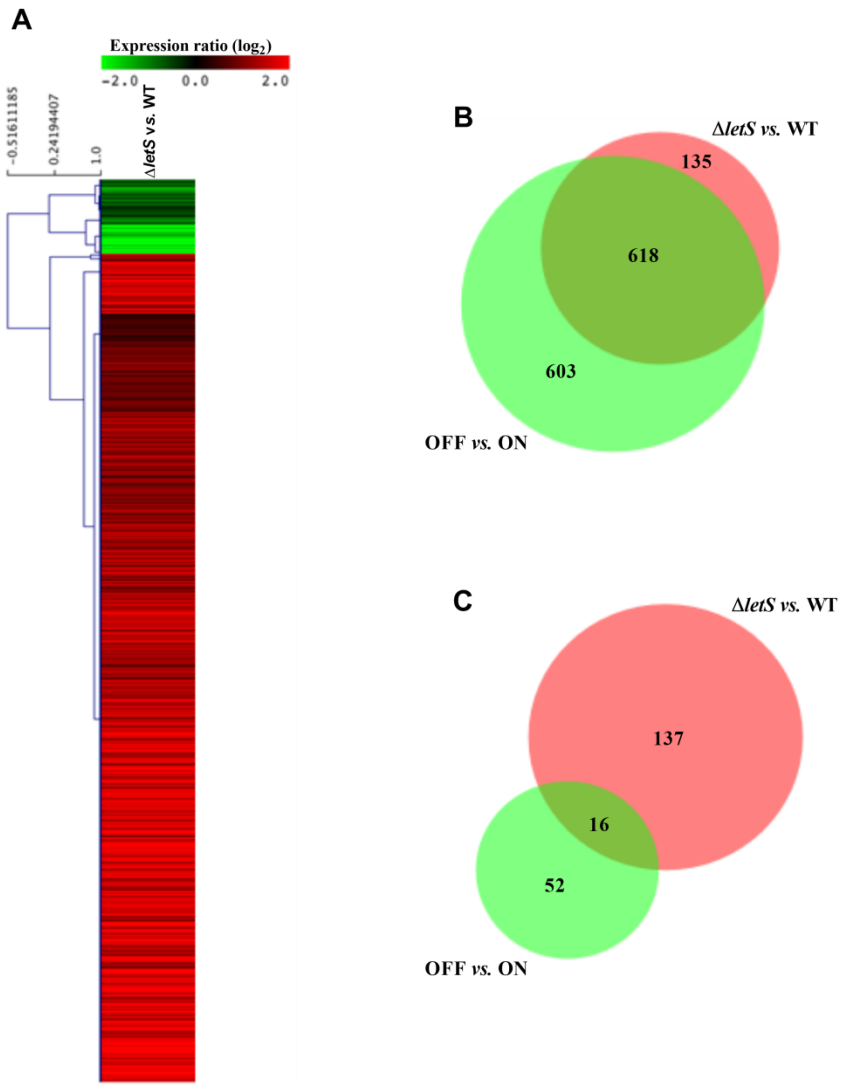

Figure 4.4 Transcriptomic effect of the deletion of *letS* in water. A) Heat map showing genes differentially expressed in the *letS* mutant compared to the WT (ratio to control value of ±2-fold with a *P value of* <0.05). Genes that are upregulated in the *letS* mutant are shown in red; genes that are downregulated are shown in green. The number of genes up- (B) or down-regulated (C) that are shared between Δ*letS vs.* WT and OFF *vs.*ON groups are shown in Venn diagrams.

Table 4.4 Select genes downregulated in Δ*letS* *vs.* WT and OFF *vs.* ON

Lpg #	Gene Product	Gene	log_2 (*letS*/*WT*)*	log_2 (OFF/ON)*
		rsmZ	-14.43	-1.32
		rsmY	-3.69	-4.21
Lpg1337	Flagellar protein FliS	*fliS*	-2.77	-1.72
Lpg1170	Pyruvate formate lyase-activating enzyme PflA	*pflA*	-2.32	-1.38
Lpg0605	Nitrogen fixation protein (Fe-S cluster formation)	nifU	-2.28	-1.36
Lpg0894	Cytokinin oxidase (cytokinin dehydrogenase)		-2.92	-1.77
Lpg2829	SidH (myosin-like protein) Icm/Dot Effector	*sidH*	-2.81	-1.04
Lpg1169	hypothetical (dioxygenase)		-1.04	-1.05
Lpg1080	dGTP triphosphohydrolase		-2.70	-1.10
Lpg1925	ORF of Unknown Function		-3.50	-1.92
Lpg0995	ORF of Unknown Function		-1.36	-2.20
Lpg2458	Sensory box histidine kinase		-3.33	-1.15
Lpg0879	Two component response regulator with GGDEF domain		-2.92	-1.49
Lpg0627	Type IV pilin	*pilE3*	-2.86	-1.43
Lpg0628	Type IV fimbrial biogenesis PilY1-related protein		-2.26	-1.09
Lpg1949	Icm/Dot Effector	*lem17*	-1.34	-1.31

* Only significant values are shown.

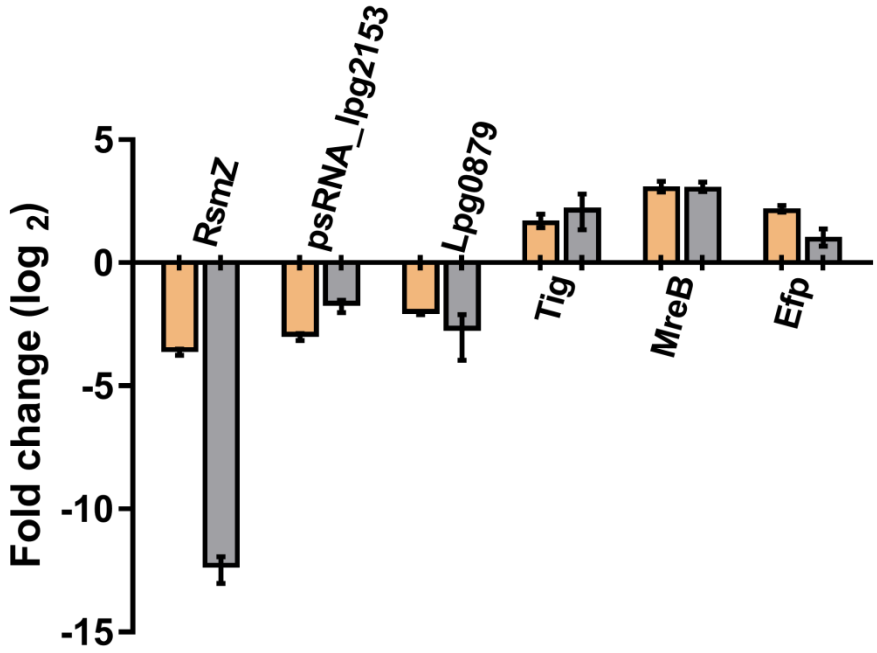

Figure 4.5: qPCR validation of microarray analysis. Three up-regulated and three down-regulated genes were chosen from the transcriptomic analysis. Fold change of gene expression in the *letS* mutant is compared to that of the WT. Orange bars represent qPCR values. Grey bars represent microarray values.

In general, the absence of *letS* resulted in the upregulation of a large number of genes (Fig 4.4A). The genes that were differentially and significantly expressed in both Δ*letS* and OFF, relative to the WT and ON respectively, were categorized into clusters of orthologous groups (COGs) to elucidate the cellular functions that were most affected by LetA/S regulation in water (Fig 4.6). Noticeably, genes associated with exponential growth or nutrient rich conditions were highly upregulated in the absence of *letS* (Fig 4.6). These COGs include "Translation", "Amino acid metabolism", "Lipid metabolism" and "Energy metabolism". Notably, LetS mediates the downregulation of 38 genes belonging to the "Translation" category upon water exposure (Fig 4.6). Of these, 30 encode 30S or 50S ribosomal proteins. It is also noteworthy that the absence of *letS* caused the transcript of *rpoD*, the housekeeping sigma factor, to be highly and significantly upregulated in water (Table 4.3).

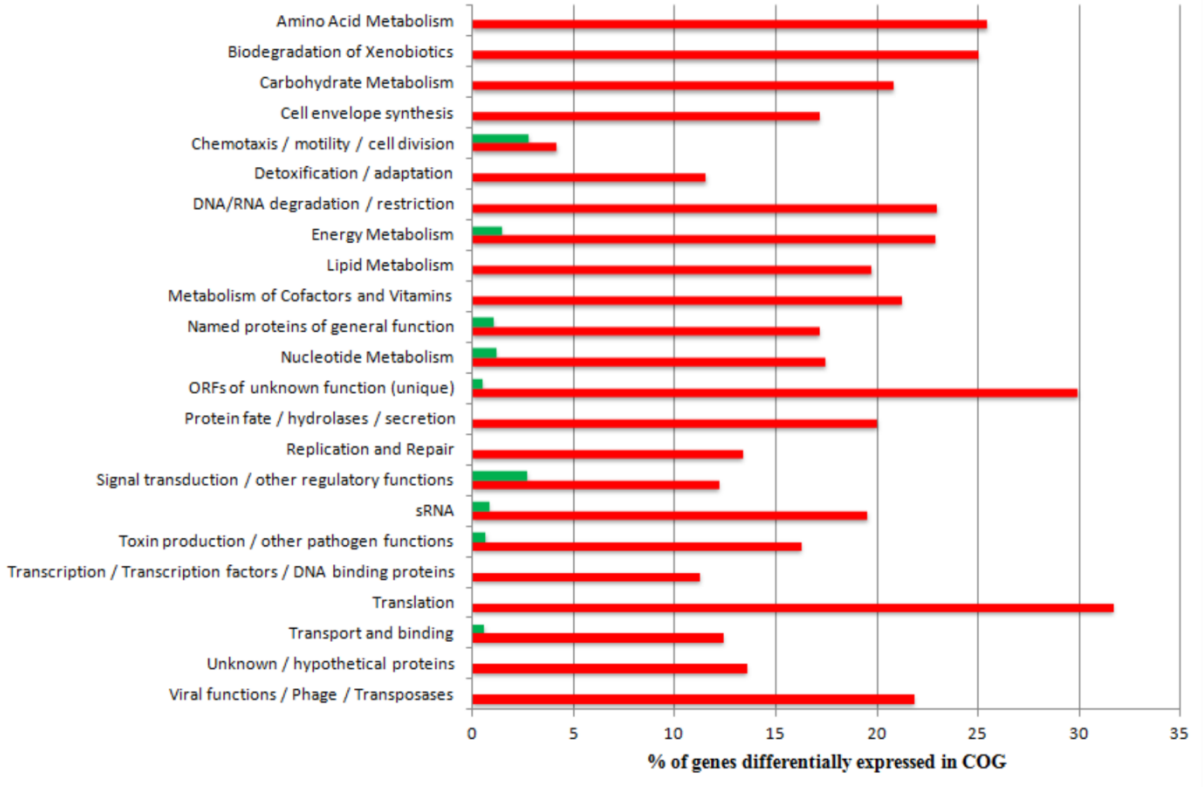

Figure 4.6: Clusters of orthologous groups (COGs) analysis. COG analysis of genes that were differentially expressed upon water exposure. Data represents the differentially expressed genes that are common to both Δ*letS vs.* WT and OFF *vs.* ON. Red bars indicate the percentage of genes upregulated in each COG, while green bars indicate the percentage of genes that are downregulated in each COG category.

Table 4.3: Select genes upregulated in Δ*letS* *vs.* WT and OFF *vs.* ON

Lpg #	Gene Product	Gene	log_2 (*letS/WT*)*	log_2 (OFF/ON)*
Amino Acid Metabolism				
Lpg0932	Shikimate kinase		1.05	1.48
Lpg1610	Glutamate-5-kinase (gamma-glutamyl kinase)	*proB*	1.72	1.33
Lpg2278	4-hydroxyphenylpyruvate dioxygenase (legiolysin)	*hpd*	2.12	1.45
Lpg0890	Cystathionine beta-lyase (cystathionine gamma lyase)	*metC*	1.53	1.40
Lpg2951	Cystathionine beta synthase (cysteine synthase)		1.62	1.98
Lpg0725	Serine hydroxymethyltransferase	*glyA3*	2.60	2.80
Carbohydrate Metabolism				
Lpg2887	Phosphomannose isomerase GDP mannose pyrophosphorylase	*rfbA*	1.57	2.14
Lpg0939	2-dehydro-3-deoxy-phosphogluconate aldolase	*eda*	1.82	1.71
Lpg0417	6-phosphogluconolactonase	*pgl*	1.91	2.46
Lpg0805	Phosphoenolpyruvate synthase		2.32	2.85
Lpg2352	Malate dehydrogenase	*mdh*	1.39	2.28
Lpg2792	Triosephosphate isomerase (TIM)	*tpiA*	2.72	2.66
Lpg0138	Glyceraldehyde 3-phosphate dehydrogenase	*gap*	1.44	2.40
Cell Envelope Synthesis				
Lpg1753	UDP-N-acetylmuramate:L-alanyl-gamma-D-glutamyl-meso-diaminopimelate ligase (murein peptide ligase)	*mpl*	1.83	2.15
Lpg0840	polysialic acid capsule expression protein (carbohydrate isomerase) (KpsF/GutQ		1.45	2.08

	family protein)			
Lpg2544	Membrane-bound lytic murein transglycosylase A	*mltA*	1.12	1.87
Lpg0748	LPS biosynthesis protein		2.10	2.35
Lpg0811	Rod shape determining protein MreB (regulator of FtsI)	*mreB*	3.07	2.41
Motility & Cell Division				
lpg2891	Sporulation initiation inhibitor protein Soj, chromosome partitioning protein ParA	soj	1.38	1.45
lpg1553	Septum site determining protein MinC	*minC*	1.05	1.86
Lpg1724	Septum site-determining protein MinD	*minD*	1.24	1.86
Detoxification / Adaptation				
Lpg0047	Chloramphenicol acetyltransferase		1.54	1.22
Lpg0426	Cold shock protein CspH	*cspD*	1.04	1.71
Lpg1060	Cold shock domain family protein		1.02	2.62
Lpg1971	Organic hydroperoxide resistance protein		1.81	2.97
Lpg2967	Superoxide dismutase	*sodB*	1.82	1.72
Lpg1861	ATP-dependent Clp protease, proteolytic subunit ClpP	*clpP*	1.41	2.15
Lpg1423	TPR domain protein (heat shock protein) N-acetylglucosaminyl transferase		1.93	1.58
DNA/RNA Degradation				
Lpg1373	Ribonuclease HII	*rnhB*	1.37	1.11
Lpg1383	Ribonuclease HI	*rnhA*	1.00	1.73
Lpg1869	Ribonuclease III (dsRNA-specific ribonuclease) (RNAse III, dsRNA)	*rnc*	2.04	2.24
Lpg2004	S-adenosylmethionine:tRNA ribosyltransferase-isomerase	*queA*	1.96	2.09
Lpg2012	Ribonuclease PH (RNAse PH)	*rph*	2.33	2.62
Energy Metabolism				

Lpg2981	ATP synthase epsilon chain, ATP synthase F1 epsilon subunit	*atpC*	2.25	2.74
Lpg2982	ATP synthase F1 subunit beta	*atpD*	1.62	2.12
Lpg2986	ATP synthase F0, B subunit	*atpF*	1.12	1.75
Lpg2779	NADH dehydrogenase I, K subunit	*nuoK*	1.56	1.32
Lpg2787	NADH dehydrogenase I, C subunit	*nuoC*	1.99	2.18
Icm/Dot Genes Effectors				
Lpg0483	LegA12	*LegA12*	2.25	2.07
Lpg2283	Small ORF (132aa)	*CelLp6*	1.43	2.42
Lpg0621	SidA	*sidA*	1.44	1.01
Lpg0963	ORF		2.25	1.94
Lpg1110	ORF	*lem5*	2.12	2.65
Lpg2298	Inclusion membrane protein A	*legC7/ ylfA*	1.52	2.07
Lpg2793	LepA, interaptin	*lepA*	1.24	2.27
Lpg2999	Eukaryotic homolog	*legP*	1.22	2.14
Lipid Metabolism				
Lpg0102	β-ketoacyl synthase	*fabF*	1.84	1.30
Lpg1395	3-oxoacyl-(acyl carrier protein) reductase	*fabG*	2.07	1.44
Lpg1854	NADH dependent enoyl ACP reductase	*fabI*	1.26	1.92
Lpg2228	3-oxoacyl (acyl carrier protein) synthase III		1.57	2.03
Lpg0729	Phosphatidylglycerophosphatase A (PgpA)	*pgpA*	1.73	2.54
Lpg0920	Phosphatidylglycerophosphatase B (Pap2)		1.34	2.90
Lpg1414	Glycerol kinase		1.88	1.48
Nucleotide Metabolism				
Lpg0218	Phosphoribosylaminoimidazole carboxylase, catalytic subunit PurE	*purE*	1.92	1.69
Lpg1181	CTP synthase PyrG	*pyrG*	1.52	2.99
Lpg1411	Adenylate kinase	*adK*	1.67	2.06
Lpg1676	Phosphoribosylformylglycinamidine	*purQ*	1.21	1.60

	synthase I			
Lpg1678	Phosphoribosylformylglycinamidine synthase II	*purL2*	1.33	2.10
Protein Fate & Secretion				
Lpg0316	Preprotein translocase, SecE subunit	*secE*	1.12	1.28
Lpg1362	Type II protein secretion LspG	*gspG*	1.60	1.81
Lpg1463	Preprotein translocase; secretion protein SecA	*secA*	2.70	2.49
Lpg1871	Signal peptidase I	*lepB-1*	1.62	1.56
Lpg2002	Transmembrane protein YajC, preprotein translocase subunit	*yajC*	1.64	1.62
Lpg2791	Preprotein translocase, SecG subunit	*secG*	2.30	2.94
Replication & Repair				
Lpg0356	Single strand binding protein	*ssb*	2.09	2.07
Lpg0691	DNA topoisomerase IV, subunit B	*parE*	1.49	2.76
Lpg1417	DNA gyrase, A subunit	*gyrA*	2.31	2.35
Lpg1576	Holliday junction DNA helicase RuvB	*ruvB*	1.47	1.96
Lpg1801	RecA bacterial DNA recombination protein	*recA*	1.74	1.68
Virulence Related Genes				
Lpg0704	Enhanced entry protein EnhA	*enhA*	1.29	1.25
Lpg0791	Macrophage infectivity potentiator (mip)	*mip*	1.27	1.83
Lpg2564	LvrA		1.69	1.27
Lpg0447	LphA (DotK) (OmpA family protein)	*lphA*	1.80	2.51
Lpg0448	IcmM (DotJ)	*icmM*	1.37	1.75
Lpg0450	IcmK (DotH) (TraN)	*icmK*	1.13	1.24
Lpg2674	DotD (TraH)	*dotD*	1.08	2.12
Lpg1862	Trigger factor TF (FKBP-type peptidyl prolyl cis-trans isomerase)	*tig*	2.62	1.64
Lpg2702	Stringent starvation protein A	*sspA*	1.42	1.77
Transcription				

Lpg2624	Transcription elongation factor GreA	greA	1.66	3.10
Lpg2934	Transcription termination factor Rho		1.52	1.68
Lpg0232	Transcriptional regulator np20 (Fur family)	*np20*	2.26	2.39
Lpg0542	DNA binding protein Fis	*fis*	2.15	2.63
Lpg1743	Fis transcriptional activator	*fis*	1.05	1.00
Lpg2361	RNA polymerase sigma 70 factor (sigma factor RpoD)	*rpoD*	1.45	2.00
Translation				
Lpg0287	Translation elongation factor P (EF-P)	*efp*	1.28	1.60
Lpg0339	50S ribosomal protein L14	*rplN*	1.05	1.58
Lpg0341	50S ribosomal protein L5		1.18	1.74
Lpg1592	30S ribosomal protein S6	*rpsF*	3.92	3.25
Lpg1711	Ribosome recycling factor	*frr*	1.34	2.09
Lpg1713	Translation elongation factor Ts (EF-Ts)	*tsf*	2.51	2.27
Lpg1714	30S ribosomal protein S2	*rpsB*	1.62	1.39
Lpg2713	Translational initiation factor IF-3	*infC*	2.80	2.65
Transport & Binding				
Lpg1277	ABC transporter ATP binding protein	*abcT3*	2.80	2.64
Lpg2245	C4-dicarboxylate transport protein (Na+/H+ dicarboxylate symporter)	*dctA*	1.51	1.66
Lpg2321	Serine transporter	*sdaC*	1.79	2.75
Lpg2475	Hydrogenase nickel incorporation protein HypB	*hypB*	1.81	1.59
Lpg2476	Hydrogenase nickel incorporation protein HypA	*hypA*	2.71	2.88
Lpg2658	Ferrous iron transporter A	*feoA*	1.89	1.79
Lpg2878	Cobalt/magnesium uptake transporter	*corA*	1.39	2.30
Small Regulatory RNA				
	LprC		1.08	2.02
	Lpr0035		1.65	1.91

	LprD		1.67	2.26

* Only significant values are shown.

4.4.4 ppGpp as a candidate for the activating signal of the LetA/S cascade

Lp responds to water by shutting down major metabolic groups, such as replication, transcription, translation and amino acid metabolism (Li et al. 2015). Deletion of *letS* seems to render *Lp* unable to mount such a response, which would likely result in overconsumption of internal resources. The transcriptomic response of Δ*letS* described in this work shows similarity to the response of an *rpoS* insertion mutant exposed to water (Trigui et al. 2014). Given the phenotypic overlap between the activation of the stringent response and the LetA/S cascade both *in vitro* (Byrne and Swanson 1998, Hammer and Swanson 1999, Hammer et al. 2002) and *in vivo* (Dalebroux et al. 2009, Hammer et al. 2002), we hypothesized that LetS responds to the alarmone, ppGpp when *Lp* experiences starvation, as suggested previously (Dalebroux and Swanson 2012). The NCBI Conserved Domain Database (CDD) was used to identify protein domains within the 910 amino acid sequence of *letS* (lpg1912) that could be responsible for environmental signal recognition (Annex 2 Doc 1). Figure 4.7A is a graphical representation of LetS topology as predicted by the bioinformatic tools described herein. The transmitter (T), receiver (R) and histidine phosphotransfer (HPT) domains that are characteristic of tripartite sensor kinases are depicted by blue boxes. Importantly, the N-terminus contains two regions that are conserved signal sensing domains; DUF2222 and HAMP. The former is located between residues 37-180 while the latter spans residues 185-241 (Annex 2 Doc 1).

To position the putative signal sensing domains according to LetS topology, the location of the transmembrane helices that tether LetS to the membrane were localized using the TMHMM and TOPCONS software. As previously reported (Edwards et al. 2010), two transmembrane domains were found on the N-terminus which anchors LetS into the inner membrane (Fig 4.7A, Annex 2 Doc 2 and 3). The transmembrane helices are predicted to be located between residues 15 to 34 and 182 to 204 according to TMHMM (Annex 2 Doc 2), or 11 to 31 and 181 to 201 according to TOPCONS (Annex 2 Doc 3). Accordingly, the DUF2222 domain is found between the two transmembrane helices, while the N-terminus of the HAMP domain overlaps the second transmembrane helix by 19 residues (TMHMM) or 16 residues

(TOPCONS) (Fig 4.7A). Therefore, an approximate 30% the HAMP domain lies within the membrane, and 70% in the cytoplasm. Given the locations of the DUF2222 and HAMP domains, it is possible that, in water, LetS is activated by an extracellular (via the DUF2222 region) or intracellular (via the HAMP region) environmental stimulus or by changes in membrane tension (sensed by the HAMP domain embedded within the membrane). Here, we investigated the possibility that ppGpp may act as an intracellular signal that activates the LetA/LetS cascade in response to water exposure as previously postulated (Molofsky and Swanson 2003, 2004).

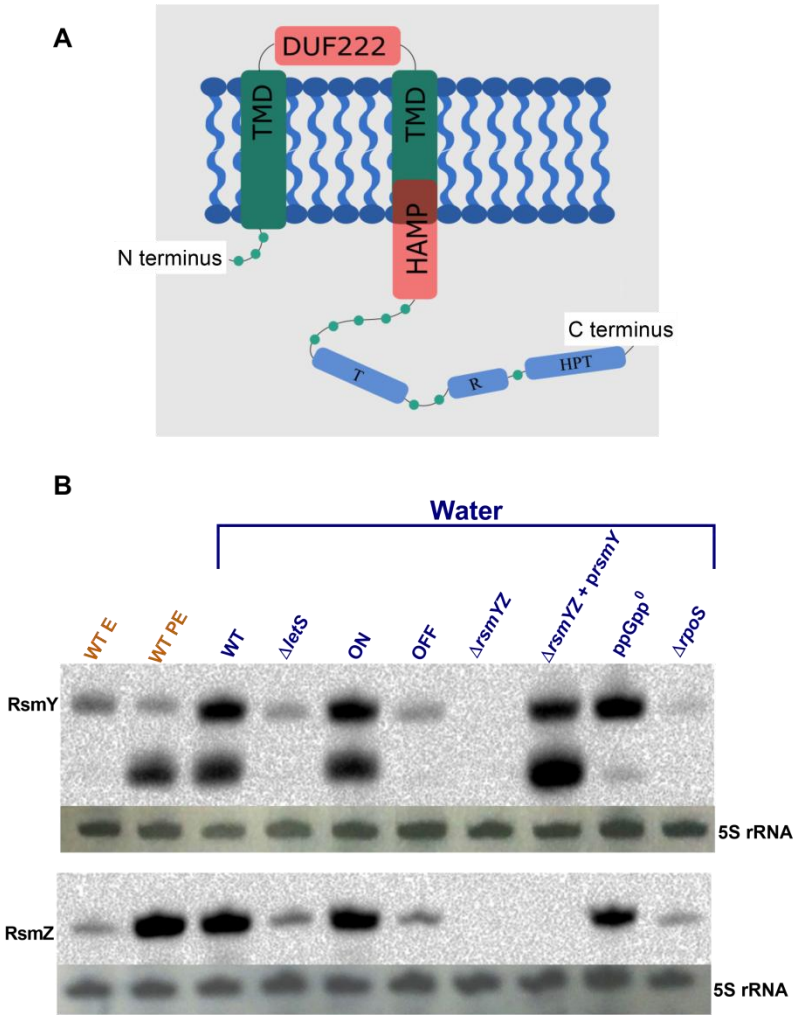

Figure 4.7: LetS topology and the impact of the stringent response on RsmY/Z expression. (A) Topology of the LetS protein was determined using the NCBI CDD Web server as well TMHMM v.2.0 and TOPCON software. Transmembrane domains (TMD) are represented by green boxes. Two putative signal sensing domains (pink boxes) are also predicted; a DUF222 domain is located between the two TMD, and a HAMP domain that overlaps the C-terminus of the second TMD. The transmitter (T), receiver (R) and phosphotransfer (HPT) domains that are involved in signal transduction are represented by light blue boxes. (B) The sRNAs RsmY (top) and RsmZ (bottom) under LetS control were probed to determine their levels in water and the influence of ppGpp on their expression. First two lanes represent the WT strain grown in rich broth to the exponential (E) or the post-exponential (PE) phase. The remaining wells represent the respective strains exposed to water for 2 hours. 1µg of RNA was loaded into each well. Gel images representing the loading controls are beneath the respective Northern blots.

Expression of the small RNAs (sRNAs) RsmY and RsmZ was used as an indicator of the activation of LetS. Northern blotting was used to detect RsmY/Z. A Δ*relAspoT* strain ($ppGpp^0$), incapable of producing the cellular alarmone ppGpp and defective in survival in water (Trigui et al. 2014), was used to determine the effect of ppGpp on RsmY/Z production. The WT strain grown in rich medium was used as a positive control for reported growth-phase dependent increases in RsmY/Z production. Probes for RsmY detected two bands (Fig 4.7B), similar to the reported appearance of double bands for RsmZ in *Pseudomonas fluorescens* and RsmY in *P. aeruginosa* (Kay et al. 2006, Reimmann et al. 2005, Valverde et al. 2003). The top band at approximately 110 nucleotides was considered the active sRNA as per previous reports (Hovel-Miner et al. 2009, Sahr et al. 2009). Corroborating previous reports (Hovel-Miner et al. 2009, Rasis and Segal 2009), the levels of RsmZ increase when cells enter the post-exponential phase (Fig 4.7B – bottom panel). This expected increase was, however, absent in RsmY (Fig 4.7 – top panel). We postulate that the increase in RsmY may occur later during the post-exponential phase. Nevertheless, both sRNAs were strongly expressed when WT *Lp* was exposed to water. Δ*letS* and OFF expressed both sRNAs at basal levels while ON recovered WT level expression in water. The expression of RsmY/Z was abolished in the double mutant (Δ*rsmYZ*); RsmY expression was recovered to WT levels when *rsmY* is expressed *in trans*. The absence of the alternative sigma factor, RpoS also resulted in a quasi-complete abolition of RsmY/Z expression, corroborating previous findings that the activation of this system is RpoS-dependent (Hovel-Miner et al. 2009). Importantly, the ppGpp null mutant had WT levels of the RsmY transcript and a slightly lower level of RsmZ compared to the WT, suggesting that the alarmone was dispensable for LetS activation in water and expression of the effector sRNAs.

4.5 Discussion

Legionella pneumophila (*Lp*) is a resident of natural and man-made water systems and uses aerosols generated by the latter to infect human hosts (Fields et al. 2002). The present study investigates the hypothesis that the LetA/LetS two-component system (TCS) and its downstream regulatory cascade are crucial for the survival of *Lp* in water distribution systems. *Lp* leads a biphasic lifestyle, alternating between the replicative phase and transmissive phase during infection, and between the exponential and post-exponential phase in broth culture (Bruggemann et al. 2006, Byrne and Swanson 1998). The former stages are characterized by nutrient abundance in the immediate environment of the bacterium, allowing for maximum replication

potential. In contrast, the hallmark of the transmissive/post-exponential stage is nutrient deprivation causing visible morphological changes, a transcriptomic shift, stress resistance and virulence phenotypes (Byrne and Swanson 1998, Hayashi et al. 2010, Molofsky and Swanson 2004). The LetA/LetS two-component system is established as a key tool for the differentiation of *Lp* both in artificial medium and inside host cells in response to nutrient deprivation (Bachman and Swanson 2004b, Gal-Mor and Segal 2003b, Lynch et al. 2003, Rasis and Segal 2009, Sahr et al. 2009). Its role in adaptating to low nutrient conditions led us to test the importance of LetS under the starvation condition of water.

The removal of *letS* from the *Lp* genome and disruption of the regulatory cascade under LetS did not affect the growth rate of the mutant on solid medium (data not shown). Pyomelanin production, known to be mediated by the LetS-LetA-RsmYZ cascade (Hammer et al. 2002, Molofsky and Swanson 2003, Sahr et al. 2009), was significantly impaired in the *letS* mutant (Fig 4.3A). However, this characteristic post-exponential phase trait may be irrelevant when *Lp* is exposed to water. The *lly* (Lpg2278/*hpd*) gene responsible for production of homogentisic acid (HGA), the precursor of pyomelanin (HGA-melanin) (Chatfield and Cianciotto 2007, Flydal et al. 2012), is repressed by LetS in water (Table 4.3). Similarly, *feoA* expression is also repressed (Table 4.3). Pyomelanin, which is a ferric reductase, and the FeoA/B ferrous iron uptake system are thought to cooperate to acquire iron from the host cell (Chatfield and Cianciotto 2007, Manske and Hilbi 2014, Robey and Cianciotto 2002). As such, pigment production seems to be positively regulated by LetS only in broth culture, and possibly inside the host, where nutrients become increasingly scarce upon entering the stationary phase. In contrast, the same pathway is negatively regulated by LetS in water where nutrients, including iron, are almost completely absent. In the latter condition, expression of iron acquisition systems may be futile and inefficient. Indeed, four other genes (Lpg0232, Lpg0124/, Lpg0746, Lpg0467) related to iron acquisition (Cianciotto 2015), which includes the Fur transcriptional regulator np20, were found to be repressed by LetS in water (Table 4.3).

Importantly, and in-line with the principal goal of this study, an intact LetA/LetS system is required for the successful survival of *Lp* in water, and this, independently of temperature (Fig 4.1 and S4.1). We corroborate previous studies showing that the LetA/LetS cascade exerts its regulatory activity via the two small RNAs, RsmYZ during the post-exponential phase of growth

in broth (Fig 4.7B) (Sahr et al. 2009). Loss of the function of LetS, LetA or RsmYZ also significantly impacts the virulence of *Lp* in amoeba and in macrophages (Bachman and Swanson 2004b, Hammer et al. 2002, Lynch et al. 2003). Moreover, virulence defects, *in vitro* phenotypes and gene expression patterns that are associated with mutations of the LetA/LetS system are linked to the later stages of growth in broth and during infection when nutrient levels are depleted (Hammer et al. 2002, Lynch et al. 2003). In accordance with these studies, our data show that the LetA/LetS TCS is activated when *Lp* is exposed to water, leading to the expression of RsmY and RsmZ (Fig 4.7). Interestingly, the survival advantage conferred by LetS depends on its expression prior to water exposure (Fig 4.1). As a result, induction of *letS* in the ON strain after *Lp* was suspended in water failed to recover the WT survival rate (Fig 4.1A), while induction on agar prior to water exposure corrected the survival defect observed in the mutant (Fig 4.1B). This suggests that the transcriptomic changes initiated by the LetA/LetS cascade occur and are needed rapidly upon water exposure. It is noteworthy that survival experiments in water were conducted using *Lp* grown on agar plates for 3 days. At the time of harvest, most of the cell population is considered to be in the post-exponential phase of growth. Assuming that *in vitro* growth resembles the *in vivo* life cycle of *Lp*, at least in the context of LetA/LetS, the activation of LetS on agar plates should resemble the activation of LetS during the intracellular transmissive phase in amoeba. Thus, the state of the bacterium prior to water exposure mimics, at least partially, its intracellular state before it escapes the host into the water environment.

Having confirmed the importance of LetS and its downstream cascade for the survival of *Lp* in water, a transcriptomic analysis was undertaken to establish its regulon in response to water. LetS is a tripartite sensor kinase whose ortholog, BvgS, in *Bordetella* is known to respond to multiple environmental stimuli activating specific regulons (Beier and Gross 2008, Dupré et al. 2015). We, therefore, expected to observe differences between the LetS regulon of *Lp* in water and the previously published regulon in the post-exponential phase (Edwards et al. 2013, Lynch et al. 2003, Sahr et al. 2009). Over time, WT *Lp* exposed to water progressively shuts down transcription relative to exponential growth (Li et al. 2015). At 2 hours, approximately 54% of differentially expressed genes in *Lp* are downregulated while the remaining are upregulated (Li et al. 2015). In stark contrast, Figure 4.4 shows that an overwhelming majority of differentially expressed genes are upregulated (97%) in the absence of *letS*. In the post-exponential phase, approximately equal numbers of genes are positively and negatively

influenced by the LetA/LetS cascade (Sahr et al. 2009). Therefore, it seems that, in water, LetS is a major repressor of genes encoding functions that are unnecessary for the survival in water.

The RNA-binding protein CsrA that lies downstream of the LetA/S cascade is known to favor replicative phase gene expression, while repressing transmissive phase gene expression (Molofsky and Swanson 2003). CsrA can affect its target mRNAs differently depending on the binding site. CsrA binding promotes replicative phase genes by enhancing the stability and expression of mRNA required during growth (Romeo et al. 2013). In the case of *Lp*, CsrA is known to directly bind replicative genes including ribosomal transcripts, thereby stabilizing them and allowing for continued translation (Sahr et al. 2017). At the same time, CsrA can repress transmission by blocking the ribosome binding site of transmissive phase transcripts, inhibiting translation and/or by increasing their degradation via RNase E (Romeo et al. 2013). The negative effect of CsrA on transmissive regulators like RpoS and LqsR, some of which is mediated through direct binding, is documented (Sahr et al. 2009, Sahr et al. 2017). The WT strain exposed to water will activate the LetA/S cascade, increasing the level of RsmY/Z. The sRNAs will bind CsrA, destabilizing mRNAs that favour replication. In parallel, the transcripts repressed by CsrA will be activated. In contrast to the WT, the *letS* mutant strain is locked in replicative mode since CsrA is constitutively active, despite exposure to a starvation condition. As such, the switch to a survival mode is not initiated, leading to the quick exhaustion of limited nutrients, and eventual demise.

The importance of LetS repression is highlighted by the Clusters of orthologous groups (COGs) that are most affected by its deletion. Other than ORFs of unknown function, translation and amino acid metabolism were the COGs that had the highest percentage of genes silenced by LetS activation (Fig 4.6). These were closely followed by Energy Metabolism, Metabolism of Cofactors and Vitamins, Carbohydrate Metabolism, Lipid Metabolism, Transcription Factors, and Replication and Repair (Fig 4.6). The functions associated with these COGs are important during nutrient abundance, and water exposure results in a general downregulation of these categories (Li et al. 2015). This abnormal transcriptome locking the Δ*letS* cells in replicative mode, also caused an elongated morphology compared to the WT (Fig 4.2). Strikingly, 30 ribosomal genes, which are associated with the replicative phase, were highly upregulated in the absence of *letS*. In contrast, only five ribosomal genes are reported to bind CsrA directly (Sahr et

al. 2017). The Fis1 transcriptional regulator, which is repressed by *Lp* in response to water at 2 and 6 hours (Li et al. 2015), is under LetS control. We report that LetS negatively influenced two out of three Fis proteins (Lpg0542/Fis1 and Lpg1743/Fis2). Fis transcripts are stabilized by CsrA (Table 4.3) (Sahr et al. 2017), which suggests that their apparent upregulation is a side effect of increased stability. Another notable regulator that was repressed by LetS is the housekeeping sigma factor, RpoD. Regulation of RpoD by LetA/LetS or by CsrA has not been reported in *Lp* (Molofsky and Swanson 2003, Sahr et al. 2009, Sahr et al. 2017). As such, the effect of CsrA on *rpoD* may be indirect. Therefore, the general downregulation of gene expression upon LetS activation in water is mediated by relieving the direct and indirect effects of CsrA binding to target mRNAs.

The most significant difference between the post-exponential phase transcriptome of LetS and that in water is the marked absence of gene upregulation. Only 16 genes were significantly induced by LetS, including the two sRNAs that are directly regulated by LetA (Table 4.4). Regulators involved in virulence phenotypes including RpoS, LetE (Lpg0537) and LqsR (Lpg2732) were not affected by LetS. Moreover, the LetA/LetS control of flagella genes is well established in broth cultures (Bachman and Swanson 2004b, Edwards et al. 2010, Hammer et al. 2002, Nevo et al. 2014, Sahr et al. 2009). We report that none of the flagella-associated genes were differentially expressed in the absence of *letS* in water with the exception of *fliS.* It is noteworthy that a time course transcriptomic analysis upon water exposure shows that flagella genes are maximally induced at 6 hours after exposure to water at 25^{o}C (Li et al. 2015). Although it is prudent to keep in mind that the initial transcriptome of *Lp* in water was conducted at a lower temperature than the condition used here, the majority of motility gene regulation may still occur at a later time point, and possibly by other regulators. Indeed, control of flagellar gene expression seems to be tightly regulated in *Lp* both cooperatively and independently by several high profile regulatory entities including RpoS, RpoN, FleQ, LetS and LqsR (Albert-Weissenberger et al. 2010, Jacobi et al. 2004, Nevo et al. 2014, Sahr et al. 2009).

Molofsky & Swanson (2003) reported that deletion of *letA* caused significant heat shock sensitivity in the post-exponential phase. Similarly, we found Δ*letS* to be heat sensitive in water compared to the WT (Fig 4.3B). As such, resistance to heat shock observed in the post-exponential phase is thought to be conferred by genes under LetS control (Lynch et al. 2003,

Molofsky and Swanson 2003). The microarray results however, do not show regulation of specific heat shock response-related proteins or the heat shock response sigma factor, RpoH, by the LetA/LetS cascade. It is possible that heat shock genes are further downstream in the cascade, i.e. controlled by CsrA or by a regulator under CsrA repression, and as a result, these effects are not visible in the microarray data. However, the heat shock response is a rapid response initiated within the first few minutes of exposure to this stress, whose transcriptomic effects subside quickly thereafter (Schumann 2016, Yura et al. 1993). Therefore, it is likely that at the 2 hour time point at which the transcriptomes were compared, the heat shock-related transcriptomic changes were missed, if they were indeed activated at 42°C. Alternatively, the general sensitivity of the *letA* and *letS* mutants to various stresses in the post-exponential phase (Lynch et al. 2003, Molofsky and Swanson 2003) and heat shock in water (Fig 4.3B) may be a result of differences in cell structure and therefore, a by-product of the cell's inability to adapt to the respective environments. Finally, LetS may mediate resistance to heat shock by inducing a specialized regulon that may require other regulators, in which case, the influence of the sensor kinase on the transcriptome cannot be revealed in the current study.

The general transcriptomic shift observed when the gene coding for the alternative sigma factor RpoS was mutated in *Lp* (Trigui et al. 2014) was similar to the transcriptome of the *letS* mutant. A general repression of multiple systems is consistent with induction of the stringent response. Bacteria deploy the stringent response (SR) network governed by the cellular alarmone ppGpp when challenged with nutrient deprivation or starvation; which is a key signal in growth phase differentiation (Dalebroux and Swanson 2012, Steinchen and Bange 2016). SR is characterized by a rapid downshift in the synthesis of stable RNAs, such as rRNAs and tRNAs (Potrykus and Cashel 2008). For the most part, ɤ-proteobacteria sense amino acid or fatty acid deprivation and synthesize ppGpp using RelA, a synthase, and SpoT, a dual-acting hydrolase with weak synthase activity (Dalebroux and Swanson 2012, Potrykus and Cashel 2008). During SR, ppGpp amd DksA, a transcription factor, act directly on RNA polymerase to guide transcription of target genes, thereby aiding survival of the bacterium (Dalebroux and Swanson 2012, Potrykus and Cashel 2008). ppGpp positively affects RpoS levels in the cell under starvation conditions (Battesti et al. 2011, Dalebroux and Swanson 2012). A previous study from our lab established that *Lp* induces the stringent response as a response to water exposure (Trigui et al. 2014). Given the similar transcriptomic response of the *rpoS* and *letS* mutants in water, the

LetA/LetS system was proposed to be integrated into the SR during water exposure. As such, we postulated that the same activating signal initiates the LetS cascade.

This hypothesis was tested by a Northern blot probing for the direct downstream targets of LetS/A, RsmY and RsmZ (Fig 4.7B). Unlike RsmZ, RsmY did not increase expression during the post-exponential. However, both sRNAs increased their transcript levels upon exposure to water, in a LetA/S-dependant manner (Fig 4.7B). The ppGpp^0 strain (Δ*relA*Δ*spoT*) used in this study does not produce ppGpp (Trigui et al. 2014), and was used to determine the effect of the cellular alarmone on RsmY/Z production. We show that the ppGpp^0 strain was able to produce WT levels of the RsmY and only slightly lowered the expression of RsmZ (Fig 4.7B). This suggests that the transcriptomic changes initiated by the SR are largely independent of those mediated by LetS and that the survival defect of ppGpp^0 is not likely to be caused by a lack of LetS response and vice-versa. We also confirm previous reports that RsmY/Z expression is RpoS-dependent (Hovel-Miner et al. 2009). It is also possible that ppGpp collaborated with RpoS by binding to RNA polymerase, contributing to the transcriptional activation of *letS* and/or RsmY/Z. However, the evidence at hand suggests that the activation of the sensor kinase is independent of the stringent response in *Lp*. Therefore, The LetA/LetS-RsmY/Z-CsrA and the SR response seem to be parallel response systems that both mediate survival under nutrient-deprived conditions. It is likely that the LetS sensor kinase responds to a variety of stresses to fine tune the transcriptomic response to the challenge at hand (Edwards et al. 2010). In broth cultures, LetA/LetS was required to increase flagella expression in response to nicotinic acid and expression of several virulence traits in response to free fatty acids (Edwards et al. 2013, Edwards et al. 2009). It is unclear whether these are direct signals in broth and whether they represent possible activation signals in water. Recently, the *Lp* quorum sensing molecule, *Legionella* autoinducer-1 (LAI-1) was shown to increase RsmY/Z levels in broth (Schell et al. 2016); however, it is unlikely to be a viable signal for the activation of LetS in water, because of its low metabolic activity, and likely low production of LAI-1. As such, the environmental signal initiating the LetS/A cascade is yet to be determined.

In conclusion, we report that the LetA/LetS-RsmY/Z-CsrA regulatory cascade is essential for the successful survival of *Lp* in water. In contrast to reports conducted in broth cultures, LetS almost exclusively acts to repress genes related to growth, with RsmY/Z being the prominent

exceptions. While there is overlap between the regulons and crosstalk between members of the SR and the LetA/LetS TCS, activation of the two systems seem largely independent of each other.

4.6 Acknowledgements

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Table 4.1: Strains used in this study

Strain Name	Relevant Genotype[a]	Source or Reference
Legionella pneumophila		
JR32	Philadelphia-1; Sm^r; r^- m^+	(Sadosky et al. 1993)
KS79 (WT)	JR32 Δ*comR*	(Hales and Shuman 1999b)
Δ*letS* (GAH338)	KS79 Δ*letS*::Kn^r	(Hovel-Miner et al. 2009)
Δ*letS* + p*letS* (SPF39)	Δ*letS* + pMMB207c *Ptac-letS*; Cm^r	(Hovel-Miner et al. 2009)
Δ*rsmYZ* (SPF41)	KS79 Δ*rsmYZ*; Kn^r, Gm^r	This study
Δ*rsmYZ* + p*rsmY* (SPF291)	Δ*rsmYZ* + pXDC39-p*rsmY*; Kn^r, Gm^r, Cm^r	This study
Δ*rpoS*	JR32 *rpoS*::Tn*903*dGent; Gm^r	(Hales and Shuman 1999b)
Δ*relA*Δ*spoT* ($ppGpp^0$)	KS79 Δ*relA*::*aacC1 spot::aptII*; Gm^r Km^r	(Trigui et al. 2014)
Plasmid Name		
pBBR1MCS-5	pBBR1MCS Gm^r	(Kovach et al. 1995)
pSF6	DH5α, pGEMT-easy-*rrnb*	(Faucher et al. 2011)
pMMB207c	RSF1010 derivative, IncQ, $lacI^q$ Cm^r P*tac* *oriT* Δ*mobA*	(Chen et al. 2004)
pXDC39	pMMB207c Δ*Ptac*, Δ*lacI* Cm^r	(Li et al. 2015)
prsmY	pXDC39-*rsmY*	This study

[a]Sm^r, streptomycin resistance; Cm^r, chloramphenical resistance; Gm^r, gentamicin resistance; Km^r, kanamycin resistance.

Table 4.2: Primers and oligonucleotide probes used in this study

Primers	Sequence 5' – 3'	Source or Reference
rsmY-F	CGGGATCCGCTCCTGGAAAGGTGTTATGC	This study
rsmY-R	CCCAAGCTTAAAGAGGTATACTGGTAAATTGG	This study
rsmZ-F	GGATATGAGTCGTGCAAATGG	This study
rsmZ-R	TTCGCAGTCATCCGTATAAGA	This study
psRNA_lpg2153-F	TAATGAATAGTACAAAGCTGTGGCA	This study
psRNA_lpg2153-R	AAGCAGCAGCTTGATGAGAAA	This study
lpg0879-F	GCGATCGCTTCTCTGTCTATT	This study
lpg0879-R	CATGGTTGCTAACATCGTTCTATC	This study
tig-F	TGGTTCTGGTTCGATGATTCC	This study
tig-R	TCCTTCCCAGCCAAATCTTTAT	This study
mreB-F	GTCTGTGTTCCTTGTGGTTCTA	This study
mreB-R	CAGCCATAGGCTCCTCAATAAG	This study
efp-F	CTGATGTGGCTGACGTAGAAA	This study
efp-R	TTGTGCTGCATCGGCTAATA	This study
rsmY-NB	biotin-GCAGCGAAGTACATCCTTTGTACTGGTCCCTTAGTTGACTTCCTGTCAGACATATCC	(Hovel-Miner et al. 2009)
rsmZ-NB	biotin-CGCAGTCATCCGTATAAGAACTTGCGTTCTTATTGTCATCCTGACAAATC	(Hovel-Miner et al. 2009)
rsmY-BF	TTGATTACTGCATTAGGCTGTGG	This study
rsmY-BR	GCTCTAGACAAACATCACTCCAGTTCAACAATAC	This study
rsmY-BRKN	CAATGTAACCCGGCCAAGCTCAAACATCACTCCAGTTCAACAATAC	This study

rsmY-EFKN	GCTCGATGAGTTTTTCTAAGGATCCT TAAGAAGTGGCATTGTCTTCGTC	This study
rsmY-EF	GCTCTAGATTAAGAAGTGGCATTGTC TTCGTC	This study
rsmY-ER	CCTGCTGAATGGTATCCTCATG	This study
rsmZ-BF	GGCATATTATATGACGACAATCCTG	This study
rsmZ-BR	GCTCTAGAGGGCTGAGTCCCTGGCTT AC	This study
rsmZ-BRGT	GCAAGGCGACAAGGTGCTGATGGGC TGAGTCCCTGGCTTAC	This study
rsmZ-EFGT	CGTTCAAGCCGAGATCGGCTTCAGC GCAAGGTGTTAAATCACAC	This study
rsmZ-EF	GCTCTAGAAGCGCAAGGTGTTAAAT CACAC	This study
rsmZ-ER	AGTGGATTAGATGCAGAACCAGAAG	This study

CONNECTING TEXT

As part of the microbiota inhabiting man-made water systems, *Lp* naturally encounters various disinfectants. The stress resistance of *Lp* strains isolated from a given water distribution system tend to reflect the biocides used in that system. In parallel to identifying genes involved in surviving the starvation condition encountered in water, I investigated putative regulators of specific stress response regulons. Of particular interest were genes involved in overcoming stresses encountered within water distribution systems. One promising candidate was the OxyR gene, a putative oxidative stress response regulator. Therefore, I tested the fitness of an *oxyR* mutant. While corroborating previously published results regarding its regulatory capacity, I uncovered a novel phenotype for the *oxyR* mutant during its characterization. OxyR has an important role in increasing the survival of *Lp* faced with oxidative stress in water.

Contributions of authors: Nilmini Mendis contributed to experimental design, conducted experiments, analyzed data and wrote the manuscript. Hana Trigui contributed to the experimental design and the execution of protein gels. Adrianna Tsang conducted the initial survival experiment and constructed pSF47. Mariam Saad constructed SPF172 and SPF173. Sebastien Faucher contributed to the experimental design, writing and editing the manuscript.

This chapter will be submitted for publication.

CHAPTER 5: Characterization of the roles of OxyR in the intracellular pathogen *Legionella pneumophila*

5.1 Abstract

The intracellular pathogen *Legionella pneumophila* (*Lp*) is a strict aerobe, surviving and replicating in environments where it frequently encounters reactive oxygen species, such as the nutrient-poor water environment and inside host cells. In many proteobacteria, the oxidative stress response is regulated by the LysR-type regulator OxyR; however, the importance of the OxyR homologue in *Lp* is still unclear. Therefore, we undertook the characterisation of the phenotypes associated with the deletion of OxyR in *Lp*. In the presence of a yet-unidentified component in commercial agar, the loss of OxyR caused a severe growth defect on charcoal yeast extract (CYE) agar plates. Supplementing the growth medium with anti-oxidants that neutralize reactive oxygen species (ROS) or introducing *oxyR* gene *in trans* rescued the observed growth defect. While OxyR does not exert transcriptional control over traditional anti-oxidative genes that target hydrogen peroxide (H_2O_2), the *oxyR* mutant was more sensitive to H_2O_2 stress than the wild-type during the post-exponential phase. OxyR was dispensable for growth in rich broth, in amoeba and in human cultured macrophages, and for the survival of *Lp* in water. Nevertheless, *Lp* required the transcriptional regulator to survive a mild H_2O_2 stress during survival in water.

5.2 Introduction

Legionella pneumophila (*Lp*) is a Gram-negative, opportunistic pathogen causing the severe pneumonia Legionnaires' disease in humans (Brenner et al. 1979, Steinert et al. 2002). A ubiquitous inhabitant of natural water bodies (lakes, rivers, ponds), *Lp* is transmitted to humans by inhalation of contaminated aerosols emitted by various water distribution systems (van Heijnsbergen et al. 2015). In water, *Lp* infects and replicates within protozoa, and preferentially invades and multiplies inside alveolar macrophages upon entry into the lung (Horwitz and Silverstein 1980, Steinert et al. 2002).

Man-made water distribution systems and the host cells, in which *Lp* multiplies, both present multiple environmental stresses, that include starvation, thermal stress, cold shock, inhibitory concentrations of metal ions, osmotic stress and pH stress (Charoenlap et al. 2015, Dukan and Nystrom 1999, Eason and Fan 2014, Fliermans et al. 1981, Follmann et al. 2009, Iuchi and Weiner 1996, Koubar et al. 2011, McDougald et al. 2002, Pagnier et al. 2012, States et al. 1985). Oxidative stress, in the form of reactive oxygen species (ROS), is one such obstacle that *Lp* must overcome to successfully survive. This is further true since *Lp* is a strict aerobe, a lifestyle that inherently generates oxidative stress (Gonzalez-Flacha and Demple 1997, Imlay 2003, Iuchi and Weiner 1996, Lesser 2006). For example, exponential phase *Escherichia coli* is known to produce 14μM of H_2O_2 in its cytoplasm (Seaver and Imlay 2001). It is noteworthy that despite the negative effects of excessive production of ROS, they are also involved in signal transduction (Lesser 2006). Conditions such as starvation, oxidative biocides as well as exposure to UV light and low pH are conditions that are commonly encountered by microorganisms that inhabit man-made water distribution systems which generate oxidative stress (Charoenlap et al. 2015, Dukan and Nystrom 1999, Follmann et al. 2009, McDougald et al. 2002). As such, oxidative stress would be frequently encountered by *Lp*. Therefore, its anti-oxidative arsenal may be important for both survival and virulence, and consequently, subject to tight regulation.

The ability to cope with both endogenous and exogenous oxidative stress is an important virulence determinant for many microorganisms, such as *Lp*, that infiltrate phagocytes (Eason and Fan 2014, Horwitz 1983b, Robinson 2009). To successfully replicate within the host, *Lp* manipulates host cell trafficking to halt endosome maturation, creating an intracellular niche called the *Legionella*-containing vacuole (LCV) (Hardiman et al. 2012, Horwitz 1983b, Horwitz and Silverstein 1980, Joshi et al. 2001). This modified vesicle is isolated from the endocytic

pathway, which results in inhibition of fusing with lysosomes and delayed phagosome acidification (Horwitz 1983b, Joshi et al. 2001, Sturgill-Koszycki and Swanson 2000). Despite this modification process isolating the LCV from the degradative effects of lysosomal enzymes and ROS, *Lp* is still reported to experience some oxidative stress upon entry into at least two host cells, namely primate alveolar macrophages and amoeba (Halablab et al. 1990, Jacobs et al. 1984). In contrast, NADPH oxidase activation, that catalyzes the production of superoxide, was abolished in the U937 macrophage-like cells which inhibits the oxidative burst (Harada et al. 2007). The extent of ROS exposure may, therefore, be host cell dependent.

Lp harbours two alkyl hydroperoxide reducatse systems, two bi-functional catalase-peroxidases and two superoxide dismutases for efficient neutralization of ROS (Bandyopadhyay and Steinman 1998, 2000, LeBlanc et al. 2006, Sadosky et al. 1994, St. John and Steinman 1996). The two catalase-peroxidases (KatA and KatB) of *Lp* have been shown to aid in replication inside cultured and primary macrophages, and *Acanthamoeba catellanii* during the first three days during infection, but does not impede the final bacterial counts (Bandyopadhyay et al. 2003, Bandyopadhyay and Steinman 1998, 2000). This would suggest that intracellular *Lp* is likely subject to ROS produced by the host cell during the initial stages of infection (Eason and Fan 2014). Despite inducing the expression of the AhpC alkyl hydroperoxide reductase systems (AhpC1 and AhpC2D) during infection (Rankin et al. 2002), mutation of *ahpC1* or *ahpC2D* does not produce any defect in intracellular growth (LeBlanc et al. 2006). The compensatory increase in Ahp and catalase-peroxidase expression when one member is mutated explains the lack of virulence defects observed upon mutation of a single gene (Bandyopadhyay et al. 2003, LeBlanc et al. 2006). While the anti-oxidative stress encountered within the host is minimized by effectors secreted by *Lp* using the type IV secretion system, it is likely that the anti-oxidative defenses are still crucial for the intracellular multiplication and survival.

Furthermore, late stages of infection are known to occur in mature acidified phagosomes which are reported to achieve pH 5.5 (Sturgill-Koszycki and Swanson 2000). pH stress has been linked to oxidative stress response in *Corynebacterium glutamicum*, whereby acidic condition have been shown to trigger anti-oxidative genes (Follmann et al. 2009). While the expression of typical anti-oxidative genes have been reported to remain unchanged within a human host cell model (Faucher et al. 2011), the overlap between pH stress and the oxidative response leaves the possibility of alternative anti-oxidative responses, other than ROS-neutralizing enzymes, being

activated during the infection cycle (Follmann et al. 2009). These lines of evidence suggest a potentially important role for the regulation of oxidative stress in the life cycle of *Lp.*

Bacteria possess genetic tools to manage their anti-oxidative response. To this effect, the characterization of genes that are regulators of oxidative stress resistance has been the subject of research in several pathogens (Burbank and Roper 2014, Gundogdu et al. 2015, Kajfasz et al. 2012, Palma et al. 2005, Reen et al. 2013, Wei et al. 2012, Yesilkaya et al. 2013). One of the most extensively studied regulators of oxidative stress in bacteria is OxyR, a LysR Type Transcriptional Regulator (LTTR) (Imlay 2015), first identified in *Salmonella enterica* serovar Typhimurium (Christman et al. 1985, Christman et al. 1989). It is composed of a characteristic N-terminal DNA-binding domain, and regulates oxidative stress-related genes in *Escherichia coli*, *Vibrio cholerae* and *Pseudomonas aeruginosa* among others (Kong et al. 2004, Tao et al. 1991, Wei et al. 2012). The *E. coli* OxyR protein is activated when a reactive cysteine residue is oxidized by H_2O_2, forming a dilsulfide bond (Zheng et al. 1998). This oxidation allows a conformational change of the OxyR tetramer allowing it to bind to DNA as a transcription factor (Imlay 2015). While OxyR primarily responds to H_2O_2, it may theoretically be generally responsive to electrophiles (Imlay 2015). The breadth of the OxyR regulon varies between bacterial species, but includes traditional anti-oxidative enzymes such as catalase, peroxidise and superoxide dismutase (Burbank and Roper 2014, Charoenlap et al. 2015, Heo et al. 2010, Seo et al. 2015). Other target genes include the DNA-binding protein Dps, the small RNA OxyS, quorum sensing, DNA repair, metal ion transport, sulfur metabolism, protein nitrosylation and iron homeostasis genes (Hassett et al. 2000, Heo et al. 2010, Imlay 2015, Kim and Holmes 2012, Liu et al. 2011, Milse et al. 2014, Seo et al. 2015, Seth et al. 2012, Storz et al. 1989, Tartaglia et al. 1989, Wang et al. 2012, Wei et al. 2012).

Recent work has elucidated some molecular mechanisms and targets of OxyR-dependant regulation in *Lp*. *In vitro* studies by LeBlanc et al. (2008) showed the binding of OxyR to AhpC2D, an alkyl hydroperoxidase system. The study also reported putative OxyR binding sites throughout the *Lp* genome (LeBlanc et al. 2008). More recently, Tanner et al. (2016b) confirmed the aforementioned binding to AhpC2D, and provided *in vivo* proof of direct OxyR binding CpxRA operon, a two-component system involved in regulating Icm/Dot effectors, and IcmR, a chaperone required for effector translocation through the Icm/Dot system. All three genes were found to be negatively affected by OxyR (LeBlanc et al. 2008, Raychaudhury et al. 2009, Tanner

et al. 2016b). To our knowledge, significant phenotypes that are mediated by OxyR in response to exogenous oxidative stress in *Lp* have never been reported. In this work, we characterized the *oxyR* mutant of *Lp*. The role of OxyR and its downstream targets on intracellular multiplication and survival in water are tested. Our results conclusively implicate a role for OxyR when *Lp* is challenged with an oxidative stress stress in water.

5.3 Materials & Methods

5.3.1 Bacterial Strains and Media

The wild-type strain KS79 is a Δ*comR* mutant of the JR32 strain rendering it competent. JR32 is a salt-sensitive, streptomycin-resistant, restriction negative mutant of *Lp* strain Philadelphia 1 (Sadosky et al. 1993). Δ*oxyR* was constructed by allelic exchange with a kanamycin cassette and is a kind gift of Galadriel Hovel-Miner (Hovel-Miner et al. 2009). The *dotA*$^-$ strain, used as a negative control in intracellular multiplication (ICM) assays, is a transposon insertion mutant carrying a mutation in the type IVb secretion system that is essential for intracellular multiplication in *Lp* (Sadosky et al. 1993). A complete list of strains can be found in Table 5.1. Bacterial strains stored at -80^{o}C in 10% glycerol were grown on CYE (charcoal yeast extract) agar supplemented with 0.25mg/ml L-cysteine and 0.4mg/ml ferric pyrophosphate. DIFCO agar was used as the solidifying agent. AYE broth (CYE without agar and charcoal) was used as the liquid medium. The defined water medium Fraquil was used for water exposure experiments (Li et al. 2015, Mendis et al. 2015).

5.3.2 Construction of Plasmids and Complemented Strains

The *oxyR* gene was amplified with its own promoter from the KS79 wild-type strain using primers 11 and 12 (Table 5.2). The pXDC39 vector and the amplicon were then digested with XbaI (New England Biolabs) and purified using a QIAGEN MiniElute Purification kit. The digested vector and insert were ligated overnight using T4 DNA Ligase (New England Biolabs) at 16^{o}C. The recombinant plasmid was then transformed into *E. coli* DH5α. The transformed population was incubated at 37^{o}C shaking for 90 minutes before plating on 25ug/ml chloramphenicol plates. Colonies that grew on antibiotic plates were patched and tested by PCR for insertion of the *oxyR* gene into the vector. The recombinant plasmid was extracted using a QIAGEN Plasmid Extraction kit and introduced into the *oxyR* mutant to produce the complement

(SPF173). The same plasmid construct was introduced into KS79 (SPF172) producing a merodiploid strain.

5.3.3 Growth of Strains on Anti-Oxidant Supplemented Agar

CYE-grown KS79, Δ*oxyR*, SPF172 and SPF173 were suspended in AYE broth at an OD_{600} of 0.1. These cultures were serially diluted in 96-well dilution plates and spotted on CYE plates with and without anti-oxidant supplements. CYE agar was supplemented with 1% α-ketoglutarate, 2000 units of catalase, 0.1% sodium pyruvate or 1mM $FeSO_4$ when necessary.

5.3.4 Polyacrylamide Gel Electrophoresis (PAGE) and Staining

The wild-type and *oxyR* mutant strains were grown in CYE agar and suspended in AYE or water at an OD_{600} of 2.5. Samples exposed to water were incubated for 24 hours at room temperature before protein extraction. Samples were centrifuged and washed twice with phosphate-buffered saline (PBS) buffer. The bacterial pellets were suspended in 500µl of PBS and sonicated for approximately 15 minutes until the PBS solution was clear. Cell lysates were then centrifuged at 12,000 RCF for 10min in a cold centrifuge and the supernatant was transferred into a fresh tube. The protein concentration in the supernatant was quantified by Bradford assay (Biorad) using a bovine serum albumin (BSA) standard curve. 500µg of protein was separated on a polyacrylamide gel composed of a 7.5% acrylamide resolving layer and a 4% acrylamide stacking layer. 50µg of purified bovine catalase was used a positive control. The gels were then bathed in a 0.03% H_2O_2 solution for 20 minutes before staining with a 1% ferric cyanide and 1% ferric chloride solution to visualize the bands corresponding to the catalase-peroxidases as previously described (Wayne and Diaz 1986).

5.3.5 Construction of GFP Reporter Plasmids and Promoter Activity Assay

To construct the green fluorescent protein (GFP) reporter plasmid pSF78, the pXDC31 and pXDC39 plasmids were extracted using the QIAprep Spin Miniprep kit. The purified plasmids were digested overnight using SacI and XmnI (New England Biolabs) at 37°C. The promoterless GFP fragment released from pXDC31 was gel purified using the QIAquick Gel Extraction kit from Qiagen. The digested pXDC39 was purified using the QIAquick PCR Purification kit. The GFP fragment was ligated and transformed into *E. coli* DH5α. After plating, the resulting colonies were patched and plasmid extracted to verify insertion of the GFP into pXDC39 (pSF78) by PCR using primers #1 and #2 (Table 5.2).

pSF78 was electroporated into the KS79 and Δ*oxyR* backgrounds as negative controls. For each of the genes that were tested for control by OxyR, approximately 500 base pairs upstream of the translation start site were amplified with XbaI restriction sites on both ends using primers #3-10 respectively. An XbaI restriction site was used as it lies upstream of the GFP gene in the pSF78. The amplified promoter regions and pSF78 were cut with XbaI for three hours at 37°C. To prevent pSF78 from self-ligating, the restriction reaction was treated with 1ul of alkaline calf intestinal phosphatase (New England Biolabs) after 2 hours of digestion and allowed to proceed for an additional hour. The digested plasmid and promoter regions were purified using the QIAquick PCR Purification kit. The plasmid and the respective promoter fragments were ligated overnight and transformed into *E. coli*. Then, the plasmid was extracted and verified for the correct orientation of insertion using primer #1 and the reverse primers of the respective fragments. Each plasmid construct was introduced into both KS79 and Δ*oxyR* strains.

Strains containing the reporter plasmids were then grown on CYE agar. One colony was suspended in water and the fluorescence of 5000 cells was measured immediately with a Millipore Guava easyCyte flow cytometer. Each strain was tested independently four times.

5.3.6 Water Exposure Experiments

Water experiments were done in the artificial water medium, Fraquil, as described previously (Li et al. 2015, Mendis et al. 2015). Briefly, *Lp* strains cultured on CYE agar at 37^{o}C for 3 days were suspended in Fraquil at an OD_{600nm} of 0.1. One millilitre of this bacterial suspension was mixed with 4ml of fresh DFM in a 25cm^2 culture flasks (Sarstedt) and incubated at 25^{o}C.Survival of the strains in water was monitored using CFU counts. Fraquil is composed of 0.25μM $CaCl_2$•$2H_2O$, 0.15 μM $MgSO_4$•$7H_2O$, 0.15 μM $NaHCO_3$, 10nM K_2HPO_4, 0.1 μM $NaNO_3$, 10nM $FeCl_3$•$6H_2O$, 1nM $CuSO_4$•$5H_2O$, 0.22nM $(NH_4)_6Mo_7O_{24}$•$4H_2O$, 2.5nM $CoCl_2$•$6H_2O$, 23nM $MnCl_2$•$4H_2O$, 4nM $ZnSO_4$•$7H_2O$ (Mendis et al. 2015).

5.3.7 Intracellular Multiplication Assays

Intracellular multiplication was measured in the amoeba, *Acanthamoeba castellanii*, and in THP-1-derived human macrophages using a multiplicity of infection (MOI) of 0.1. *A. castellanii* were cultured in peptone yeast glucose (PYG) broth (20g proteose peptone, 1g yeast extract, 0.1M glucose, 0.4mM $MgSO_4$, 0.05mM $CaCl_2$, 0.1mM sodium citrate, 0.005mM $Fe(NH_4)_2(SO_4)_2$, 0.25mM Na_2HPO_4 and 0.25mM KH_2PO_4, adjusted to pH 6.5 with HCl). One

day before infection, $2.5x10^5$ cells in 1ml of PYG were seeded into each well of a 24-well plate and left to incubate at 30°C. One hour prior to infecting the amoeba with *Lp*, the media in each well was replaced with 1ml of AC buffer (PYG without proteose peptone, yeast extract and glucose). THP-1 monocytes were cultured in RPMI (GIBCO) supplemented with L-glutamine and 5% FBS. For the THP-1 infection, $5x10^5$ cells treated with 10^{-7}M phorbol myristate acetate (PMA) were seeded into a 24-well plate in 1ml of RPMI 3 days prior to infection, and incubated at 37°C in 5% CO_2. One hour prior to infecting the macrophages with *Lp*, the media in each well was replaced with fresh RPMI. The wild type KS79 was used as a positive control, while a *dotA* mutant deficient in intracellular multiplication served as a negative control. Strains grown on CYE agar were suspended in AYE broth at an OD_{600} of 0.1, further diluted 10 fold to obtain an approximate OD_{600} of 0.01 and 2µl of this final solution was used to infect macrophages and amoeba. Growth inside cells was measured by CFU counts every 24h.

5.3.8 Susceptibility to Exogenous H_2O_2

Lp grown on CYE were suspended in AYE broth at an OD_{600} of 0.1 and grown to exponential phase (E) and post-exponential phase (PE) at 37°C shaking. For each growth phase, a sample was first removed and the number of cells was determined by CFU counts. Then, aliquots from each growth phase were treated with 1mM and 10mM H_2O_2. The susceptibility of each strain was tested in triplicate by CFU counts and sampled 10, 30 and 60 minutes after addition of H_2O_2. To test sensitivity to H_2O_2 in water, *Lp* strains cultured on CYE agar at 37°C for 3 days were suspended in Fraquil at an OD_{600nm} of 0.1. One millilitre of this bacterial suspension was mixed with 4ml of fresh Fraquil in a $25cm^2$ cell culture flasks (Sarstedt) and incubated at 25°C for two hours. At the end of this acclimatization period, each strain was treated with 1mM H_2O_2. CFU counts were performed immediately before and 24 hours after H_2O_2 treatment.

5.4 Results

5.4.1 Deletion of *oxyR* prevents the formation of isolated colonies

A screening of regulatory mutants for their potential role in adapting to and surviving a nutrient-poor water environment led to the discovery of an apparent severe survival defect of the Δ*oxyR* strain. Surprisingly, absence of this transcriptional regulator caused a complete survival defect at the first time point (t=0) (Fig 5.1A), only growing in the undiluted samples in

concentric circles. At the time of plating, the strains had been exposed to the freshwater medium for approximately 40 minutes. Total loss of viability is unlikely after the given incubation time in water. Importantly, colony forming units (CFUs) are essential for the characterization of the mutant, and the inability of the *oxyR* mutant to form CFUs needed to be addressed. An alternative cause, other than cellular death, was sought to explain the lack of CFUs of Δ*oxyR* strain upon water exposure. When inoculated on solid CYE medium, the Δ*oxyR* strain is unable to form small isolated colonies like the wild-type, and mostly presents itself as a lawn in the quadrant of initial inoculation (Fig 5.1B & C). In addition, the few isolated colonies that Δ*oxyR* formed were comparatively larger than the wild-type colonies (Fig. 5.1B and C). Loss of OxyR is known to decrease the culturability of *Shewanella oneidensis*, *Vibrio vulnificus*, *Pseudomonas aeruginosa* among others (Hassett et al. 2000, Kong et al. 2004, Shi et al. 2015). Furthermore, this inability is attributable to the presence of reactive oxygen species (ROS) or their accumulation in media over time. Therefore, we tested the ability of anti-ROS supplemented CYE agar to correct the growth defect of Δ*oxyR*. Accordingly, the wild-type, mutant, complemented and merodiploid strains grown on CYE plates were suspended in AYE broth and CFU counts were used to enumerate bacteria on agar supplemented with one of the following anti-oxidative molecules: sodium pyruvate, α-ketoglutarate or catalase. As expected, there was a visible defect in the culturability of the *oxyR* mutant strain on CYE medium in the absence of anti-oxidant molecules (Fig 5.2). This resulted in a 400 fold difference in the culturability between the wild-type and mutant strain. The complemented Δ*oxyR* (SPF173) and the merodiploid strain (SPF172) showed wild-type culturability levels after suspension, independent of anti-oxidant presence (Fig 5.2). Therefore, supplementation of CYE agar with anti-ROS compounds increased the recovery of the mutant strain to wild-type levels. Moreover, the complemented strain grew as well as the wild-type, confirming that the growth defect of the mutant is caused by the absence of *oxyR*.

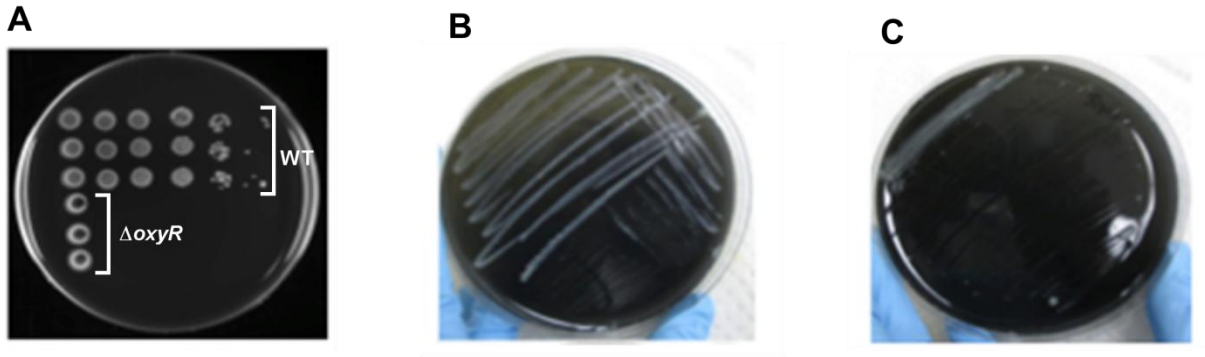

Figure 5.1: The *oxyR* mutant strain is unable to form isolated colonies on CYE agar. A) The wild-type (WT) and *oxyR* mutant strain were suspended in water, serially diluted and plated on CYE agar for CFU counts. Colony morphology of the wild-type (B) and Δ*oxyR* (C) strains on CYE plates after three days at 37^{o}C.

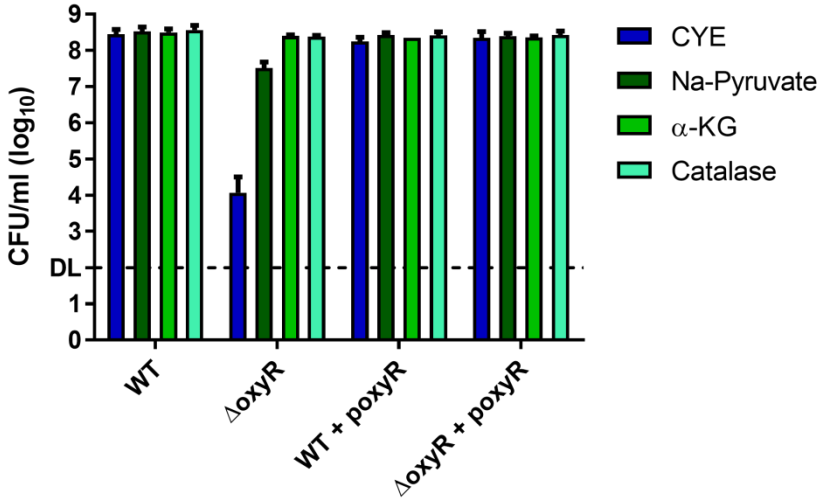

Figure 5.2: Addition of antioxidants to CYE agar plates. Antioxidant molecules alleviate the growth phenotype of the *oxyR* mutant strain. The wild-type (WT), *oxyR* mutant, complemented (Δ*oxyR* + p*oxyR*) and merodiploid (WT + p*oxyR*) strains were suspended in AYE broth and CFU counts were performed on CYE plates with or without an antioxidant molecule (sodium pyruvate, α-ketoglutarate or catalase). DL, detection limit; α-KG, α-ketoglutarate; Na-pyruvate, sodium pyruvate. Data shown represent the mean and SD of three biological replicates.

In stark contrast to the impaired growth of Δ*oxyR* on solid media, the mutant grew comparably to the wild-type in broth (Fig 5.3). Given that agar is often cited as a source of toxic compounds (Ezraty et al. 2014, Gould et al. 1944, Ley and Mueller 1946, Sands and Bennett 1966), the growth of *Lp* on CYE solidified with agarose was compared to growth on CYE solidified with agar (Fig 5.4). The substitution by agarose allowed the *oxyR* mutant strain to form colonies to levels comparable to the wild-type and the complemented strain (Fig 5.4), supporting the hypothesis that a compound in the agar is causing the growth defect of the *oxyR* mutant.

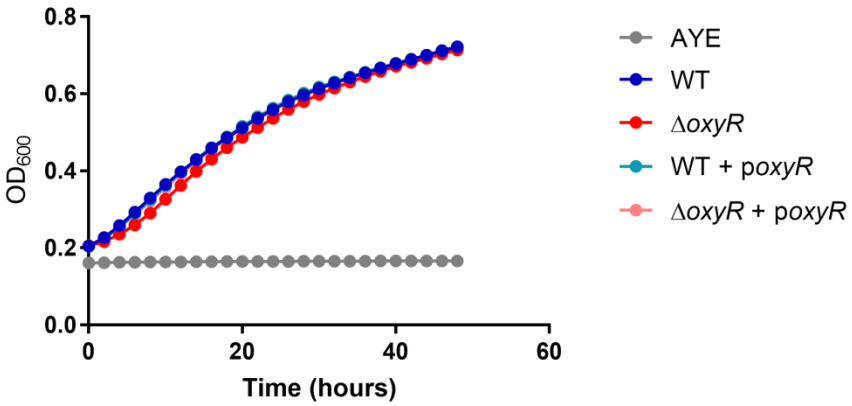

Figure 5.3: OxyR is dispensable for *Lp* growth in rich AYE broth. The wild-type (WT), *oxyR* mutant, complemented (Δ*oxyR* + p*oxyR*) and merodiploid (WT + p*oxyR*) strains were inoculated into AYE broth and their growth was monitored using the optical density at 600nm (OD_{600}) over a period of 48 hours. Data shown represent the mean and SD of readings from three biological replicates.

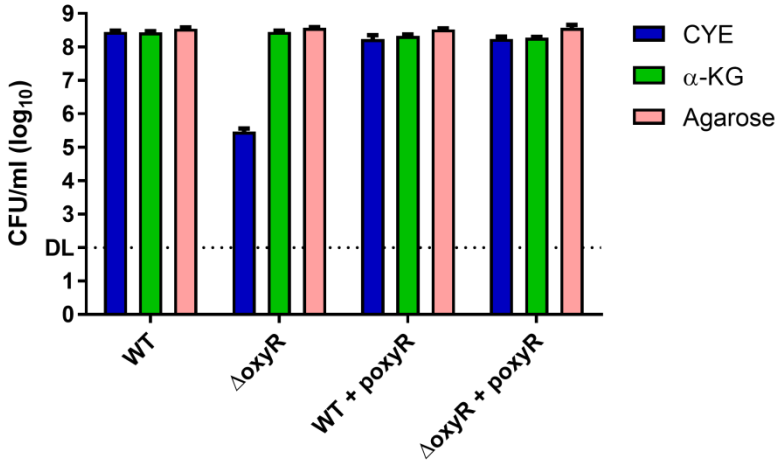

Figure 5.4: Δ*oxyR* is sensitive to a compound found on agar. The wild-type (WT), *oxyR* mutant, complemented (Δ*oxyR* + p*oxyR*) and merodiploid (WT + p*oxyR*) strains were suspended AYE broth. CFU counts were performed on CYE plates made with agar, with agarose or supplemented with α-ketoglutarate. DL, detection limit; α -KG, α-ketoglutarate. Data shown represent the mean and SD of three biological replicates.

5.4.2 Δ*oxyR* is more susceptible to hydrogen peroxide in PE phase

The molecule(s) in commercial agar that cause the observed oxidative stress is unknown; however, the growth defect can be neutralized with anti-oxidative agents (Fig 5.2). Therefore, we postulated that the *oxyR* mutant may be sensitive to hydrogen peroxide. To test this hypothesis, the wild-type, Δ*oxyR*, complemented strain and the merodiploid strain grown to E or PE phase were exposed to either 1mM or 10mM H_2O_2. *Lp* was found to be more resistant to H_2O_2 in PE phase than in E phase (Fig 5.5). In fact, the mutant strain was also slightly more resistant than the wild-type strain to 1 mM H_2O_2 during E phase (Fig 5.5A). All strains were equally sensitive to 10 mM H_2O_2 during E phase, reaching the detection limit at 30 minutes post-treatment (Fig 5.5B). In contrast, during PE phase, all strains were resistant to 1mM H_2O_2 with no changes in CFU counts for the wild-type (Fig 5.5C). The *oxyR* mutant was slightly more sensitive than the wild-type when exposed to 10mM H_2O_2 (Fig 5.5D). Therefore, OxyR-dependant oxidative stress resistance is affected by the growth phase of *Lp*.

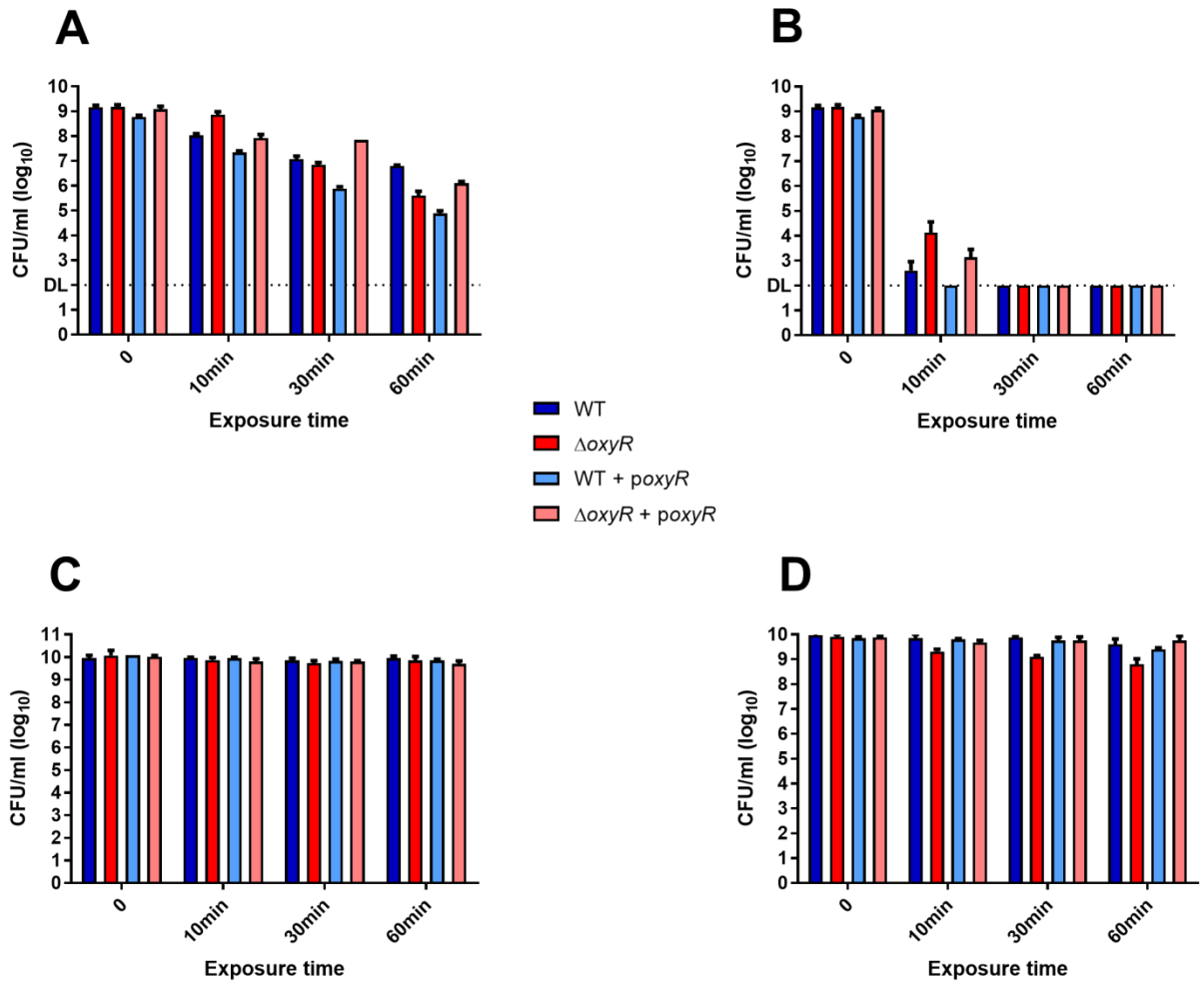

Figure 5.5: The *oxyR* mutant strain is more susceptible to H_2O_2 in the post-exponential phase. The wild-type (WT), *oxyR* mutant, complemented (Δ*oxyR* + p*oxyR*) and merodiploid (WT + p*oxyR*) strains were grown in AYE broth to exponential phase (A and B) and to post-exponential phase (C and D), and exposed to 1 mM (A and C) and 10 mM (B and D) H_2O_2. CFU counts were performed before addition of H_2O_2 and after 10 min, 30 min and 60 min of exposure. DL, detection limit. Data shown represent the mean and SD of three biological replicates. Student T-test was used to access significant differences compared to the wild-type: * $p<0.05$; ** $p<0.005$.

5.4.3 OxyR does not positively regulate typical antioxidant genes

The sensitivity of the *oxyR* mutant to H_2O_2 stress may indicate that 1) antioxidative genes that directly neutralize H_2O_2 are downregulated in the mutant, or 2) the mutant lacks expression of genes that prevent or repair damage imparted by H_2O_2 action. Previous studies using the Lp02 strain have established that OxyR does not bind traditional antioxidative genes that neutralize H_2O_2 in *Lp* (LeBlanc et al. 2008, Tanner et al. 2016b). To corroborate this regulatory pattern in the JR32 parental strain used here, the regulation of six traditional anti-oxidative genes was tested on solid medium. The activity of the two catalase-peroxidases of *Lp*, KatA and KatB, in the wild-type strain were compared to that of Δ*oxyR* (Fig 5.6A). A native PAGE gel was used to migrate total protein extracts of the wild-type and mutant cultured on CYE medium. After exposing the gel to H_2O_2, it was stained with a mixture of ferric cyanide and ferric hydrochloride to reveal the catalase activities of KatA and KatB (Fig 5.6A). The KatA and KatB band intensities were similar in the wild-type compared to the mutant, suggesting similar activities.

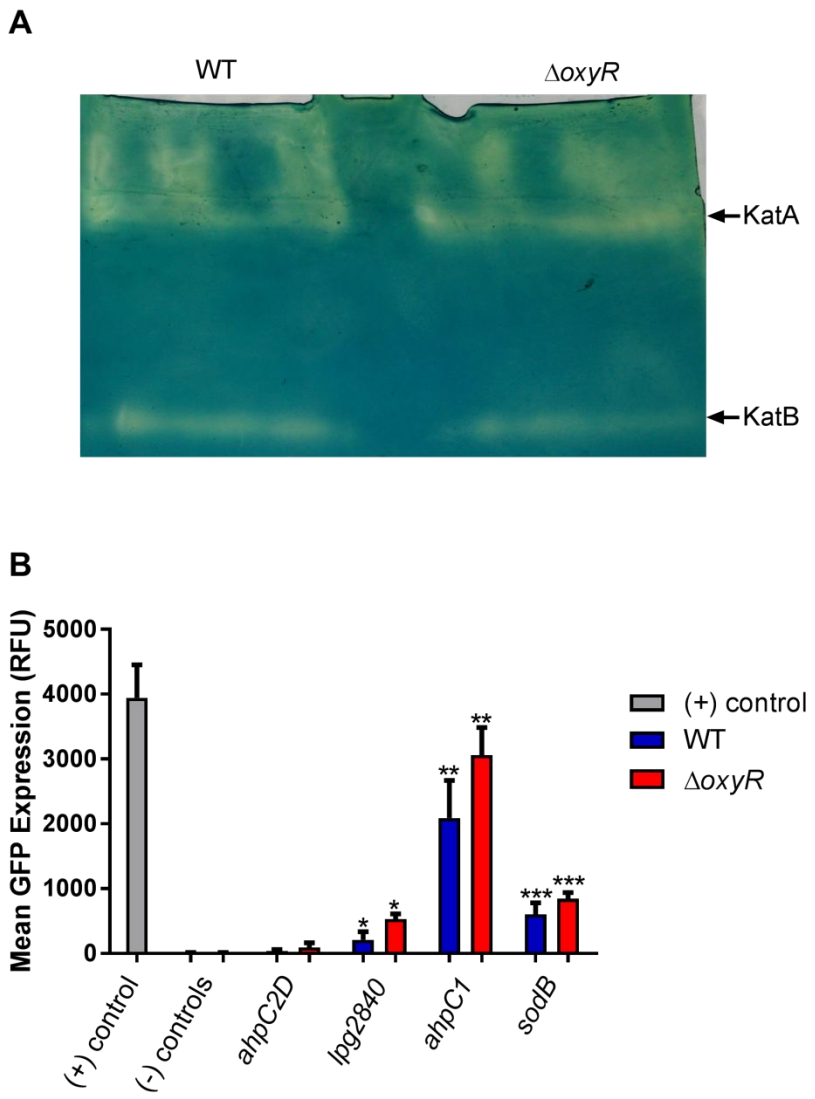

Figure 5.6: Activity and expression of classical antioxidative genes are not affected by OxyR. A) Whole protein extracts from the wild-type (WT) and the *oxyR* mutant strains grown on CYE agar (left) and exposed to water for 24 hours (right) were migrated and stained for catalase activity on a native PAGE gel. Two bands corresponding to the two catalase-peroxidases of *Lp* are shown. B) The promoter activity of *ahpC2D, lpg2840, ahpC1* and *sodB* was monitored using a GFP reporter introduced into the wild-type (WT) and *oxyR* mutant strains. Strains were grown on CYE plates, suspended in water and the GFP fluorescence of 5000 cells was measured immediately by flow cytometry. The values show the mean of four independent replicates.

To investigate whether other genes commonly associated with oxidative stress were under the control of OxyR, the promoter activity of four anti-oxidative genes was tested using a GFP transcriptional fusion. The promoter regions of three alkyl hydroperoxidase genes (*lpg2350/ahpC2*, *lpg2840* and *lpg2965/ahpC1*) and *sodB* were cloned into pSF78, upstream of a promoterless GFP gene. Each construct was introduced into both the wild-type strain and Δ*oxyR* to measure the activation of the respective promoters using GFP production. *lpg2350* codes for AhpC2 of the AhpC2D operon and *lpg2965* for AhpC1, both of which are alkyl hydroperoxide reductase systems (LeBlanc et al. 2006). *lpg2840* encodes a putative peroxiredoxin of the AhpC/TSA family of proteins. *sodB* encodes a cytoplasmic superoxide dismutase (Sadosky et al. 1994). Since the Δ*oxyR* growth defect phenotype was observed on agar plates, we tested activation of these promoters when the wild-type and Δ*oxyR* were grown on CYE agar. The promoters of *lpg2840*, *ahpC1* and *sodB* are negatively affected by the absence of *oxyR* (Fig 5.6B). Expression of GFP driven from the promoter of *ahpC2D* was minimal and no difference was observed. Taken together, our data supports previous reports that OxyR is a negative regulator of traditional antioxidant genes (LeBlanc et al. 2008, Tanner et al. 2016b), and that the growth defect observed in the *oxyR* mutant is not due to insufficient expression of these genes. Given the complex nature of OxyR-mediated regulation and the deviation from established regulatory patterns associated with other OxyR homologs, the following experiments were designed to establish phenotypic effects that accompany loss of OxyR in *Lp*.

5.4.4 Intracellular multiplication of *Lp* in host cells is not affected by the absence of *oxyR*

Since *Lp* undergoes oxidative stress inside host cells during infection and requires catalases (Bandyopadhyay et al. 2003), we tested the capacity of the Δ*oxyR* mutant to multiply inside two host cell types; THP-1 cultured human macrophages (Fig 5.7A) and *A. castellanii* (Fig 5.7B), a natural host of *Lp* in the environment (Rowbotham 1980). The loss of *oxyR* did not cause an intracellular multiplication defect in either of the host cells tested, when CFU counts were performed on CYE plates supplemented with catalase. The mutant was able to grow inside the host cells to the same extent as the wild-type, complemented strain and the merodiploid strains (Fig 5.7). OxyR is, therefore, dispensable for the intracellular growth of *Lp* in the two host cells that were used.

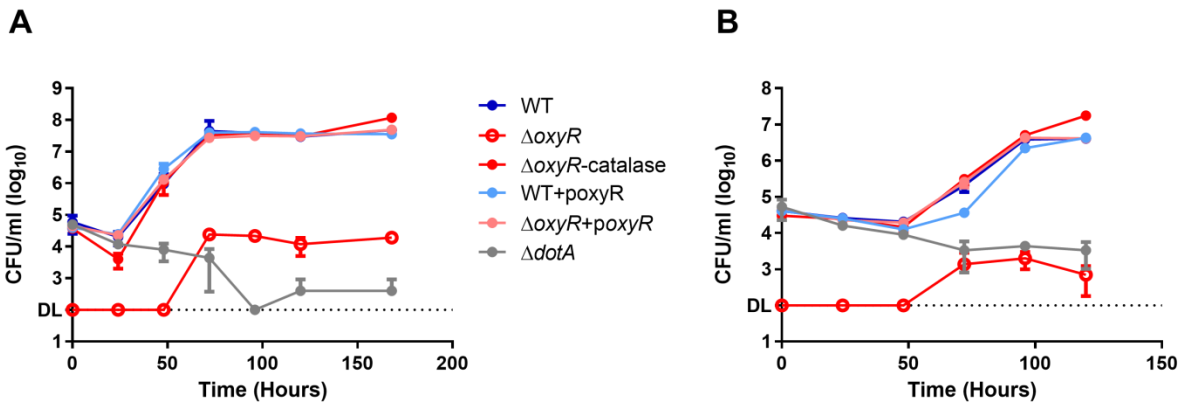

Figure 5.7: The *oxyR* mutant strain does not show an intracellular growth defect. The wild-type (WT), *oxyR* mutant, complemented (Δ*oxyR* + p*oxyR*; SPF173) and merodiploid (WT + p*oxyR*; SPF172) strains grown on CYE agar were suspended in AYE broth and used to infect THP-1-derived macrophages (A) and *Acanthamoeba castellanii* cells (B) at an MOI of 0.1. Daily CFU counts were performed to monitor intracellular multiplication. Δ*oxyR* counts were done on both CYE and CYE supplemented with catalase. DL, detection limit. Data shown represent the mean and SD of three biological replicates.

5.4.5 Deletion of *ΔoxyR* does not impact the survival of *Lp* in water

The long-term survival of the wild-type strain, the mutant, the complemented strain and the merodiploid strain in Fraquil was re-evaluated using CYE plates supplemented with catalase. Over the course of 60 days, the mutant did not demonstrate a survival defect in water compared to the wild-type (Fig 5.8A). Therefore, deletion of *oxyR* does not affect the survival of *Lp* in water.

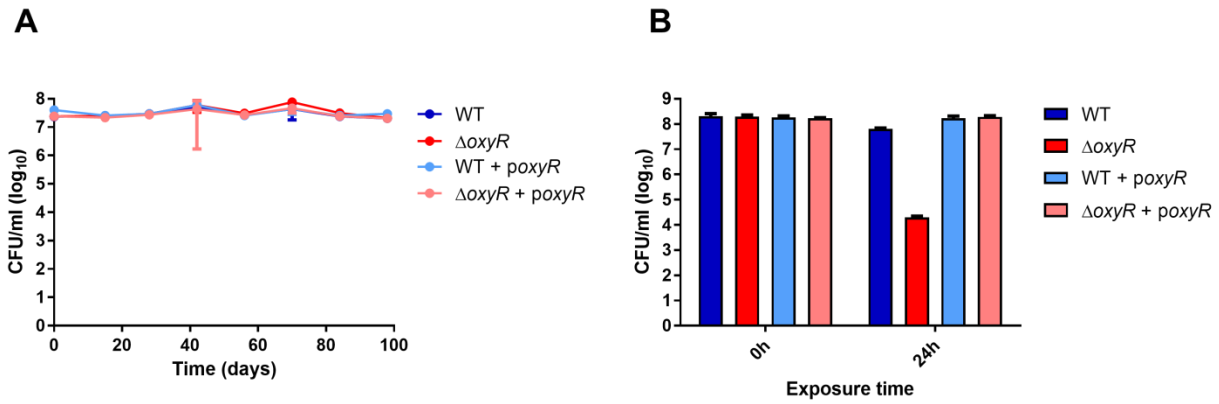

Figure 5.8: The *oxyR* mutant is sensitive to H_2O_2 in water. (A) The wild-type (WT), *oxyR* mutant, complemented (Δ*oxyR* + p*oxyR*) and merodiploid (WT + p*oxyR*) strains were suspended in Fraquil. CFU counts were performed weekly to track survival. CFU counts for Δ*oxyR* were monitored using both standard CYE supplemented with α-ketoglutarate. (B) The WT, Δ*oxyR*, complemented and merodiploid strains were allowed to acclimate to water for 2 hours and treated with 1mM H_2O_2. CFU counts were enumerated after 24 hours of treatment. DL, detection limit. Data shown represent the mean and SD of three biological replicates.

5.4.6 OxyR positively influences resistance to oxidative stress in water

In order to simulate oxidative stress encountered by *Lp* in water distribution systems, we treated water-exposed *Lp* strains to a 1mM H_2O_2 stress. The wild-type strain, the mutant, the complemented strain and the merodiploid strain were suspended in water and allowed to acclimate for 2 hours. At the end of this first incubation period, each strain was challenged with 1mM H_2O_2. After 24 hours of exposure to hydrogen peroxide, the survival of Δ*oxyR* was found to be significantly impaired compared to the wild-type in the order of two magnitudes (Fig 5.8B). The observed defect is corrected in the complemented strain. The merodiploid strain reflects survival similar to the wild-type. It would seem that OxyR has a significant impact on the survival of *Lp* against oxidative stress in the water environment.

5.6 Discussion

Legionella pneumophila (*Lp*) is an opportunistic human pathogen that is markedly adept at surviving for extended periods of time in, at times harsh, aquatic environments; persisting despite thermal and disinfection treatments (Alleron et al. 2008, Mendis et al. 2015, Paszko-Kolva et al. 1992, Turetgen 2008, van der Kooij et al. 2005). While *Lp* preferentially infect alveolar macrophages in the context of human infection, its natural environmental hosts include a variety of amoeba, ciliates and nematodes (Horwitz 1983b, Steinert et al. 2002, Swanson and Hammer 2000). The major regulators that allow its survival in man-made water distribution systems and that contribute to the bacterium's virulence are, therefore, of particular interest to better understand the seemingly inevitable colonization of anthropogenic systems by *Lp*. To this end, mutants of key regulator genes were suspended in the defined freshwater medium Fraquil and tested for survival by performing CFU counts as described previously (Li et al. 2015, Mendis et al. 2015, Trigui et al. 2014). Using mutational studies, several genes important for the survival of *Lp* in water, including RpoS, RelA, SpoT, BdhA and LasM, were identified (Li and Faucher 2016, Li et al. 2015, Trigui et al. 2014). In the present article, we report the characterization of a regulatory mutant, lacking the putative oxidative stress response regulator, OxyR.

In contrast to the wild-type that forms isolated colonies on CYE agar, the *oxyR* mutant grows in patches (Fig 5.1A and B), suggesting that the cumulative action of a compound collectively secreted by a large number of cells is able to overcome the growth defect that was observed when Δ*oxyR* is diluted. Alternatively, given that the *Lp* anti-oxidative enzymes, catalase-peroxidases and superoxide dismutases, are compartmentalized in the cytoplasm or the periplasm (Bandyopadhyay and Steinman 1998, 2000, Sadosky et al. 1994, St. John and Steinman 1996), dead cells may release sufficient amounts of each to allow for dense growth observed in the first quadrant (Fig 5.1B). Rich media used for the cultivation of *Legionella* are known to accumulate ROS during autoclaving or exposure to light, and activated charcoal is added to alleviate this stress in solid media (Hoffman et al. 1983, Pine et al. 1986). Despite the presence of charcoal, the Δ*oxyR* strain used in the present study was unable to form isolated colonies on standard CYE agar (Fig 5.1). This is reminiscent of a growth phenotype that was observed in a *Vibrio vulnificus oxyR* mutant, which was corrected by the addition of anti-oxidative molecules to the growth medium (Kong et al. 2004). A growth defect similar to what is

observed here was recently reported by a group studying the molecular regulatory pattern of OxyR in the Lp02 strain and the defect was corrected upon addition of α-ketoglutarate to the culture medium (Tanner et al. 2016b). Similarly, supplementing CYE plates with catalase, sodium pyruvate or α-ketoglutarate fully recovered the colony forming capacity of the Δ*oxyR* strain originating from both rich medium and from water (Fig 5.2). Sodium pyruvate could only partially recover culturability when the mutant was suspended in rich medium (Fig 5.2). Both sodium pyruvate and α-ketoglutarate have been, and still are used to relieve additional oxidative stress from various bacteriological media (Bogosian et al. 2000, Imazaki and Kobori 2010, McDonald et al. 1983). Notably, α-ketoglutarate is routinely used in CYE agar to help the recovery of *Lp* from environmental sources (Pine et al. 1986). In general, α-ketoglutarate is also a common addition to CYE medium used by most *Legionella* laboratories when employing lab-adapted strains. The parental strain used in this study, JR32, does not require its addition for enhanced growth. We, therefore, omitted α-ketoglutarate in routine CYE. Given the positive effect of supplementation with antioxidants, oxidative stress was ascertained to be the cause of the growth defect of the *oxyR* mutant on agar. Toxic effects of commercial agar has been previously observed for *Haemophilus influenza* and *Neisseria gonorrheae* (Dukes and Gardner 1961, Ley and Mueller 1946). Rebers et al. (1989) noted that replacing agar with agarose enhanced the culturing of *Pasteurella* species. Indeed, replacement of agar with agarose in CYE plates rescued the growth phenotype of the *oxyR* mutant in *Lp* (Fig 5.4). A recent study found that in *Shewanella oneidensis*, the *oxyR* mutation rendered the bacterium unculturable at low cell densities, much like what is observed in here with *Lp* (Shi et al. 2015). The authors successfully demonstrate that both catalase and Fe^{2+} is able to neutralize H_2O_2 in the medium that arrests bacterial growth (Shi et al. 2015). In our case, supplementing CYE with Fe^{2+} did not contribute significantly to correcting the plating defect (Fig S5.1). This suggests that other oxidation products in addition to H_2O_2 may contribute to the oxidative stress encountered on agar plates. Indeed, Hoffman et al. (1983) proposed that activated charcoal used in CYE medium prevents photo-oxidative reactions (including oxidation of cysteine producing additional H_2O_2) and adsorbs free fatty acid which is a source of oxidative stress.

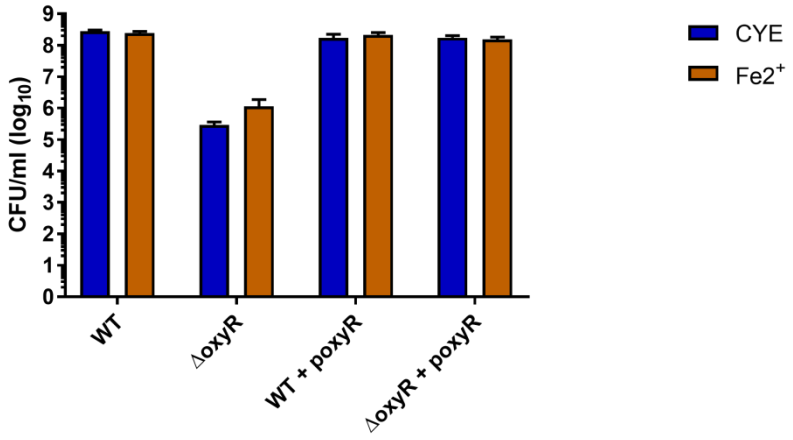

Figure S5.1: Supplementation with Fe^{2+}. The Δ*oxyR* plating defect is not corrected by the addition of Fe^{2+} to agar. The wild-type (WT), *oxyR* mutant, complemented (Δ*oxyR* + p*oxyR*) and merodiploid (WT + p*oxyR*) strains were suspended AYE broth. CFU counts were performed on standard CYE plates and plates supplemented with 1mM Fe^{2+}. Data shown represent the mean and SD of three biological replicates.

The *Lp* genome encodes two bi-functional catalase-peroxidase genes, KatA and KatB (Bandyopadhyay and Steinman 1998, 2000). Using transcriptional fusions, it was established that only a modest increase of at *katA* expression was observed in response to exogenous H_2O_2, while KatB expression remained unchanged (Bandyopadhyay and Steinman 1998, 2000). Mutation of either one resulted in increased sensitivity to exogenous H_2O_2 compared to the wild-type, while Δ*katA* demonstrated a more pronounced sensitivity than Δ*katB* (Bandyopadhyay and Steinman 2000). The transcription of both genes increases during the exponential phase, peaking in the late exponential phase; however, the activities of KatA and KatB are at maximum capacity in the stationary phase (Bandyopadhyay and Steinman 2000). We probed a native protein gel for catalase activity of the two catalase-peroxidases in *Lp* produced on CYE plates (Fig 5.6A). As previously reported, the KatA band showed a higher catalytic activity in both rich media and in water (Bandyopadhyay and Steinman 1998). However, the activities of KatA and KatB were not changed in Δ*oxyR* (Fig 5.6A), similar to *Streptomyces coelicolor* where OxyR does not control the gene coding for catalase, *catA* (Hahn et al. 2002). This also corroborate findings by LeBlanc et al. reporting the lack of OxyR-binding motifs in the promoter regions of the Kat genes (LeBlanc et al. 2008). Interestingly, we report a significant increase in the activity of the

periplasmic catalase-peroxidase, KatA, when *Lp* is exposed to water (Fig S5.2); which correlates with previous studies that report an overlap between the starvation and anti-oxidative responses (Dukan and Nystrom 1999, McDougald et al. 2002). A GFP transcriptional fusion was used to further determine the effect of OxyR regulation on four other anti-oxidative genes (Fig 5.6B). A previous study of *Lp* OxyR found that the binding of OxyR to the promoter region of *ahpC2D* reduces its expression (LeBlanc et al. 2008). LeBlanc et al. (2008) also found that a 1mM H_2O_2 stress abolished binding of OxyR to the *ahpC2D* promoter; however, since this binding was reversed when OxyR was artificially reduced, the authors concluded that OxyR was unresponsive to H_2O_2. On CYE agar plates, the condition tested in this study, the *ahpC2D* promoter was expressed at such a low level, comparable to the negative control, that regulation by OxyR was unclear (Fig 5.6B). Compared to *ahpC2D*, the promoter region of *ahpC1* was highly active in the WT strain on CYE (Fig 5.6B). The compensatory behaviour of the alkyl hydroperoxide reductase systems is consistent with a previous report that found a similar pattern dependent on growth phase (LeBlanc et al. 2006). After three days of growth on CYE agar, the *Lp* population is considered to be mainly composed of cells in the post-exponential phase. As a result, the higher expression of *ahpC1*compared to *ahpC2D* observed in Fig 5.6B is consistent with a higher expression of the latter in exponential phase, and the former in the post-exponential phase of growth in broth (LeBlanc et al. 2006). Moreover, the *oxyR* mutant negatively affected three out of four genes tested here (Fig 5.6B). This negative regulation diverges from previous reports that failed to find OxyR-binding motifs in the promoter regions of the genes tested here and lack of transcriptional activation of *ahpC1* (LeBlanc et al. 2008). Divergence from the canonical *E. coli* system is also seen in other bacterial species. The observed negative regulation may be explained by indirect regulation by OxyR; alternatively, while the GFP levels between the wild-type and the *oxyR* mutant were statistically significant, the differences between strains may not be biologically significant.

Next, we attempted to unveil the importance of OxyR under conditions that *Lp* would encounter naturally. Mutations in the *oxyR* gene resulting in a reduced antioxidant arsenal in other bacterial species have been implicated in virulence attenuation (Burbank and Roper 2014, Erickson et al. 2011, Johnson et al. 2013, Whitby et al. 2012). We, therefore, tested ICM capacity of the *oxyR* mutant in *Acanthamoeba castellanii*, a freshwater amoeba, and in THP-1 cultured human macrophages (Fig 5.7). The mutant was able to grow inside the respective host

cells to the same extent as the wild-type, suggesting that the OxyR regulon was dispensable for infection in the models and conditions that were tested herein, which confirm previous findings (Tanner et al. 2016b). We also tested whether starvation conditions impacted the ICM behaviour of the mutant towards human host cells by exposing the strain to water 24 hours prior to infection; growth within the host was similar to *Lp* that originated from rich medium (Fig S5.3). Interestingly, a recent report found attenuation of intracellular multiplication (ICM) in the freshwater amoeba, *A. castellanii* upon over-expression of *oxyR* in the Lp02 strain of *Lp* (Tanner et al. 2016b). The authors achieved over-expression by introducing the *oxyR* gene with its own promoter into the wild-type strain. Introduction *in trans* using the RSF1010 cloning vector resulted in a 10-fold increase in OxyR protein levels, confirmed by immunoblotting (Tanner et al. 2016b). This over-expression is consistent with the copy number of RSF1010, estimated to be 10-13 copies per cell (Katashkina et al. 2007). In the current study, another derivative of the RSF1010 plasmid, pXDC39, was used to produce the merodiploid strain (SPF172, wild-type+p*oxyR*) which also contains approximately 10-12 copies per cell (Morales et al. 1991). While expression of *oxyR* in the merodiploid was not tested, we report that an extra copy of the gene did not lead to changes in ICM in the host cells tested (Fig 5.7). This discrepancy may be due to strain differences, as reported *oxyR* regulation by CpxR was conflicting in the JR32 wild-type compared to the Lp02 wild-type (Feldheim et al. 2016, Tanner et al. 2016b). It is noteworthy that an *oxyR* mutant in *Haemophilus influenzae* showed no defect during infection in two of the models tested with the exception of competition studies (Whitby et al. 2012). It is therefore, possible that the cultured host cell strains tested here are permissive for infection compared to primary macrophages or environmentally adapted amoebae. Infection of bone marrow-derived macrophages and a competition experiment between the wild-type and the *oxyR* mutant is needed to confirm this hypothesis.

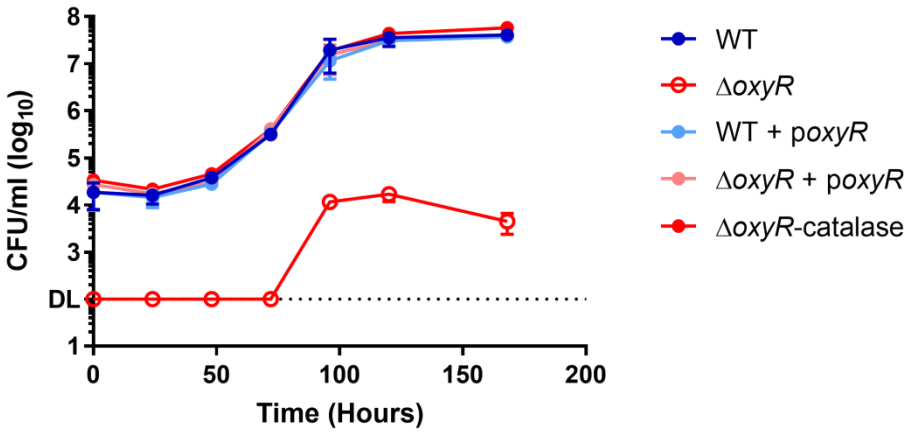

Figure S5.3: Intracellular growth after water exposure. The *oxyR* mutant strain does not show an intracellular growth defect in human cells after exposure to water. The wild-type (WT), *oxyR* mutant, complemented (Δ*oxyR* + p*oxyR*; SPF173) and merodiploid (WT + p*oxyR*; SPF172) strains grown on CYE agar were suspended in water for 24 hours and used to infect THP-1-derived macrophages at an MOI of 0.1. Daily CFU counts were performed to monitor intracellular multiplication. Δ*oxyR* counts were done on both CYE and CYE supplemented with catalase. DL, detection limit. Data shown represent the mean and SD of three biological replicates.

H_2O_2 is the most common stress that is known to activate OxyR (Zheng et al. 1998). In *Lp,* OxyR was reported to be unresponsive to micromolar concentrations H_2O_2; however, milimolar levels of the oxidant induced an irreversible conformational change of OxyR (LeBlanc et al. 2008). As a result, the authors concluded that *Lp* OxyR was locked in an active conformation (LeBlanc et al. 2008). Nevertheless, OxyR was found to positively influence a small regulatory RNA (LprA) which is induced upon H_2O_2 exposure during the exponential phase (Faucher et al. 2010). The DNA-binding protein Dps is known to confer resistance to H_2O_2 in *Lp,* both *in vivo* and *in vitro* through iron sequestration, which Fenton reaction-mediated hydroxyl radical formation (Park et al. 2006, Yu et al. 2009), and its expression is proposed to be under OxyR control given the identification of an OxyR-binding motif in its promoter region (LeBlanc et al. 2008). With the goal of obtaining a global view of the OxyR regulon under a known oxidative stress, we tested the sensitivity of the *oxyR* mutant strain to different levels of

H_2O_2, in rich media and in water. The transcription of *oxyR* is known to increase during the post-exponential phase of growth (Bruggemann et al. 2006, Faucher et al. 2010, LeBlanc et al. 2008, Weissenmayer et al. 2011), even though protein dosing has proven little increase between exponential and post-exponential growth (Tanner et al. 2016b). In agreement with previously reported data, we found that *Lp* is significantly more resistant to H_2O_2 in PE phase than in E phase (Hales and Shuman 1999b). Moreover, deletion of *oxyR* increases the susceptibility of *Lp* to H_2O_2 in PE phase only (Fig 5.5D).

Thus far, a positive role for OxyR-mediated regulation of the anti-oxidative response has been elusive. Starvation in bacteria is known to induce a response that overlaps with the response to oxidative stress (McDougald et al. 2002). Indeed, starved *E. coli* cells are reported to require the antioxidant regulon to counter protein oxidation (Dukan and Nystrom 1999). Therefore, we tested the survival of the wild-type and mutant strains in a defined water medium (Fraquil) over a period of 98 days. Water induces the stringent response upon starvation in *Lp* (Trigui et al. 2014). In this case, the *oxyR* mutant survived in water without a decrease in viability, mirroring the wild-type (Fig 5.8A). However, an intact OxyR protein was invaluable in surviving a 1mM H_2O_2 stress in water (Fig 5.8B). It would, therefore, seem that while it is unlikely that OxyR participates in the initial response to starvation and survival, its presence if crucial for surviving the additional stress posed by ROS in an aquatic milieu. To date, the OxyR protein in *Lp* has been characterized as a negative regulator of AhpC2D (LeBlanc et al. 2008), and a conditional negative regulator of the CpxRA two component system and IcmR, a chaperon protein that is involved in the assembly of the type IV secretion system (Raychaudhury et al. 2009, Tanner et al. 2016b). Despite a previous study reporting that the *Lp* OxyR homolog is unresponsive to H_2O_2 *in vitro* (LeBlanc et al. 2008), evidence presented here supports a role for this transcriptional regulator in managing oxidative stress. Whether OxyR regulates genes that act directly on H_2O_2, or on by products of H_2O_2 damage is yet to be determined. To our knowledge, this study encompasses the first set of data demonstrating a positive role of OxyR in mediating oxidative stress in *Lp* and an accompanying phenotypic effect.

Pseudomonas aeruginosa OxyR positively controls the expression of one of three catalases (*katB*) and alkyl hydroperoxide reductases in response to oxidative stress (Ochsner et al. 2000). Auto-oxidation of the liquid medium used to grow *P. aeruginosa* produced an approximate 1.2μM/min H_2O_2, which was found to inhibit growth of diluted Δ*oxyR* cultures

(Hassett et al. 2000). This observed sensitivity was despite retention of wild-type levels of KatA activity, the major catalase gene of *P. aeruginosa* which is not governed by the transcriptional regulator (Hassett et al. 2000, Ochsner et al. 2000). The authors postulate that extracellular oxidative stress, which cannot be neutralized by cytoplasmic KatA, impacts a highly sensitive periplasmic protein. Interestingly, exposure to H_2O_2, paraquat and menadione stresses during aerobic growth was shown to oxidize the β subunit of the ATP synthase and affect its functioning (Tamarit et al. 1998). A similar H_2O_2-induced damage may cause hypersensitivity of the *oxyR* mutant in *Lp*. LeBlanc et al. (2008) reported OxyR motifs upstream genes that are part of the electron transport chain. In further support of a periplasmic target for exogenous ROS, mutating the periplasmic *katA* rendered *Lp* more sensitive to exogenous H_2O_2 than mutating cytoplasmic *katB* (Bandyopadhyay and Steinman 2000). The *E. coli* OxyR protein is reported to regulate multiple metabolic genes involved in biosynthetic pathways, metal ion transport proteins and cell wall synthesis proteins in addition to traditional anti-oxidative genes in response to oxidative stress (Imlay 2015, Seo et al. 2015). Reflecting part of the *E. coli* regulon, OxyR-binding regions were also found upstream of genes involved in metal ion transport, efflux pumps and DNA repair (LeBlanc et al. 2008). Metal ions are important for the function of superoxide dismutases and can regulate the rate of ROS production (LeBlanc et al. 2008, Storz 2016). Taken together, these collective observations suggests that transcriptomic changes in response to exogenous oxidative stress in rich media and, more importantly, in water may be a result of direct OxyR control of genes that are highly susceptible to oxidation by exogenous ROS, or by OxyR-directed expression of genes that indirectly protect vulnerable cellular components. In order to test these hypotheses, a transcriptome of the *oxyR* mutant's response to H_2O_2 in water is currently being undertaken in our lab.

Our data together with previous studies suggest that the expression of *Lp* classical anti-oxidant genes, *katA, katB* and *ahpC2D* are OxyR-independant. Indeed, *Mycobacterium tuberculosis,* another respiratory tract pathogen, has lost the function of its *oxyR* gene completely through evolution, leaving a pseudogene in its place (Deretic et al. 1995). In contrast, OxyR negatively regulates *ahpC1* and *sodB* on agar. Noticeably, the activity of KatA increased when *Lp* was exposed to water. In conclusion, we found that OxyR is dispensable for intracellular growth and survival in an aquatic milieu; however, it significantly contributes to *Lp* survival when faced with oxidative stress in the post-exponential and in water.

5.7 Acknowledgements

We would like to thank Galadriel Hovel-Miner for providing the Δ*oxyR* strain. This study was funded by Discovery Grant 418289-2012 from the Natural Sciences and Engineering Research Council of Canada (NSERC) and a John R. Evans Leaders Fund–Funding for research infrastructure from the Canadian Foundation for Innovation to SPF. NM is the recipient of a PhD scholarship from Fond de Recherche du Québec – Nature et Technologie.

Table 5.1: List of strains used in this study

Strain/Plasmid Name	Relevant Genotype	Source or Reference
Legionella pneumophila		
JR32	Restriction negative, Str	(Sadosky et al. 1993)
KS79	JR32 Δ*comR*	(Hales and Shuman 1999b)
GAH054 (Δ*oxyR***)**	KS79 Δ*oxyR*, Kn	(Faucher et al. 2010)
LELA3118 (Δ*dotA*)	JR32 Δ*dotA*, Kn	(Sadosky et al. 1993)
JR32-GFP	pXDC31-GFP	This study
SPF172	KS79 + pSF47, Cm	This study
SPF173	GAH054 + pSF47, Kn, Cm	This study
SPF203	KS79 pSF57, Cm	This study
SPF205	Δ*oxyR* pSF57, Kn, Cm	This study
SPF206	Δ*oxyR* pSF55, Kn, Cm	This study
SPF207	KS79 pSF56, Cm	This study
SPF208	KS79 pSF55, Cm	This study
SPF209	Δ*oxyR* pSF56, Kn, Cm	This study
SPF210	KS79 pSF58, Cm	This study
SPF212	Δ*oxyR* pSF58, Kn, Cm	This study
SPF213	KS79 pSF78, Cm	(Li et al. 2015)
SPF214	Δ*oxyR* pSF78, Cm	This study
Escherichia coli		
DH5α	F$^-$*endAlhsdR17* (r^- m^+) *supE44* *thi-1* λ- *recAlrelAl*Δ(*argF-*	(Sadosky et al. 1993)

	lacZYA)U169 Φ80d*lacZ*ΔM15 *deoR gyrA96*Nal^r	
Plasmids		
pXDC31	pMMB207c*Ptac*-GFP, Cm	(Hovel-Miner et al. 2009)
pXDC39	pXDC31 without *Ptac*, Cm	(Li et al. 2015)
pSF47	pXDC39-*oxyR*, Cm	This study
pSF55	pXDC39-GFP-Prom *sodB*, Cm	This study
pSF56	pXDC39-GFP-Prom *lpg2965*, Cm	This study
pSF57	pXDC39-GFP-Prom *lpg2350*, Cm	This study
pSF58	pXDC39-GFP-Prom *lpg2840*, Cm	This study
pSF78	pXDC39-GFP, Cm	This study

Str: Streptomycin; Kn: Kanamycin; Cm: Chloremphenical

Table 5.2: List of primers used in this study

#	Primer Name	Primer Sequence
1	pXDC39-F	5’-GCT TCC ACA GCA ATG GCA TCC-3’
2	GFP-R	5’-TGT CGA CAG GTA ATG GTT GTC-3’
3	lpg2350-F	5’-GCTCTAGA CCAATTGAGC AAAGGGTTTT C-3’
4	lpg2350-R	5’-GCTCTAGAAATTAATCATAATAAAAATTATTAATCCATTTTG-3’
5	lpg2840-F	5’-GCTCTAGA CGT GTTTATCCAC AGTTACCTC-3’
6	lpg2840-R	5’-GCTCTAGA TCTCTGTCTTTGATCGTGTCTC-3’
7	lpg2965-F	5’-GCTCTAGA GATCGTTA AGGTAGGGTA AAATA-3’
8	lpg2965-R	5’-GCTCTAGA TCTATAATCTCCATTAGCGGTG-3’
9	sodB-F	5’-GCTCTAGA CTTGAC GAATATCAGG ATTGG-3’
10	sodB-R	5’-GCTCTAGA GATTAAAAGTCTTTAGTTTATCAGGATAAG-3’
11	oxyR-F	5’-GCTCTAGATCCGATGGCACAAAGAGT-3’
12	oxyR-R	5'-GCTCTAGAAAGCTCATCCAGCTAAGC-3'

CHAPTER 6: Discussion and Future Directions

6.1 Establishing Fraquil as a water model and the relationship of *Lp* to temperature, pH and trace metal concentrations

In this doctoral work, I attempt to identify some of the genetic determinants that underlie the ability of *Lp* to survive not only strict starvation in water, but also additional stresses encountered within potable water distribution systems. Previous studies attempting to determine the viability and culturability of *Lp* in water systems have employed sterilized water originating from faucets or natural sources (Murga et al. 2001, Paszko-Kolva et al. 1992, Söderberg et al. 2008, van der Kooij et al. 2016). However, geographical and seasonal variations that influence water composition fueled the need for a reproducible water model to study a bacterium whose only mode of transmission is aerosols generated from man-made water distribution systems. To date, a reproducible water model to study a water-borne pathogen does not exist. The goal of the first chapter of this thesis was to establish a working freshwater model, namely Fraquil, that could be used as a stable and reproducible medium to study the behaviour and, more importantly, the genes that are required for the survival of *Lp* in water distribution systems.

As a first step in establishing Fraquil as a viable model freshwater system for the study of *Legionella*, I tested the survival of *Lp* under three environmental conditions that are commonly associated with naturally existing *Legionella*; temperature, pH and trace metal content. In doing so, I hoped to corroborate survival patterns associated with temperature observed in the literature, as well as demonstrate differential survival dependent on pH and trace metal concentrations. *Lp* is commonly isolated at higher densities from hot water distribution systems, and temperature is often cited as a risk factor for *Legionella* colonization (Borella et al. 2005a). Despite high *Legionella* concentrations associated with temperatures between 35-50^{o}C in hot water system, temperatures of 37^{o}C and 42^{o}C caused a rapid decline of planktonic *Lp* in Fraquil alone (Fig 3.1). Therefore, the long-term persistence of *Lp* in nature is certainly influenced by protection within biofilm and inside host cells, which are both absent in Fraquil. It would also be worthwhile to investigate the influence of these two factors on the survival of *Lp* at varying temperatures. A previous study found that *Lp* survived in tap water for over 500 days (Paszko-Kolva et al. 1992). One of the key findings of my study was that a 15^{o}C incubation temperature allowed the best survival of *Lp* in Fraquil, with only a minor loss

of CFU counts after 207 days (Fig 3.4A). The small volume (5ml) used for this experiment did not allow a longer experimentation period since evaporation halted further testing. The use of the bioreactor that is currently available in Dr. Faucher's laboratory would be useful in testing the limit of *Lp* survival at 15^{o}C with a larger volume of water. Steinert et al. (1997) reported that *Lp* lost culturability after 125 days at 20^{o}C. Similarly, I found that CFU counts at 25^{o}C reached the detection limit after 176 days (Fig 3.4A). Using Live/Dead staining, I also found that *Lp* entered a VBNC state when incubated in water at 4^{o}C (Annex Fig S1A). This correlates with previous studies that report loss of culturability of environmental samples (Borella et al. 2005a, Hussong et al. 1987, Wadowsky et al. 1985). Multiple attempts to resuscitate these VBNC *Lp* using various stimuli were unsuccessful. During the last attempt, I discovered that diluting the water sample prior to resuscitation resulted in a shift of the *Lp* population towards being stained as "dead" (Annex Fig S1B). The dilution step was used to ensure that any resuscitation that would be observed precludes cells that were below the detection limit. I propose further investigating the resuscitation of 4^{o}C VBNC cells without performing the dilution step, or the use of cell-free supernatant of water-exposed *Lp* for dilution.

In the first chapter, I also tested the effect of several trace metal concentrations and pH on the survival of *Lp* (Fig 3.2). Fraquil contains a relatively low level of trace metals compared to average tap water (Table 3.1). Therefore, it is representative of the lower limit of metal concentrations reported in water distribution systems (Bargellini et al. 2011, Borella et al. 2005a, Borella et al. 2005b, States et al. 1985). According to Figure 3.2, it is clear that *Lp* is able to survive in conditions of severe metal deficiency. The absence of trace metals in Fraquil did not affect the survival of *Lp*; however, the use of chelating agents would have been useful to observe the true requirement of trace metals in water. Nonetheless, the observed immunity to unusually low trace metal levels in water suggests that the correlation of *Legionella* contamination with trace metals is not due to their direct action on the bacterium. Instead, these metals may be beneficial to the other microorganisms that cohabit water systems. Alternatively, higher concentrations of the elements may be indicative of the condition of the building (age or material used for plumbing). As such, it is well established that corrosion can lead to leaching of some metals into the water and older buildings are more prone to *Legionella* contamination (Borella et al. 2005a).

In contrast to trace metals, low pH levels negatively affected the survival of *Lp*, reducing CFU counts below the detection limit in as little as one week. The use of acids for disinfection is rare. One such disinfection method is hydrogen peroxide-peracetic acid treatment, which is relatively recent (Ditommaso et al. 2005). One of the major concerns in the use of peracetic acid is that the construction of the water distribution system needs to be considered before employing this disinfection method, since acidity can damage copper, galvanized iron, steel, brass and bronze elements (Rutala et al. 2008).

In addition to survival under the respective conditions, I endeavoured to study any changes to the virulence potential of *Lp* subsequent to water exposure. The transcriptome of *Lp* exposed to water shows that some virulence genes are upregulated compared to the exponential phase (Li et al. 2015); however, I found that the intracellular multiplication (ICM) of *Lp* exposed to water did not differ significantly from cells cultured on rich medium (Fig 3.1-3.3). Moreover, the exposure time does not seem to influence the ICM potential of *Lp*. Indeed, one of the most significant discoveries in Chapter 1 was the ability of *Lp* to retain its virulence potential after over 200 days in water (Fig 3.4). It would, therefore, seem that ICM potential is neither enhanced nor depleted upon long-term exposure to the nutrient-poor aquatic environment, as long as the cells are cultivable. Unexpectedly, I found that exposure to pH 4 in water consistently resulted in higher CFU counts at the end of an infection experiment (Fig 3.3). It is noteworthy that in order to ensure uniform multiplicities of infection (MOI) for each condition, CFU counts were performed three days prior to the start of the infection assay. The increased CFU counts subsequent to pH 4 exposure may be a result of; 1) an enhanced ability to infect and/or multiply within host phagosome or 2) a slightly higher MOI due to the formation of VBNC cells under this condition. I propose enumerating cells by flow cytometry and Live/Dead staining as an alternative to CFU counts to answer the question at hand. If pH 4 is found to increase the ICM capacity of *Lp*, it would be worthwhile to investigate the transcriptome of the bacteria subject to this condition. Not only would such a study reveal the transcriptional program induced under acid stress, it could potentially reveal novel virulence factors expressed when *Lp* senses other shock treatments.

In line with the work in Chapter 3, I tested the behavior of *Lp* in response to Fraquil, local tap water and lake water. The tap water originated from the faucet located in the laboratory, while the lake was sourced from Lake Saint-Louis, bordering the city of Sainte-

Anne-de-Bellevue. The tap and lake water were filter sterilized using a 0.2µ filter prior to use. Fraquil elicited a similar survival curve to that of tap and lake water over the course of 200 days at room temperature (Annex Fig S2). This supports Fraquil as a suitable surrogate for local water.

Finally, keeping in mind that a lab-adapted strain was used throughout Chapter 3, the differences in the survival rate of environmental and clinical *Lp* strains should be investigated to ensure reciprocal behaviour and further validation of Fraquil as a viable proxy for the study of *Legionella*. One study using environmental and clinical strains found that found that *Lp* survived in sterile tap water at 24^{o}C for at least 600 days (James et al. 1999), a significantly longer survival rate than what is observed in Chapter 3. Moreover, I found that an environmental strain isolated from the cooling tower responsible for the 2012 Quebec City outbreak was significantly more resistant to copper and H_2O_2 than a laboratory WT strain (Annex Fig S3). Whether a similar pattern is observed in strains exposed to water is yet to be determined.

6.2 Contribution of the LetA/LetS two-component system to survival in water

Having established Fraquil (herein referred to as water) as a suitable model system, my next objective was to identify regulators that have an impact on the survival of *Lp* in water and when exposed to stresses commonly encountered in water distribution systems. Using regulatory mutants of the Faucher lab strain bank, I tested six mutants including two sRNAs for their capacity to survive the starvation condition encountered in water. The *letS* and *rsmYZ* mutants had the most severe survival defect (Fig 4.1, Annex Fig S4). LetS is a sensor kinase that functions in conjunction with its cognate response regulator LetA to aid in the transition of *Lp* into the transmissive phase (*in vitro* and *in vivo*) (Hammer et al. 2002). RsmY and RsmZ are two sRNAs that are transcribed when LetA/S is activated, which antagonize the mRNA-binding protein, CsrA (reviewed in Chapter 2.8.3) (Sahr et al. 2009). The LetA/LetS two component system (TCS) has been proposed to be directly linked to the stringent response (SR) (Dalebroux and Swanson 2012). During the SR, the alternative sigma factor, RpoS, guides the transcription of genes that promote survival under nutrient poor conditions (Hovel-Miner et al. 2009, Trigui et al. 2014). Given that the SR was found to be essential for the survival of *Lp* in water (Trigui et al. 2014), it was not surprising that LetA/LetS contributed to

survival as well. It has long been proposed that the cellular alarmone, ppGpp, which activates the SR also constitutes the activation signal for LetS. I found that, in water, the absence of ppGpp did not affect RsmY/Z production. This leads to the conclusion that, even though the SR and the LetA/LetS TCS act in concert, the former does not act as an activation signals for the latter during adaptation to the water environment. This does not, however, preclude crosstalk between the two systems. Indeed, RpoS is required for the expression of RsmY/Z in the post-exponential phase (Fig 4.7) (Hovel-Miner et al. 2009). Inducing the expression of *letS* in an *rpoS* mutant restores WT levels of RsmY/Z, suggesting that RpoS positively impacts the transcription of *letS* (Hovel-Miner et al. 2009). Moreover, LetA was shown to increase *rpoS* transcription (Lynch et al. 2003); however an independent study found that a *letA* mutation did not affect the *rpoS* transcript levels in the exponential phase (Bachman and Swanson 2004a). As such, the precise relationship between RpoS and LetA/LetS remains unclear. The transcriptomic analysis of Δ*letS* in water suggests that RpoS transcripts are not affected by the TCS. It is noteworthy that the *rpoS* transcript levels are not a good indication of the actual protein level; RpoS transcription is highest in the late exponential phase, but the stationary phase contains the highest protein levels coinciding with the lowest transcripts (Bachman and Swanson 2004a, Hales and Shuman 1999b, Lynch et al. 2003). Given that the transcript level is highest during growth, any effect of LetA/LetS would be absent in the water environment. If LetA/LetS does indeed contribute to the increase in transcripts resulting in higher RpoS during water exposure; then, the transcriptome of the *letS* mutant in water must also partially represent a defect in the SR. I propose dosing the RpoS protein levels in the *letS* mutant that is exposed to water in order to answer this question.

In the matter of the LetS activation signal, there are two putative signal sensing domains; DUF2222, located in the periplasm, and HAMP, which is found mostly in the cytoplasm with the N-terminal embedded within the membrane (Fig 4.6). Partial deletion of these domains and mutations of individual residues will aid in determining whether one or both of the signaling domains are required, and which is most essential for signal transduction in water. Recently, treatment of exponential phase *Lp* with acetic acid or propionic acid was found to cause differentiation through disruptions in the fatty acid biosynthesis pathway (Edwards et al. 2009). Some of the transmissive phase traits expressed upon treatment were LetA/LetS dependent. Given that fatty acids are incorporated into the bacterial membrane, it is

possible that LetS responds to changes in membrane composition or tension (Cronan and Thomas 2009). I propose testing this hypothesis using a reporter plasmid which produces GFP when the RsmY or RsmZ promoter region is activated. Membrane disrupting agents can be used to detect the activation of the LetA/S cascade in response to changes in the membrane. A more laborious method for detecting the LetS activation signal would require the use of inverted membrane vesicles (Schell et al. 2014). These could be treated with membrane disrupting agents or molecules that may bind and phosphorylate LetS, thereby activating it.

The transcriptome of the *letS* mutant revealed that the TCS is a major repressor of replicative phase genes. This repression is likely mediated both directly through CsrA and indirectly through regulators under CsrA control, given the reported targets of CsrA (Sahr et al. 2017). Microarray analysis cannot differentiate between direct and indirect affects. Immuno-precipitation of CsrA in the WT and the *letS* mutant exposed to water, followed by sequencing could help tease out direct targets of CsrA. Genes that were most affected by this repressive action of LetS are involved in the use of amino acids and carbohydrates, energy metabolism, cell division, translation and transcription (Fig 4.5). In contrast to previous reports of LetS in broth cultures, the vast majority of differentially expressed genes were downregulated by LetS, while a meagre 3% were upregulated (Fig 4.4). In fact, a key characteristic of the transmissive phase is motility and the expression of virulence-related genes (Byrne and Swanson 1998). It is noteworthy that genes related to these two functions were either not regulated or repressed in water; this suggests that LetS initiates a response to water that is specific and significantly different to that in broth. Indeed, flagella and Icm/Dot effector genes were markedly absent in the LetS regulon when *Lp* is exposed to water. At least 26 Icm/Dot effectors are reported to be positively controlled by LetA/S in broth (Nevo et al. 2014, Rasis and Segal 2009). Upon water exposure, 46 effectors were repressed by LetS, and only two were upregulated. According to the most recent CsrA-binding study, this repression is both directly and indirectly mediated by CsrA (Sahr et al. 2017). Similarly, the lack of flagellar gene control is a major deviation from the known regulatory pattern of LetS in broth cultures, especially since the expression of flagellin has been used as marker for the activation of the TCS (Edwards et al. 2010, Fettes et al. 2001, Hammer et al. 2002). In addition to structural flagellar genes and the sigma factor driving the transcription of these genes (FliA), the following genes that are reported to

contribute to flagella regulation showed lack of regulation by LetS in water; LqsR, LetE and GGDEF-EAL domain proteins.

For the purpose of a robust analysis, only genes that were differentially expressed in both the *letS* mutant and the uninduced complement (OFF) compared to the WT and the induced complement (ON) respectively were considered. The Clusters of orthologous groups (COG) analysis of the *letS* mutant and OFF were similar, with two exceptions. The expression pattern of genes in the COGs relating to motility and signal transduction were different between Δ*letS* and OFF. Both COGs are implicated in the regulation of flagella. Therefore, I address these differences here. In the *letS* mutant, most of the differentially expressed genes in the former category were under-expressed compared to the WT. In contrast, the majority of genes in this category were upregulated in the OFF strain compared to ON. The LetA/S system in *Lp* is known to induce transcription of the major flagellar subunit (flagellin) coded by *flaA* in broth (Gal-Mor and Segal 2003b, Hammer et al. 2002, Lynch et al. 2003, Sahr et al. 2009). This positive regulation is confirmed only in the *letS* mutant; the homolog of *flaA* in the *Lp* Philadelphia-1 strain *fliC* (Lpg1340) was significantly down-regulated by more than two-fold. In addition, 31 out of 46 structural flagella genes investigated by Sahr et al. (2009) were positively influenced by LetA in the post-exponential phase. Comparably and upon water exposure, I report that 21 of the same genes are positively influenced by LetS. Indeed, of the 23 down-regulated genes belonging to the "Chemotaxis/Motility/Cell division" COG in the Δ*letS vs.* WT transcriptome, 20 genes are involved in flagellar regulation and synthesis. Strikingly, only 1 flagella gene was down-regulated in the OFF strain. GGDEF-EAL domain protein-coding genes are part of the "Signal Transduction" COG. These proteins maintain intracellular c-di-GMP levels, thereby playing a role in flagellar expression (Levi et al. 2011). Ten GGDEF-EAL domain containing proteins, some of which are reported to be under positive regulation by LetA (Levi et al. 2011, Sahr et al. 2009), were also significantly under-expressed in Δ*letS* compared to the WT exposed to water. Only one of these genes was down-regulated in the OFF strain. It is tempting to suggest that leaky transcription of *letS* in the OFF strain allows low RsmY/Z expression, which is sufficient counteract some of the regulatory effects of CsrA. If true, this would also suggest that flagella-related genes are the most sensitive to LetA/LetS activation. It is difficult to ascertain whether the aforementioned discrepancies are due to leaky transcription without confirming the expression of the flagellum in water. Electron microscopy could be

employed to visualize the strain used in this study after exposure to water in order to confirm true regulation of the flagellum by LetS. It is noteworthy that a time course transcriptomic analysis of gene expression upon water exposure showed that flagella genes are maximally induced at 6 hours after exposure to water at 25^{o}C (Li et al. 2015). Although it is prudent to keep in mind that the initial transcriptome of *Lp* in water was conducted at a lower temperature than the conditions used here, the majority of motility gene regulation may still occur at a later time point.

Finally, a GGDEF-EAL protein named CsrD has been identified and added as part of the extended regulatory network to the LetS/LetA homolog in *E. coli*, BarA/UvrY (Suzuki et al. 2006). CsrD is involved in the degradation of the CsrB sRNA antagonizing CsrA, and is negatively regulated by the latter (Esquerré et al. 2016, Suzuki et al. 2006, Vakulskas et al. 2016). BLAST analysis of Lpg0829 in *Lp* Philadelphia-1 shows 23% identity and 78% query cover with an E value of 5^{e-26} when compared to CsrD of *E. coli*. Moreover, Levi et al. (2011) established that the GGDEF domain in Lpg0829 (cdgS9) was non-functional with respect to c-di-GMP production, similar to CsrD in *E. coli* (Suzuki et al. 2006). Finally, over-expression of Lpg0829 in WT *Lp* caused a severe defect in intracellular multiplication in macrophages, similar to mutants of the LetA/LetS cascade (Levi et al. 2011). Assuming that Lpg0829 is regulated similarly to CsrD in *E. coli*, its over-expression would cause increased degradation of RsmY/Z and continued repression of transmission phase traits that are required for successful infection. In light of our microarray data that show its significantly reduced expression in *letS* compared to the WT, I propose Lpg0829 as a viable candidate for the CsrD homolog in *Lp*. Nevertheless, further experimentation is required to confirm its role in the regulatory framework of the LetA/S-RsmY/Z-CsrA cascade.

6.3 The role of OxyR in the survival of *Lp* in water

In the final chapter of this doctoral work, I pursue the characterization of another regulator, namely OxyR. The early characterization attempts revealed that the *oxyR* mutant exhibited a significant growth defect on standard agar plates (Fig 5.1). This could be circumvented by the addition of anti-oxidant molecules or by replacing the agar in the medium with agarose (Fig 5.2 and Fig 5.4). Charcoal that is added to the standard *Legionella* growth medium is known to adsorb free fatty acids, as well as neutralize excess H_2O_2 and superoxide

molecules produced by the medium over time (Hoffman et al. 1983). At this time, the nature of the molecules that causes the growth defect in the mutant is unclear. The observed phenotype may simply be caused by an excess of reactive oxygen species.

I conducted a microarray to determine the transcriptomic differences between the *oxyR* mutant and the WT strain in the post-exponential phase when *Lp* is exposed to a 10mM H_2O_2 stress (Annex Table S1). I found that a total of 204 genes were significantly downregulated in the *oxyR* mutant compared to the wild-type after exposure to H_2O_2. This indicates that OxyR is a positive regulator of the response to oxidative stress, albeit not of the typical antioxidant genes as none of them were differentially expressed. Notably, genes of the TCA cycle and the respiratory chain were differentially expressed in the *oxyR* mutant, compared to the wild-type (Annex Table S1). These genes were under-expressed in the mutant in response to the H_2O_2 stress, suggesting that a reduction of energy generation when facing oxidative stress may be the cause for the growth defect observed on agar plates. Careful inspection of the transcriptomic response of the *oxyR* mutant strain also revealed that many putative efflux pumps, such as *lpg0638, lpg0652, lpg0720, lpg1454, lpg2178* and *lpg2512* were repressed upon exposure to H_2O_2 (Annex Table S1). While this microarray still needs to be validated by qPCR, a previous study predicted efflux pumps and respiratory genes to be direct targets of OxyR based on OxyR-binding sites in their promoter regions (LeBlanc et al. 2008). Based on the aforementioned transcriptome, another hypothesis can be put forth to explain the observed growth defect on solid medium: a product in agar may be oxidized by reactive oxygen species, generating a toxic compound. The efflux pumps controlled by OxyR may aid in expelling this product, thereby avoiding its accumulation within the cell and suspending cell growth.

Despite being a virulence determinant in other bacteria, the mutation of *oxyR* in *Lp* has not produced a significant phenotypic defect to-date. In fact, the first study attempting to characterize the mutant reported that the regulator did not respond to H_2O_2, suggesting an alternative role for this protein in *Lp* (LeBlanc et al. 2008). A more recent study linked OxyR to the CpxR regulatory pathway involved in virulence (Tanner et al. 2016b). The most significant contribution of my study is linking OxyR to the survival of an oxidative stress in water (Fig 5.8B). The WT mutant survived a 1mM H_2O_2 stress in water significantly better than Δ*oxyR*. The use of silver stabilized H_2O_2 is reported to be successful and proposed as a novel disinfection technique (Ditommaso et al. 2016, Martin et al. 2015, Shuval et al. 2009). Moreover, the toxic

effects of chlorine are suggested to be partially mediated through the generation of peroxides and ROS (Charoenlap et al. 2015). Therefore, the mechanisms of oxidative stress resistance of *Lp* is a relevant study matter. A preliminary test was used to study the sensitivity of *Lp* to residual chlorine levels (at 1mg/ml); however, I found no differences between the WT and mutant strains at this concentration (Annex Fig S5). Given that the WT population was not affected at the chlorine concentration tested, I propose testing the sensitivities of the strains at higher chlorine levels that are equivalent to shock treatments as the next step (Orsi et al. 2014). If OxyR is indeed involved in surviving chlorination in water, it would be worthwhile to study the transcriptomic response of the mutant under this condition, or alternatively, under H_2O_2 stress in water.

6.4 A small RNA implicated in water survival

During the course of this doctoral work, I attempted to characterize several mutant strains. One of them produced a phenotype that should be further investigated. Lpr0001 is a trans-encoded sRNA first identified by Weissenmayer et al. (2011). This sRNA presented itself as potentially important for the survival of *Lp* in water based on two findings: 1) it was upregulated in *Lp* exposed to water (Annex Fig S6A) (Li et al. 2015) and 2) according to a Chip-Chip analysis by a colleague, its transcription seemed to be RpoS-dependent (Annex Fig S6B). The RNA predator software predicted that this sRNA coded between nucleotides 18080 to 18215 targeted a putative DedA family protein. In *E. coli*, proteins belonging to this family are associated with growth at elevated temperatures (Boughner and Doerrler 2012). As such, I tested whether Δ*lpr0001* was impaired for survival at 42^{o}C, but the mutant did not exhibit a survival defect compared to the WT. However, I found that deletion of *lpr0001* significantly impaired the survival of heat shock in water (Annex Fig S7). This defect was complemented when the sRNA was introduced into the mutant *in trans*. Given the use of heat shock treatment as a disinfection method, this is an interesting discovery that warrants further investigation to elucidate the binding targets of the sRNA under this condition.

CHAPTER 7: Summary and Conclusion

The aim of this doctoral work is to expand the understanding of the genetic tools used by *Lp* to survive the nutrient-deprivation and environmental stresses that it encounters within anthropogenic water systems. To this end, I have established a reproducible freshwater model for studying *Lp* in water. Using Fraquil, I confirmed the long-term survival capacity of *Lp*; however, I also found that *Lp* is able to maintain its virulence potential for at least 6 months in water, despite the absence of host cells. It would seem that during their survival in water, the virulence potential of the bacterium is not affected. This data highlights the resilience of *Lp* in the face of extreme starvation. Beyond this initial validation of Fraquil as suitable freshwater proxy, it presents a useful tool for future work in Dr. Faucher's lab, but also for other research groups that study the behaviour of *Lp* and other water-borne bacterial pathogens. A stable medium allows for comparisons between data originating from independent groups. Moreover, given its minimal composition, Fraquil allows for a gradual and controlled increase of the complexity of the water model, according to the users needs. A defined medium such as Fraquil is essential in order to evaluate the contribution of individual environmental factors to the survival of *Lp*, but also to study the effect of combinations of environmental factors in a controlled manner.

For the purpose of this doctoral work, I used Fraquil to identify regulatory genes that are involved in the survival of *Lp*. I screened regulatory mutants to identify key pathways that may contribute to surviving the nutrient-poor condition encountered in water. This resulted in the identification of a key two-component system, LetA/S, as an essential tool for the adaptation and subsequent survival of *Lp* in the nutrient-poor water environment. The work presented here elucidates its role in water as a major repressor of gene expression. I also show that the stringent response is activated independently of LetS in water, even though crosstalk between the two systems is apparent. The LetA/S cascade and the SR have been linked to differentiation of *Lp* in broth and in host cells. The LetA/S transcriptome in water shows that the response to water is distinct from that *in vitro* or *in vivo*. As a tri-partite sensor kinase, it is not surprising that the transcriptomic pattern in water deviates from that observed in a spent rich environment. The transcriptome of the *letS* mutant in water provides insight into the fine-tuned trasncriptomic response of *Lp* according to its environment, but also raises questions in regards to its activation signals; which should be addresed in future studies.

I also aimed to identify regulatory elements that contributed to overcoming specialized stresses encountered within the water environment. One such regulator that is characterized in Chapter 5 is the putative oxidative stress response regulator, OxyR. Previous studies failed to associate a significant phenotypic defect to loss of OxyR. Despite the lack of control that OxyR exerts on traditional anti-oxidative genes such as catalase and alkyl hydroperoxide reductase, the intact protein was found to be essential for surviving and exogenous oxidative stress in water. While the identification of this important role for OxyR is exciting, we are conducting a transcriptomic analysis to better elucidate the mechanisms by which OxyR confers oxidative stress resistence to *Lp*. The regulon of OxyR in this aquatic bacterium will certainly expand the anti-oxidative pathways employed by bacteria.

The work presented here contributes to fundamental research that aims to understand the mechanisms underlying the survival of starvation and other stresses that can be encountered in water. While it unveils some of the genetic pathways that are used by *Lp* to survive, it could certainly hint towards mechanisms used by other pathogenic and/or aquatic bacteria in similar conditions. Better understanding of the role of genetic elements can lead to identifying species that are likely to persist in anthropogenic systems over others. Importantly, pathways essential for survival have the potential to be used as targets for control and disinfection procedures.

REFERENCES

Abbott, Z.D., Yakhnin, H., Babitzke, P. and Swanson, M.S. (2015) *csrR*, a Paralog and Direct Target of CsrA, Promotes *Legionella pneumophila* Resilience in Water. mBio 6(3), e00595-00515.

Abdel-Nour, M., Duncan, C., Low, D.E. and Guyard, C. (2013) Biofilms: The Stronghold of *Legionella pneumophila*. Int J Mol Sci 14(11), 21660-21675.

Abu-Zant, A., Asare, R., Graham, J.E. and Abu Kwaik, Y. (2006) Role for RpoS but Not RelA of *Legionella pneumophila* in Modulation of Phagosome Biogenesis and Adaptation to the Phagosomal Microenvironment. Infect Immun 74(5), 3021-3026.

Abu-Zant, A., Santic, M., Molmeret, M., Jones, S., Helbig, J. and Abu Kwaik, Y. (2005) Incomplete Activation of Macrophage Apoptosis during Intracellular Replication of *Legionella pneumophila*. Infect Immun 73(9), 5339-5349.

Abu Kwaik, Y. (1996) The phagosome containing *Legionella pneumophila* within the protozoan *Hartmannella vermiformis* is surrounded by the rough endoplasmic reticulum. Appl Environ Microbiol 62(6), 2022-2028.

Abu Kwaik, Y., Gao, L.-Y., Stone, B.J., Venkataraman, C. and Harb, O.S. (1998) Invasion of Protozoa by *Legionella pneumophila* and Its Role in Bacterial Ecology and Pathogenesis. Appl Environ Microbiol 64(9), 3127-3133.

AFNOR. (2010) NF T90-471 Qualité de l'eau—Détection et quantification des *Legionella* et/ou *Legionella pneumophila* par concentration et amplification génique par réaction de polymérisation en chaîne en temps réel (RT-PCR): Association Française de Normalisation.

Ager, B.P. and Tickner, J.A. (1983) The Control of Microbiological Hazards Associated with Air-Conditioning and Ventilation Systems. Ann Occup Hyg 27(4), 341-358.

Ahlen, C., Aas, M., Krusnell, J. and Iversen, O.-J. (2016) A single *Legionella pneumophila* genotype in the freshwater system in a ship experiencing three separate outbreaks of legionellosis in 6 years. Microb Ecol Health Dis 27, 10.3402/mehd.v3427.31148.

Ahmer, B.M.M., Van Reeuwijk, J., Watson, P.R., Wallis, T.S. and Heffron, F. (1999) *Salmonella* SirA is a global regulator of genes mediating enteropathogenesis. Mol Microbiol 31(3), 971-982.

Al-Bana, B.H., Haddad, M.T. and Garduño, R.A. (2014) Stationary phase and mature infectious forms of *Legionella pneumophila* produce distinct viable but non-culturable cells. Environ Microbiol 16(2), 382-395.

Al-Khodor, S., Kalachikov, S., Morozova, I., Price, C.T. and Abu Kwaik, Y. (2009) The PmrA/PmrB Two-Component System of *Legionella pneumophila* Is a Global Regulator Required for Intracellular Replication within Macrophages and Protozoa. Infect Immun 77(1), 374-386.

Al-khodor, S., Price, C.T., Habyarimana, F., Kalia, A. and Kwaik, Y.A. (2008) A Dot/Icm-translocated ankyrin protein of *Legionella pneumophila* is required for intracellular proliferation within human macrophages and protozoa. Mol Microbiol 70(4), 908-923.

Alary, M. and Joly, J.R. (1991) Risk Factors for Contamination of Domestic Hot Water Systems by Legionellae. Appl Environ Microbiol 57(8), 2360-2367.

Alary, M. and Joly, J.R. (1992) Factors Contributing to the Contamination of Hospital Water Distribution Systems by *Legionellae*. J Infect Dis 165(3), 565-569.

Albert-Weissenberger, C., Sahr, T., Sismeiro, O., Hacker, J., Heuner, K. and Buchrieser, C. (2010) Control of Flagellar Gene Regulation in *Legionella pneumophila* and Its Relation to Growth Phase. J Bacteriol 192(2), 446-455.

Alkhuder, K., Meibom, K.L., Dubail, I., Dupuis, M. and Charbit, A. (2009) Glutathione Provides a Source of Cysteine Essential for Intracellular Multiplication of *Francisella tularensis*. PLoS Pathog 5(1), e1000284.

Allard, K.A., Viswanathan, V.K. and Cianciotto, N.P. (2006) *lbtA and lbtB* Are Required for Production of the *Legionella pneumophila* Siderophore Legiobactin. J Bacteriol 188(4), 1351-1363.

Allegra, S., Grattard, F., Girardot, F., Riffard, S., Pozzetto, B. and Berthelot, P. (2011) Longitudinal evaluation of the efficacy of heat treatment procedures against *Legionella* spp. in hospital water systems by using a flow cytometric assay. Appl Environ Microbiol 77(4), 1268-1275.

Alleron, L., Khemiri, A., Koubar, M., Lacombe, C., Coquet, L., Cosette, P., Jouenne, T. and Frere, J. (2013) VBNC *Legionella pneumophila* cells are still able to produce virulence proteins. Water Res 47(17), 6606-6617.

Alleron, L., Merlet, N., Lacombe, C. and Frère, J. (2008) Long-Term Survival of *Legionella pneumophila* in the Viable But Nonculturable State After Monochloramine Treatment. Curr Microbiol 57(5), 497-502.

Alli, O.A.T., Gao, L.-Y., Pedersen, L.L., Zink, S., Radulic, M., Doric, M. and Abu Kwaik, Y. (2000) Temporal Pore Formation-Mediated Egress from Macrophages and Alveolar Epithelial Cells by *Legionella pneumophila*. Infect Immun 68(11), 6431-6440.

Allombert, J., Lazzaroni, J.-C., Baïlo, N., Gilbert, C., Charpentier, X., Doublet, P. and Vianney, A. (2014) Three Antagonistic Cyclic di-GMP-Catabolizing Enzymes Promote Differential Dot/Icm Effector Delivery and Intracellular Survival at the Early Steps of *Legionella pneumophila* Infection. Infect Immun 82(3), 1222-1233.

Altier, C., Suyemoto, M. and Lawhon, S.D. (2000) Regulation of *Salmonella enterica* Serovar Typhimurium Invasion Genes by *csrA*. Infect Immun 68(12), 6790-6797.

Altman, E. and Segal, G. (2008) The Response Regulator CpxR Directly Regulates Expression of Several *Legionella pneumophila icm/dot* Components as Well as New Translocated Substrates. J Bacteriol 190(6), 1985-1996.

Amaro, F., Wang, W., Gilbert, J.A., Roger Anderson, O. and Shuman, H.A. (2015) Diverse protist grazers select for virulence-related traits in *Legionella*. ISME 9(7), 1607-1618.

Amer, A.O. (2010) Modulation of caspases and their non-apoptotic functions by *Legionella pneumophila*. Cell Microbiol 12(2), 140-147.

Aragon, V., Kurtz, S. and Cianciotto, N.P. (2001) *Legionella pneumophila* Major Acid Phosphatase and Its Role in Intracellular Infection. Infect Immun 69(1), 177-185.

Arnosti, D.N. and Chamberlin, M.J. (1989) Secondary sigma factor controls transcription of flagellar and chemotaxis genes in *Escherichia coli*. Proc Natl Acad Sci U S A 86(3), 830-834.

Arnow, P.M., Weil, D. and Para, M.F. (1985) Prevalence and Significance of *Legionella pneumophila* Contamination of Residential Hot-Tap Water Systems. J Infect Dis 152(1), 145-151.

Arvand, M., Jungkind, K. and Hack, A. (2011) Contamination fo the cold water distribution system of health care facilities by *Legionella pneumophila*: do we know the true dimension? Euro Surveill 16(16), 19844.

Azuma, K., Uchiyama, I. and Okumura, J. (2013) Assessing the risk of Legionnaires' disease: The inhalation exposure model and the estimated risk in residential bathrooms. Regul Toxicol Pharmacol 65(1), 1-6.

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

Bachman, M.A. and Swanson, M.S. (2004a) Genetic Evidence that *Legionella pneumophila* RpoS Modulates Expression of the Transmission Phenotype in Both the Exponential Phase and the Stationary Phase. Infect Imm 72(5), 2468-2476.

Bachman, M.A. and Swanson, M.S. (2004b) The LetE Protein Enhances Expression of Multiple LetA/LetS-Dependent Transmission Traits by *Legionella pneumophila*. Infect Immun 72(6), 3284-3293.

Bandyopadhyay, P., Byrne, B., Chan, Y., Swanson, M.S. and Steinman, H.M. (2003) *Legionella pneumophila* Catalase-Peroxidases Are Required for Proper Trafficking and Growth in Primary Macrophages. Infect Immun 71(8), 4526-4535.

Bandyopadhyay, P. and Steinman, H.M. (1998) *Legionella pneumophila* Catalase-Peroxidases: Cloning of the *katB* Gene and Studies of KatB Function. J Bacteriol 180(20), 5369-5374.

Bandyopadhyay, P. and Steinman, H.M. (2000) Catalase-Peroxidases of *Legionella pneumophila*: Cloning of the *katA* Gene and Studies of KatA Function. J Bacteriol 182(23), 6679-6686.

Bandyopadhyay, P., Xiao, H., Coleman, H.A., Price-Whelan, A. and Steinman, H.M. (2004) Icm/Dot-Independent Entry of *Legionella pneumophila* into Amoeba and Macrophage Hosts. Infect Immun 72(8), 4541-4551.

Banga, S., Gao, P., Shen, X., Fiscus, V., Zong, W.X., Chen, L. and Luo, Z.Q. (2007) *Legionella pneumophila* inhibits macrophage apoptosis by targeting pro-death members of the Bcl2 protein family. Proc Natl Acad Sci 104(12), 5121-5126.

Barbaree, J.M., Fields, B.S., Feeley, J.C., Gorman, G.W. and Martin, T.M. (1986) Isolation of Protozoa from Water Associated with a Legionellosis Outbreak and Demonstration of Intracellular Multiplication of *Legionella pneumophila*. Appl Environ Microbiol 51(2), 422-424.

Bargellini, A., Marchesi, I., Righi, E., Ferrari, A., Cencetti, S., Borella, P. and Rovesti, S. (2011) Parameters predictive of *Legionella* contamination in hot water systems: Association with trace elements and heterotrophic plate counts. Water Res 45(6), 2315-2321.

Barker, J., Brown, M.R., Collier, P.J., Farrell, I. and Gilbert, P. (1992) Relationship between *Legionella pneumophila* and *Acanthamoeba polyphaga*: physiological status and susceptibility to chemical inactivation. Appl Environ Microbiol 58(8), 2420-2425.

Barker, J., Sciafe, H. and Brown, M.R.W. (1995) Intraphagocytic Growth Induces and Antibiotic-Resistant Phenotype of *Legionella pneumophila*. Antimicrob Agents Chemother 39(12), 2684-2688.

Barrabeig, I., Rovira, A., Garcia, M., Oliva, J.M., Vilamala, A., Ferrer, M.D., SabriÀ, M. and DomÍNguez, A. (2010) Outbreak of Legionnaires' disease associated with a supermarket mist machine. Epidemiol Infect 138(12), 1823-1828.

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

Battesti, A. and Bouveret, E. (2009) Bacteria Possessing Two RelA/SpoT-Like Proteins Have Evolved a Specific Stringent Response Involving the Acyl Carrier Protein-SpoT Interaction. J Bacteriol 191(2), 616-624.

Battesti, A., Majdalani, N. and Gottesman, S. (2011) The RpoS-Mediated General Stress Response in *Escherichia coli*. Annu Rev Microbiol 65, 189-213.

Bédard, E., Boppe, I., Kouamé, S., Martin, P., Pinsonneault, L., Valiquette, L., Racine, J. and Prévost, M. (2016a) Combination of Heat Shock and Enhanced Thermal Regime to Control the Growth of a Persistent *Legionella pneumophila* Strain. Pathogens 5(2), 35.

Bédard, E., Lévesque, S., Martin, P., Pinsonneault, L., Paranjape, K., Lalancette, C., Dolcé, C.-É., Villion, M., Valiquette, L., Faucher, S.P. and Prévost, M. (2016b) Energy Conservation and the Promotion of *Legionella pneumophila* Growth: The Probable Role of Heat Exchangers in a Nosocomial Outbreak. Infect Control Hosp EpidemiolS, 1-6.

Beer, K.D., Gargano, J.W., Roberts, V.A., Hill, V.R., Garrison, L.E., Kutty, P.K., Hilborn, E.D., Wade, T.J., Fullerton, K.E. and Yoder, J.S. (2015) Surveillance for waterborne disease outbreaks associated with drinking water – United States, 2011-2012. MMWR Morb Mortal Wkly Rep 64(15), 842-848.

Beier, D. and Gross, R. (2008) Bacterial Signal Transduction: Networks and Drug Targets. Utsumi, R. (ed), pp. 149-160, Springer New York, New York, NY.

Benson, R.F., Thacker, W.L., Lanser, J.A., Sangster, N., Mayberry, W.R. and Brenner, D.J. (1991) *Legionella adelaidensis*, a new species isolated from cooling tower water. J Clin Microbiol 29(5), 1004-1006.

Berjeaud, J.-M., Chevalier, S., Schlusselhuber, M., Portier, E., Loiseau, C., Aucher, W., Lesouhaitier, O. and Verdon, J. (2016) *Legionella pneumophila*: The Paradox of a Highly Sensitive Opportunistic Waterborne Pathogen Able to Persist in the Environment. Front Microbiol 7, 486.

Berk, S.G., Faulkner, G., Garduño, E., Joy, M.C., Ortiz-Jimenez, M.A. and Garduño, R.A. (2008) Packaging of Live *Legionella pneumophila* into Pellets Expelled by *Tetrahymena* spp. Does Not Require Bacterial Replication and Depends on a Dot/Icm-Mediated Survival Mechanism. Appl Environ Microbiol 74(7), 2187-2199.

Berk, S.G., Ting, R.S., Turner, G.W. and Ashburn, R.J. (1998) Production of Respirable Vesicles Containing Live *Legionella pneumophila* Cells by Two *Acanthamoeba* spp. Appl Environ Microbiol 64(1), 279-286.

Bigot, R., Bertaux, J., Frere, J. and Berjeaud, J.-M. (2013) Intra-Amoeba Multiplication Induces Chemotaxis and Biofilm Colonization and Formation for *Legionella*. PLoS ONE 8(10), e77875.

Bogosian, G., Aardema, N.D., Bourneuf, E.V., Morris, P.J. and O'Neil, J.P. (2000) Recovery of hydrogen peroxide-sensitive culturable cells of *Vibrio vulnificus* gives the appearance of resuscitation from a viable but nonculturable state. J Bacteriol 182, 5070-5075.

Bohach, G.A. and Snyder, I.S. (1983) Cyanobacterial stimulation of growth and oxygen uptake by *Legionella pneumophila*. Appl Environ Microbiol 46(2), 528-531.

Bopp, C.A., Sumner, J.W., Morris, G.K. and Wells, J.G. (1981) Isolation of *Legionella* spp. from environmental water samples by low-pH treatment and use of a selective medium. J Clin Microbiol 13(4), 714-719.

Borella, P., Guerrieri, E., Marchesi, I., Bondi, M. and Messi, P. (2005a) Water Ecology of *Legionella* and Protozoan: Environmental and Public Health Perspectives. Biotechnol Annu Rev 11, 355-380.

Borella, P., Montagna, M.T., Romano-Spica, V., Stampi, S., Stancanelli, G., Triassi, M., Neglia, R., Marchesi, I., Fantuzzi, G., Tatò, D., Napoli, C., Quaranta, G., Laurenti, P., Leoni, E., De Luca, G., Ossi, C., Moro, M. and D'Alcalà, G.R. (2004) *Legionella* Infection Risk from Domestic Hot Water. Emerg Infect Dis 10(3), 457-464.

Borella, P., Montagna, M.T., Stampi, S., Stancanelli, G., Romano-Spica, V., Triassi, M., Marchesi, I., Bargellini, A., Tato, D., Napoli, C., Zanetti, F., Leoni, E., Moro, M., Scaltriti, S., D'Alcala, G.R., Santarpia, R. and Boccia, S. (2005b) *Legionella* Contamination in Hot Water of Italian Hotels. Appl Environ Microbiol 71(10), 5805-5813.

Bosshardt, S.C., Benson, R.F. and Fields, B.S. (1997) Flagella are a positive predictor for virulence in *Legionella*. Microb Pathog 23(2), 107-112.

Bouchard, F., Veilleux, J., De Blois, C., Murray, G. and Joly, J.R. (1985) Nosocomial Legionnaires' disease in the Quebec City area. Can Med Assoc J 132, 159-160.

Boughner, L.A. and Doerrler, W.T. (2012) Multiple deletions reveal the essentiality of the DedA membrane protein family in E*scherichia coli*. Microbiology 158(5), 1162-1171.

Bouyer, S., Imbert, C., Rodier, M.-H. and Héchard, Y. (2007) Long-term survival of *Legionella pneumophila* associated with *Acanthamoeba castellanii* vesicles. Environ Microbiol 9(5), 1341-1344.

Bozue, J.A. and Johnson, W. (1996) Interation of *Legionella pneumophila* with *Acanthamoeba catellanii*: uptake by coiling phagocytosis and inhibition of phagosome-lysosome fusion. Infect Immun 64(2), 668-673.

Brassinga, A.K.C., Kinchen, J.M., Cupp, M.E., Day, S.R., Hoffman, P.S. and Sifri, C.D. (2010) Caenorhabditis is a metazoan host for *Legionella*. Cell Microbiol 12(3), 343-361.

Brenner, D.J., Steigerwalt, A.G. and McDade, J.E. (1979) Classification of the Legionnaires' Disease Bacterium: *Legionella pneumophila*, genus novum, species nova, of the Family Legionellaceae, familia nova. Ann Intern Med 90(4), 656-658.

Brieland, J., McClain, M., LeGendre, M. and Engleberg, C. (1997) Intrapulmonary *Hartmannella vermiformis*: a potential niche for *Legionella pneumophila* replication in a murine model of legionellosis. Infect Immun 65(11), 4892-4896.

Broberg, M., Lee, G.W., Nykyri, J., Lee, Y.H., Pirhonen, M., Palva, E.T. and Kivisaar, M. (2014) The Glogal Response Regulator ExpA Controls Virulence Gene Expression

through RsmA-Mediated and RsmA-Independent Pathways in *Pectobacterium wasabiae* SCC3193. Appl Environ Microbiol 80(6), 1972-1984.

Broich, M., Rydzewski, K., McNealy, T.L., Marre, R. and Flieger, A. (2006) The Global Regulatory Proteins LetA and RpoS Control Phospholipase A, Lysophospholipase A, Acyltransferase, and Other Hydrolytic Activities of *Legionella pneumophila* JR32. J Bacteriol 188(4), 1218-1226.

Brown, L., Gentry, D., Elliott, T. and Cashel, M. (2002) DksA Affects ppGpp Induction of RpoS at a Translational Level. J Bacteriol 184(16), 4455-4465.

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

Brûlet, A., Nicolle, M.-C., Giard, M., Nicolini, F.-E., Michallet, M., Jarraud, S., Etienne, J. and Vanhems, P. (2008) Fatal Nosocomial *Legionella pneumophila* Infection Due to Exposure to Contaminated Water From a Washbasin in a Hematology Unit. Infect Control Hosp Epidemiol 29(11), 1091-1093.

Buesching, W.J., Brust, R.A. and Ayers, L.W. (1983) Enhanced primary isolation of *Legionella pneumophila* from clinical specimens by low-pH treatment. J Clin Microbiol 17(6), 1153-1155.

Burbank, L. and Roper, M.C. (2014) OxyR and SoxR Modulate the Inducible Oxidative Stress Response and Are Implicated During Different Stages of Infection for the Bacterial Phytopathogen *Pantoea stewartii* subsp. stewartii. Mol Plant Microbe Interact 27(5), 479-490.

Burillo, A., Pedro-Botet, M.L. and Bouza, E. (2017) Microbiology and Epidemiology of Legionnaire's Disease. Infect Dis Clin North Am 31(1), 7-27.

Buse, H.Y. and Ashbolt, N.J. (2011) Differential growth of *Legionella pneumophila* strains within a range of amoebae at various temperatures associated with in-premise plumbing. Lett Appl Microbiol 53(2), 217-224.

Buse, H.Y., Ji, P., Gomez-Alvarez, V., Pruden, A., Edwards, M.A. and Ashbolt, N.J. (2016) Effect of temperature and colonization of *Legionella pneumophila* and *Vermamoeba*

vermiformis on bacterial community composition of copper drinking water biofilms. Microb Biotechnol, n/a-n/a.

Buse, H.Y., Lu, J., Struewing, I.T. and Ashbolt, N.J. (2014) Preferential colonization and release of *Legionella pneumophila* from mature drinking water biofilms grown on copper versus unplasticized polyvinylchloride coupons. I J Hyg Environ Health 217(2–3), 219-225.

Buse, H.Y., Schoen, M.E. and Ashbolt, N.J. (2012) *Legionellae* in engineered systems and use of quantitative microbial risk assessment to predict exposure. Water Res 46(4), 921-933.

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

Cachafeiro, S.P., Naveira, I.M. and García, I.G. (2007) Is copper–silver ionisation safe and effective in controlling legionella? J Hosp Infect 67(3), 209-216.

Campèse, C., Descours, G., Lepoutre, A., Beraud, L., Maine, C., Che, D. and Jarraud, S. (2015) Legionnaires' disease in France. Med Mal Infect 45(3), 65-71.

Capra, E.J. and Laub, M.T. (2012) The Evolution of Two-Component Signal Transduction Systems. Annu Rev Microbiol 66, 325-347.

Cargill, K.L., Pyle, B.H., Sauer, R.L. and McFeters, G.A. (1992) Effects of culture conditions and biofilm formation on the iodine susceptibility of *Legionella pneumophila*. Can J Microbiol 38(5), 423-429.

Carratalà, J. and Garcia-Vidal, C. (2010) An update on *Legionella*. Curr Opin Infect Dis 23(2), 152-157.

Carrington, G.O. (1979) Legionnaire's Disease bacillus: Inhibition by normal flora. Clin Microbiol Newslett 1(12), 7-8.

Carvalho, F.R.S., Nastasi, F.R., Gamba, R.C., Foronda, A.S. and Pellizari, V.H. (2008) Occurrence and Diversity of *Legionellaceae* in Polar Lakes of the Antarctic Peninsula. Curr Microbiol 57(4), 294-300.

Cazalet, C., Gomez-Valero, L., Rusniok, C., Lomma, M., Dervins-Ravault, D., Newton, H.J., Sansom, F.M., Jarraud, S., Zidane, N., Ma, L., Bouchier, C., Etienne, J., Hartland, E.L. and Buchrieser, C. (2010) Analysis of the *Legionella longbeachae* Genome and Transcriptome Uncovers Unique Strategies to Cause Legionnaires' Disease. PLoS Genet 6(2), e1000851.

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

Chandler , F.W., Hicklin , M.D. and Blackmon , J.A. (1977) Demonstration of the Agent of Legionnaires' Disease in Tissue. N Engl J Med 297(22), 1218-1220.

Charoenlap, N., Sornchuer, P., Piwkam, A., Srijaruskul, K., Mongkolsuk, S. and Vattanaviboon, P. (2015) The roles of peroxide protective regulons in protecting *Xanthomonas campestris* pv. *campestris* from sodium hypochlorite stress. Can J Microbiol 61(5), 343-350.

Chatfield, C.H. and Cianciotto, N.P. (2007) The Secreted Pyomelanin Pigment of *Legionella pneumophila* Confers Ferric Reductase Activity. Infect Immun 75(8), 4062-4070.

Chatterji, D., Fujita, N. and Ishihama, A. (1998) The mediator for stringent control, ppGpp, binds to the β-subunit of *Escherichia coli* RNA polymerase. Genes Cells 3(5), 279-287.

Chen, D.E., Podell, S., Sauer, J.-D., Swanson, M.S. and Saier, M.H. (2008a) The phagosomal nutrient transporter (Pht) family. Microbiology 154(1), 42-53.

Chen, J., De Felipe, K.S., Clarke, M., Lu, H., Anderson, O.R., Segal, G. and Shuman, H.A. (2004) *Legionella* Effectors That Promote Nonlytic Release from Protozoa. Science 303(5662), 1358-1361.

Chen, Y.S., Lin, Y.E., Liu, Y.C., Huang, W.K., Shih, H.Y., Wann, S.R., Lee, S.S., Tsai, H.C., Li, C.H., Chao, H.L., Ke, C.M., Lu, H.H. and Chang, C.L. (2008b) Efficacy of point-of-entry copper–silver ionisation system in eradicating *Legionella pneumophila* in a tropical tertiary care hospital: implications for hospitals contaminated with *Legionella* in both hot and cold water. J Hosp Infect 68(2), 152-158.

Chiaraviglio, L., Brown, D.A. and Kirby, J.E. (2008) Infection of Cultured Human Endothelial Cells by *Legionella pneumophila*. PLoS ONE 3(4), e2012.

Christie, P.J. and Vogel, J.P. (2000) Bacterial type IV secretion: conjugation systems adapted to deliver effector molecules to host cells. Trends Microbiol 8(8), 354-360.

Christman, M.F., Morgan, R.W., Jacobson, F.S. and Ames, B.N. (1985) Positive control of a regulon for defenses against oxidative stress and some heat-shock proteins in *Salmonella typhimurium*. Cell 41(3), 753-762.

Christman, M.F., Storz, G. and Ames, B.N. (1989) OxyR, a positive regulator of hydrogen peroxide-inducible genes in *Escherichia coli* and *Salmonella typhimurium*, is homologous to a family of bacterial regulatory proteins. Proc Natl Acad Sci U S A 86(10), 3484-3488.

Cianciotto, N.P. (2005) Type II secretion: a protein secretion system for all seasons. Trends Microbiol 13(12), 581-588.

Cianciotto, N.P. (2007) Iron Acquisition by *Legionella pneumophila*. BioMetals 20(3), 323-331.

Cianciotto, N.P. (2014) Molecular Mechanisms in Legionella Pathogenesis. Hilbi, H. (ed), pp. 81-102, Springer Berlin Heidelberg, Berlin, Heidelberg.

Cianciotto, N.P. (2015) An update on iron acquisition by *Legionella pneumophila*: new pathways for siderophore uptake and ferric iron reduction. Future Microbiol 10, 841-851.

Cianciotto, N.P. and Fields, B.S. (1992) *Legionella pneumophila mip* gene potentiates intracellular infection of protozoa and human macrophages. Proc Natl Acad Sci U S A 89(11), 5188-5191.

Cilloniz, C., Ewig, S., Gabarrus, A., Ferrer, M., Puig de la Bella Casa, J., Mensa, J. and Torres, A. (2017) Seasonality of pathogens causing community-acquired pneumonia. Respirology, n/a-n/a.

Cirillo, J.D., Cirillo, S.L.G., Yan, L., Bermudez, L.E., Falkow, S. and Tompkins, L.S. (1999) Intracellular Growth in *Acanthamoeba castellanii* Affects Monocyte Entry Mechanisms and Enhances Virulence of *Legionella pneumophila*. Infect Immun 67(9), 4427-4434.

Cirillo, J.D., Falkow, S. and Tompkins, L.S. (1994) Growth of *Legionella pneumophila* in *Acanthamoeba castellanii* enhances invasion. Infect Immun 62(8), 3254-3261.

Cloud, J.L., Carroll, K.C., Pixton, P., Erali, M. and Hillyard, D.R. (2000) Detection of *Legionella* Species in Respiratory Specimens Using PCR with Sequencing Confirmation. J Clin Microbiol 38(5), 1709-1712.

Coetzee, N., Duggal, H., Hawker, J., Ibbotson, S., Harrison, T.G., Phin, N., Laza-Stanca, V., Johnston, R., Iqbal, Z., Rehman, Y., Knapper, E., Robinson, S. and Aigbogun, N. (2012) An outbreak of Legionnaires' disease associated with a display spa pool in retail premises, Stoke-on-Trent, United Kingdom, July 2012. Euro Surveill 17(37), pii: 20271.

Coggan, K.A. and Wolfgang, M.C. (2012) Global regulatory pathways and cross-talk control *Pseudomonas aeruginosa* environmental lifestyle and virulence phenotype. Curr Issues Mol Biol 14(2), 47.

Colbourne, J.S., Dennis, P.J., Trew, R.M., Berry, C. and Vesey, G. (1988) *Legionella* and Public Water Supplies. Water Sci Technol 20(11-12), 5-10.

Collier, S.A., Stockman, L.J., Hicks, L.A., Garrison, L.E., Zhou, F.J. and Beach, M.J. (2012) Direct healthcare costs of selected diseases primarily or partially transmitted by water. Epidemiol Infect 140(11), 2003-2013.

Comas, I. (2016) *Legionella* effectors reflect strength in diversity. Nat Genet 48(2), 115-116.

Conza, L., Casati Pagani, S. and Gaia, V. (2014) Influence of climate and geography on the occurrence of *Legionella* and amoebae in composting facilities. BMC Res Notes 7, 831.

Cooper, I.R. and Hanlon, G.W. (2010) Resistance of *Legionella pneumophila* serotype 1 biofilms to chlorine-based disinfection. J Hosp Infect 74(2), 152-159.

Cooper, I.R., White, J., Mahenthiralingam, E. and Hanlon, G.W. (2008) Long-term persistence of a single *Legionella pneumophila* strain possessing the mip gene in a municipal shower despite repeated cycles of chlorination. J Hosp Infect 70(2), 154-159.

Correia, A.M., Ferreira, J.S., Borges, V., Nunes, A., Gomes, B., Capucho, R., Gonçalves, J., Antunes, D.M., Almeida, S., Mendes, A., Guerreiro, M., Sampaio, D.A., Vieira, L., Machado, J., Simões, M.J., Gonçalves, P. and Gomes, J.P. (2016) Probable Person-to-Person Transmission of Legionnaires' Disease. New Eng J Med 374(5), 497-498.

Cotter, P.A. and Miller, J.F. (1997) A mutation in the *Bordetella bronchiseptica bvgS* gene results in reduced virulence and increased resistance to starvation, and identifies a new class of Bvg-regulated antigens. Mol Microbiol 24(4), 671-685.

Cronan, J.E. and Thomas, J. (2009) Bacterial Fatty Acid Synthesis and its Relationships with Polyketide Synthetic Pathways. Method Enzymol 459, 395-433.

Cui, Y., Chatterjee, A. and Chatterjee, A.K. (2001) Effects of the Two-Component System Comprising GacA and GacS of *Erwinia carotovora* subsp. *carotovora* on the Production of Global Regulatory *rsmB* RNA, Extracellular Enzymes, and $Harpin_{Ecc}$. Mol Plant Microbe Interact 14(4), 516-526.

Cunha, B.A. (2006) The atypical pneumonias: clinical diagnosis and importance. Clin Microbiol Infect 12, 12-24.

Cunha, B.A., Burillo, A. and Bouza, E. (2016) Legionnaires' disease. Lancet 387(10016), 376-385.

Currie, S.L. and Beattie, T.K. (2015) Compost and *Legionella longbeachae*: an emerging infection? Perspect Public Health 135(6), 309-315.

Dalebroux, Z.D., Edwards, R.L. and Swanson, M.S. (2009) SpoT governs *Legionella pneumophila* differentiation in host macrophages. Mol Microbiol 71(3), 640-658.

Dalebroux, Z.D. and Swanson, M.S. (2012) ppGpp: magic beyond RNA polymerase. Nat Rev Micro 10(3), 203-212.

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

Darelid, J., Löfgren, S. and Malmvall, B.E. (2002) Control of nosocomial Legionnaires' disease by keeping the circulating hot water temperature above 55°C: Experience from a 10-year surveillance programme in a district general hospital. J Hosp Infect 50(3), 213-219.

De Buck, E., Lebeau, I., Maes, L., Geukens, N., Meyen, E., Van Mellaert, L., Anné, J. and Lammertyn, E. (2004) A putative twin-arginine translocation pathway in *Legionella pneumophila*. Biochem Biophys Res Comm 317(2), 654-661.

De Buck, E., Maes, L., Meyen, E., Van Mellaert, L., Geukens, N., Anné, J. and Lammertyn, E. (2005) *Legionella pneumophila* Philadelphia-1 *tatB* and *tatC* affect intracellular replication and biofilm formation. Biochem Biophys Res Commun 331(4), 1413-1420.

De Wulf, P., Kwon, O. and Lin, E.C.C. (1999) The CpxRA Signal Transduction System of *Escherichia coli:* Growth-Related Autoactivation and Control of Unanticipated Target Operons. J Bacteriol 181(21), 6772-6778.

DebRoy, S., Dao, J., Söderberg, M., Rossier, O. and Cianciotto, N.P. (2006) *Legionella pneumophila* type II secretome reveals unique exoproteins and a chitinase that promotes bacterial persistence in the lung. PNAS 103(50), 19146-19151.

Declerck, P., Behets, J., van Hoef, V. and Ollevier, F. (2007) Replication of *Legionella pneumophila* in Floating Biofilms. Curr Microbiol 55(5), 435-440.

Dennis, P.J., Green, D. and Jones, B.P.C. (1984) A note on the temperature tolerance of *Legionella*. J Appl Bacteriol 56(2), 349-350.

Deora, R., Bootsma, H.J., Miller, J.F. and Cotter, P.A. (2001) Diversity in the *Bordetella* virulence regulon: transcriptional control of a Bvg-intermediate phase gene. Mol Microbiol 40(3), 669-683.

Deretic, V., Philipp, W., Dhandayuthapani, S., Mudd, M.H., Curcic, R., Garbe, T., Heym, B., Via, L.E. and Cole, S.T. (1995) *Mycobacterium tuberculosis* is a natural mutant with an inactivated oxidative-stress regulatory gene:implications for sensitivity to isoniazid. Mol Microbiol 17(5), 889-900.

Devos, L., Boon, N. and Verstraete, W. (2005) *Legionella pneumophila* in the Environment: The Occurrence of a Fastidious Bacterium in Oligotrophic Conditions. Rev Environ Sci Biotech 4(1), 61-74.

Dewailly, E. and Joly, J.R. (1991) Contamination of Domestic Water Heaters with *Legionella pneumophila*: Impact of Water Temperature on Growth and Dissemination of the Bacterium. Environ Toxicol Water Qual 6(2), 249-257.

Diederen, B.M.W. (2008) *Legionella* spp. and Legionnaires' disease. J Infect 56(1), 1-12.

Dietrich, C., Heuner, K., Brand, B.C., Hacker, J. and Steinert, M. (2001) Flagellum of *Legionella pneumophila* Positively Affects the Early Phase of Infection of Eukaryotic Host Cells. Infect Immun 69(4), 2116-2122.

Diez, E., Lee, S.H., Gauthier, S., Yaraghi, Z., Tremblay, M., Vidal, S. and Gros, P. (2003) Birc1e is the gene within the Lgn1 locus associated with resistance to *Legionella pneumophila*. Nat Genet 33(1), 55-60.

Ditommaso, S., Biasin, C., Giacomuzzi, M., Zotti, C.M., Cavanna, A. and Moiraghi, A.R. (2005) Peracetic Acid in the Disinfection of a Hospital Water System Contaminated with *Legionella* Species. Infect Control Hos Epidemiol 26(5), 490-493.

Ditommaso, S., Giacomuzzi, M., Ricciardi, E. and Zotti, C.M. (2016) Efficacy of a Low Dose of Hydrogen Peroxide (Peroxy Ag+) for Continuous Treatment of Dental Unit Water Lines: Challenge Test with *Legionella pneumophila* Serogroup 1 in a Simulated Dental Unit Waterline. Int J Env Res Public Health 13, 745.

Doebbeling, B.N. and Wenzel, R.P. (1987) The Epidemiology of *Legionella pneumophila* Infections. Semin Respir Infect 2(4), 206-221.

Doleans, A., Aurell, H., Reyrolle, M., Lina, G., Freney, J., Vandenesch, F., Etienne, J. and Jarraud, S. (2004) Clinical and Environmental Distributions of *Legionella* Strains in France are Different. J Clin Microbiol 42(1), 458-460.

DSMZ, L.I.D.-G.C.o.M.a.C.C. (2015) https://www.dsmz.de/bacterial-diversity/prokaryotic-nomenclature-up-to-date/prokariotic-nomenclature-up-to-date.html.

Duda, S., Baron, J.L., Wagener, M.M., Vidic, R.D. and Stout, J.E. (2015) Lack of correlation between *Legionella* colonization and microbial population quantification using heterotrophic plate count and adenosine triphosphate bioluminescence measurement. Environ Monit Assess 187(7), 393.

Dufresne, S.F., Locas, M.C., Duchesne, A., Restieri, C., IsmaÏL, J., Lefebvre, B., LabbÉ, A.C., Dion, R., Plante, M. and LaverdiÈRe, M. (2012) Sporadic Legionnaires' disease: the role of domestic electric hot-water tanks. Epidemiol Infect 140(1), 172-181.

Dukan, S. and Nystrom, T. (1999) Oxidative Stress Defence and Deterioration of Growth-arrested *Escherichia coli* Cells. J Biol Chem 274, 26027-26032.

Dukes, C.D. and Gardner, H.L. (1961) Identification of *Haemophilus vaginalis*. J Bacteriol 81(2), 277-283.

Dunne Jr, W.M., Picot, N. and van Belkum, A. (2017) Laboratory Tests for Legionnaire's Disease. Infect Dis Clin North Am 31(1), 167-178.

Dupré, E., Herrou, J., Lensink, M.F., Wintjens, R., Vagin, A., Lebedev, A., Crosson, S., Villeret, V., Locht, C., Antoine, R. and Jacob-Dubuisson, F. (2015) Virulence Regulation with Venus Flytrap Domains: Structure and Function of the Periplasmic Moiety of the Sensor-Kinase BvgS. PLo Pathog 11(3), e1004700.

Dupuy, M., Mazoua, S., Berne, F., Bodet, C., Garrec, N., Herbelin, P., Ménard-Szczebara, F., Oberti, S., Rodier, M.-H., Soreau, S., Wallet, F. and Héchard, Y. (2011) Efficiency of water disinfectants against *Legionella pneumophila* and *Acanthamoeba*. Water Res 45(3), 1087-1094.

Eason, M.M. and Fan, X. (2014) The role and regulation of catalase in respiratory tract opportunistic bacterial pathogens. Microb Pathog 74, 50-58.

Edagawa, A., Kimura, A., Doi, H., Tanaka, H., Tomioka, K., Sakabe, K., Nakajima, C. and Suzuki, Y. (2008) Detection of culturable and nonculturable *Legionella* species from hot water systems of public buildings in Japan. J Appl Microbiol 105(6), 2104-2114.

Edelstein, P.H. (2007) *Legionella*, ASM Press, Washington, DC.

Edelstein, P.H., Beer, K.B. and DeBoynton, E.D. (1987) Influence of growth temperature on virulence of Legionella pneumophila. Infection and Immunity 55(11), 2701-2705.

Edwards, R.L., Bryan, A., Jules, M., Harada, K., Buchrieser, C. and Swanson, M.S. (2013) Nicotinic Acid Modulates *Legionella pneumophila* Gene Expression and Induces Virulence Traits. Infect Imm 81(3), 945-955.

Edwards, R.L., Dalebroux, Z.D. and Swanson, M.S. (2009) *Legionella pneumophila* couples fatty acid flux to microbial differentiation and virulence. Mol Microbiol 71(5), 1190-1204.

Edwards, R.L., Jules, M., Sahr, T., Buchrieser, C. and Swanson, M.S. (2010) The *Legionella pneumophila* LetA/LetS Two-Component System Exhibits Rheostat-Like Behavior. Infect Immun 78(6), 2571-2583.

Eisenreich, W., Dandekar, T., Heesemann, J. and Goebel, W. (2010) Carbon metabolism of intracellular bacterial pathogens and possible links to virulence. Nat Rev Micro 8(6), 401-412.

Erdoğan, H. and Arslan, H. (2016) Domestically Acquired Legionnaires' Disease: Two Case Reports and a Review of the Pertinent Literature. Balkan Med J 33(3), 350-353.

Erickson, D.L., Russell, C.W., Johnson, K.L., Hileman, T. and Stewart, R.M. (2011) PhoP and OxyR transcriptional regulators contribute to *Yersinia pestis* virulence and survival within *Galleria mellonella*. Microb Pathog 51(6), 389-395.

Esquerré, T., Bouvier, M., Turlan, C., Carpousis, A.J., Girbal, L. and Cocaign-Bousquet, M. (2016) The Csr system regulates genome-wide mRNA stability and transcription and thus gene expression in *Escherichia coli*. Sci Rep 6, 25057.

Essig, A., von Baum, H., Gonser, T., Haerter, G. and Lück, C. (2016) Microbiological Diagnosis and Molecular Typing of *Legionella* Strains During an Outbreak of Legionellosis in Southern Germany. Int J Med Microbiol 306(2), 109-114.

Euser, S.M., Pelgrim, M. and den Boer, J.W. (2010) Legionnaires' disease and Pontiac fever after using a private outdoor whirlpool spa. Scand J Infect Dis 42(11-12), 910-916.

Eylert, E., Herrmann, V., Jules, M., Gillmaier, N., Lautner, M., Buchrieser, C., Eisenreich, W. and Heuner, K. (2010) Isotopologue Profiling of *Legionella pneumophila*: ROLE OF

SERINE AND GLUCOSE AS CARBON SUBSTRATES. J Biol Chem 285(29), 22232-22243.

Ezraty, B., Henry, C., Hérisse, M., Denamur, E. and Barras, F. (2014) Commercial Lysogeny Broth culture media and oxidative stress: A cautious tale. Free Radic Biol Med 74, 245-251.

Ezzeddine, H., Van Ossel, C., Delmée, M. and Wauters, G. (1989) *Legionella* spp. in a hospital hot water system: effect of control measures. J Hosp Infect 13(2), 121-131.

Falkinham, J.O., Hilborn, E.D., Arduino, M.J., Pruden, A. and Edwards, M.A. (2015) Epidemiology and Ecology of Opportunistic Premise Plumbing Pathogens: *Legionella pneumophila, Mycobacterium avium*, and *Pseudomonas aeruginosa*. Environ Health Perspect 123(8), 749-758.

Farhat, M., Moletta-Denat, M., Frère, J., Onillon, S., Trouihé, M.-C. and Robine, E. (2012) Effects of Disinfection on *Legionella* spp., Eukarya, and Biofilms in a Hot Water System. Appl Environ Microbiol 78(19), 6850-6858.

Faucher, S., Mueller, C.A. and Shuman, H.A. (2011) *Legionella pneumophila* Transcriptome during Intracellular Multiplication in Human Macrophages. Front Microbiol 2, 60.

Faucher, S. and Shuman, H.A. (2013) Legionella: Methods and Protocols. Buchrieser, C. and Hilbi, H. (eds), pp. 567-582, Humana Press, Totowa, NJ.

Faucher, S.P., Friedlander, G., Livny, J., Margalit, H. and Shuman, H.A. (2010) *Legionella pneumophila* 6S RNA optimizes intracellular multiplication. Proc Natl Acad Sci U S A 107(16), 7533-7538.

Faulkner, G., Berk, S.G., Garduño, E., Ortiz-Jiménez, M.A. and Garduño, R.A. (2008) Passage through *Tetrahymena tropicalis* Triggers a Rapid Morphological Differentiation in *Legionella pneumophila*. J Bacteriol 190(23), 7728-7738.

Faulkner, G. and Garduño, R.A. (2002) Ultrastructural Analysis of Differentiation in *Legionella pneumophila*. J Bacteriol 184(24), 7025-7041.

Feeley, J.C., Gibson, R.J., Gorman, G.W., Langford, N.C., Rasheed, J.K., Mackel, D.C. and Baine, W.B. (1979) Charcoal-yeast extract agar: primary isolation medium for *Legionella pneumophila*. J Clin Microbiol 10(4), 437-441.

Feldheim, Y.S., Zusman, T., Speiser, Y. and Segal, G. (2016) The *Legionella pneumophila* CpxRA two-component regulatory system: new insights into CpxR's function as a dual

regulator and its connection to the effectors regulatory network. Mol Microbiol 99(6), 1059-1079.

Feldman, M. and Segal, G. (2007) A Pair of Highly Conserved Two-Component Systems Participates in the Regulation of the Hypervariable FIR Proteins in Different *Legionella* Species. J Bacteriol 189(9), 3382-3391.

Fettes, P.S., Forsbach-Birk, V., Lynch, D. and Marre, R. (2001) Overexpresssion of a *Legionella pneumophila* homologue of the E. coli regulator csrA affects cell size, flagellation, and pigmentation. Int J Med Microbiol 291(5), 353-360.

Fields, B.S. (1996) The molecular ecology of legionellae. Trends Microbiol 4(7), 286-290.

Fields, B.S., Benson, R.F. and Besser, R.E. (2002) *Legionella* and Legionnaires' Disease: 25 Years of Investigation. Clin Microbiol Rev 15(3), 506-526.

Fields, B.S., Sanden, G.N., Barbaree, J.M., Morrill, W.E., Wadowsky, R.M., White, E.H. and Feeley, J.C. (1989) Intracellular multiplication of *Legionella pneumophila* in amoebae isolated from hospital hot water tanks. Curr Microbiol 18(2), 131-137.

Fields, B.S., Shotts Jr, E.B., Feeley, J.C., Gorman, G.W. and Martin, W.T. (1984) Proliferation of *Legionella pneumophila* as an intracellular parasite of the ciliated protozoan *Tetrahymena pyriformis*. Appl Environ Microbiol 47(3), 467-471.

Flannery, B., Gelling, L.B., Vugia, D.J., Weintraub, J.M., Salerno, J.J., Conroy, M.J., Stevens, V.A., Rose, C.E., Moore, M.R., Fields, B.S. and Besser, R.E. (2006) Reducing *Legionella* Colonization of Water Systems with Monochloramine. Emerg Infect Dis 12(4), 588-596.

Flesher, A.R., Kasper, D.L., Modern, P.A. and Mason, J.E.O. (1980) *Legionella pneumophila*: Growth Inhibition by Human Pharyngeal Flora. J Infect Dis 142(3), 313-317.

Fliermans, C.B. (1996) Ecology of *Legionella*: From data to knowledge with a little wisdom. Microb Ecol 32(2), 203-228.

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

Flydal, M.I., Chatfield, C.H., Zheng, H., Gunderson, F.F., Aubi, O., Cianciotto, N.P. and Martinez, A. (2012) Phenylalanine Hydroxylase from *Legionella pneumophila* Is a Thermostable Enzyme with a Major Functional Role in Pyomelanin Synthesis. PLoS ONE 7(9), e46209.

Follmann, M., Ochrombel, I., Krämer, R., Trötschel, C., Poetsch, A., Rückert, C., Hüser, A., Persicke, M., Seiferling, D., Kalinowski, J. and Marin, K. (2009) Functional genomics of pH homeostasis in *Corynebacterium glutamicum* revealed novel links between pH response, oxidative stress, iron homeostasis and methionine synthesis. BMC Genomics 10(1), 1-23.

Fonseca, M.V., Sauer, J.-D., Crepin, S., Byrne, B. and Swanson, M.S. (2014) The *phtC-phtD* Locus Equips *Legionella pneumophila* for Thymidine Salvage and Replication in Macrophages. Infect Immun 82(2), 720-730.

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

Frangipani, E., Visaggio, D., Heeb, S., Kaever, V., Cámara, M., Visca, P. and Imperi, F. (2014) The Gac/Rsm and cyclic-di-GMP signalling networks coordinately regulate iron uptake in *Pseudomonas aeruginosa*. Environ Microbiol 16(3), 676-688.

Friedman, M. and Friedland, G.W. (2000) Medicine's 10 Greatest Discoveries, Yale University Press.

Fry, N.K., Alexiou-Daniel, S., Bangsborg, J.M., Bernander, S., Pastoris, M.C., Etienne, J., Forsblom, B., Gaia, V., Helbig, J.H., Lindsay, D., Löck, P.C., Pelaz, C., Uldum, S.A. and Harrison, T.G. (1999) A multicenter evaluation of genotypic methods for the epidemiologic typing of *Legionella pneumophila* serogroup 1: results of a pan-European study. Clin Microbiol Infect 5(8), 462-477.

Fuche, F., Vianney, A., Andrea, C., Doublet, P. and Gilbert, C. (2015) Functional Type 1 Secretion System Involved in *Legionella pneumophila* Virulence. J Bacteriol 197(3), 563-571.

Fux, C.A., Costerton, J.W., Stewart, P.S. and Stoodley, P. (2005) Survival strategies of infectious biofilms. Trends Microbiol 13(1), 34-40.

Gaia, V., Fry, N.K., Afshar, B., Lück, P.C., Meugnier, H., Etienne, J., Peduzzi, R. and Harrison, T.G. (2005) Consensus Sequence-Based Scheme for Epidemiological Typing of Clinical and Environmental Isolates of *Legionella pneumophila*. J Clin Microbiol 43(5), 2047-2052.

Gaia, V., Fry, N.K., Harrison, T.G. and Peduzzi, R. (2003) Sequence-based typing of *Legionella pneumophila* serogroup 1 offers the potential for true portability in legionellosis outbreak investigation. J Clin Microbiol 41(7), 2932-2939.

Gal-Mor, O. and Segal, G. (2003a) Identification of CpxR as a Positive Regulator of *icm* and *dot* Virulence Genes of *Legionella pneumophila*. J Bacteriol 185(16), 4908-4919.

Gal-Mor, O. and Segal, G. (2003b) The *Legionella pneumophila* GacA homolog (LetA) is involved in the regulation of *icm* virulence genes and is required for intracellular multiplication in *Acanthamoeba castellanii*. Microb Pathog 34(4), 187-194.

Gao, L.-Y. and Abu Kwaik, Y. (2000) The mechanism of killing and exiting the protozoan host *Acanthamoeba polyphaga* by *Legionella pneumophila*. Environ Microbiol 2(1), 79-90.

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

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

Garduño, R.A. (2008) Legionella pneumophila: Pathogenesis and Immunity. Hoffmann, P., Friedman, H. and Bendinelli, M. (eds), pp. 65-84, Springer US, Boston, MA.

Garduño, R.A., Garduño, E., Hiltz, M. and Hoffman, P.S. (2002) Intracellular Growth of *Legionella pneumophila* Gives Rise to a Differentiated Form Dissimilar to Stationary-Phase Forms. Infect Immun 70(11), 6273-6283.

Garduño, R.A., Garduño, E. and Hoffman, P.S. (1998a) Surface-Associated Hsp60 Chaperonin of *Legionella pneumophila* Mediates Invasion in a HeLa Cell Model. Infect Immun 66(10), 4602-4610.

Garduño, R.A., Quinn, F.D. and Hoffman, P.S. (1998b) HeLa cells as a model to study the invasiveness and biology of *Legionella pneumophila*. Can J Microbiol 44(5), 430-440.

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

Ge, J. and Shao, F. (2011) Manipulation of host vesicular trafficking and innate immune defence by *Legionella* Dot/Icm effectors. Cell Microbiol 13(12), 1870-1880.

Gião, M.S., Azevedo, N.F., Wilks, S.A., Vieira, M.J. and Keevil, C.W. (2011) Interaction of *Legionella pneumophila* and *Helicobacter pylori* with bacterial species isolated from drinking water biofilms. BMC Microbiol 11, 57-57.

Gião, M.S., Wilks, S., Azevedo, N.F., Vieira, M.J. and Keevil, C.W. (2009) Incorporation of natural uncultivable Legionella pneumophila into potable water biofilms provides a protective niche against chlorination stress. Biofouling 25(4), 345-351.

Gibson III, F.C., Tzianabos, A.O. and Rodgers, F.G. (1994) Adherence of *Legionella pneumophila* to U-937 cells, guinea-pig alveolar macrophages, and MRC-5 cells by a novel, complement-independent binding mechanism. Can J Microbiol 40, 865-872.

Gillmaier, N., Schunder, E., Kutzner, E., Tlapák, H., Rydzewski, K., Herrmann, V., Stämmler, M., Lasch, P., Eisenreich, W. and Heuner, K. (2016) Growth-related Metabolism of the Carbon Storage Poly-3-hydroxybutyrate in *Legionella pneumophila*. J Biol Chem 291(12), 6471-6482.

Ginevra, C., Lopez, M., Forey, F., Reyrolle, M., Meugnier, H., Vandenesch, F., Etienne, J., Jarraud, S. and Molmeret, M. (2009) Evaluation of a nested-PCR-derived Sequence-based typing method applied directly to respiratory samples from patients with legionnaires' disease. J Clin Microbiol 47(4), 981-987.

Glick, T.H., Gregg, M.B., Berman, B., Mallison, G., Rhodes, W.W.J. and Kassanoff, I. (1978) Pontiac Fever. An epidemic of unknown etiology in a health department: I. Clinical and epidemiological aspects. Am J Epidemiol 107(2), 149-160.

Gomez-Valero, L., Rusniok, C., Cazalet, C. and Buchrieser, C. (2011) Comparative and Functional Genomics of *Legionella* Identified Eukaryotic Like Proteins as Key Players in Host–Pathogen Interactions. Front Microbiol 2, 208.

Gong, L., Takayama, K. and Kjelleberg, S. (2002) Role of *spoT*-dependent ppGpp accumulation in the survival of light-exposed starved bacteria. Microbiology 148(2), 559-570.

Gonzalez-Flacha, B. and Demple, B. (1997) Homeostatic Regulation of Intracellular Hydrogen Peroxide Concentration in Aerobically Growing *Escherichia coli*. J Bacteriol 179(2), 382-388.

Gould, R.G., Kane, L.W. and Mueller, J.H. (1944) On the Growth Requirements of *Neisseria gonorrhoeae*. J Bacteriol 47(3), 287-292.

Goutziana, G., Mouchtouri, V.A., Karanika, M., Kavagias, A., Stathakis, N.E., Gourgoulianis, K., Kremastinou, J. and Hadjichristodoulou, C. (2008) *Legionella* species colonization of water distribution systems, pools and air conditioning systems in cruise ships and ferries. BMC Public Health 8, 390-390.

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

Grimes, D.J. (1991) Ecology of estuarine bacteria capable of causing human disease: A review. Estuaries 14(4), 345-360.

Grimont, F., Lefevre, M., Ageron, E. and Grimont, P.A. (1989) rRNA Gene Restriction Patterns of *Legionella* Species: A Molecular Identification System. Res Microbiol 140(9), 615-626.

Grosso-Becerra, M.V., Croda-García, G., Merino, E., Servín-González, L., Mojica-Espinosa, R. and Soberón-Chávez, G. (2014) Regulation of *Pseudomonas aeruginosa* virulence factors by two novel RNA thermometers. Proc Natl Acad Sci U S A 111(43), 15562-15567.

Grubmüller, S., Schauer, K., Goebel, W., Fuchs, T.M. and Eisenreich, W. (2014) Analysis of carbon substrates used by *Listeria monocytogenes* during growth in J774A.1 macrophages suggests a bipartite intracellular metabolism. Front Cell Infect Microbiol 4, 156.

Guerrieri, E., Bondi, M., Sabia, C., de Niederhäusern, S., Borella, P. and Messi, P. (2008) Effect of Bacterial Interference on Biofilm Development by *Legionella pneumophila*. Curr Microbiol 57(6), 532-536.

Guillemet, T.A., Lévesque, B., Gauvin, D., Brousseau, N., Giroux, J.P. and Cantin, P. (2010) Assessment of real-time PCR for quantification of *Legionella* spp. in spa water. Lett Appl Microbiol 51(6), 639-644.

Gundogdu, O., Teixeira Da Silva, D., Mohammad, B., Elmi, A., Mills, D.C., Wren, B.W. and Dorrell, N. (2015) The *Campylobacter jejuni* MarR-like transcriptional regulators RrpA and RrpB both influence bacterial responses to oxidative and aerobic stresses. Front Microbiol 6.

Hägele, S., Köhler, R., Merkert, H., Schleicher, M., Hacker, J. and Steinert, M. (2000) *Dictyostelium discoideum*: a new host model system for intracellular pathogens of the genus *Legionella*. Cell Microbiol 2(2), 165-171.

Hahn, J.-S., Oh, S.-Y. and Roe, J.-H. (2002) Role of OxyR as a Peroxide-Sensing Positive Regulator in *Streptomyces coelicolor* A3(2). J Bacteriol 184(19), 5214-5222.

Halablab, M.A., Bazin, M., Richards, L. and Pacy, J. (1990) Ultra-structure and localisation of formazan formed by human neutrophils and amoebae phagocytosing virulent and avirulent *Legionella pneumophila*. FEMS Microbiol Immunol 2(5-6), 295-301.

Hales, L.M. and Shuman, H.A. (1999a) *Legionella pneumophila* Contains a Type II General Secretion Pathway Required for Growth in Amoebae as Well as for Secretion of the Msp Protease. Infect Immun 67(7), 3662-3666.

Hales, L.M. and Shuman, H.A. (1999b) The *Legionella pneumophila rpoS* Gene Is Required for Growth within *Acanthamoeba castellanii*. J Bacteriol 181(16), 4879-4889.

Hammer, B.K. and Swanson, M.S. (1999) Co-ordination of *Legionella pneumophila* virulence with entry into stationary phase by ppGpp. Mol Microbiol 33(4), 721-731.

Hammer, B.K., Tateda, E.S. and Swanson, M.S. (2002) A two-component regulator induces the transmission phenotype of stationary-phase *Legionella pneumophila*. Mol Microbiol 44(1), 107-118.

Harada, E., Iida, K.-I., Shiota, S., Nakayama, H. and Yoshida, S.-i. (2010) Glucose Metabolism in *Legionella pneumophila*: Depedence on the Entner-Doudoroff Pathway and Connection with Intracellular Bacterial Growth. J Bacteriol 192(11), 2892-2899.

Harada, T., Miyake, M. and Imai, Y. (2007) Evasion of *Legionella pneumophila* from the Bactericidal System by Reactive Oxygen Species (ROS) in Macrophages. Microbiol Immunol 51(12), 1161-1170.

Harb, O.S., Gao, L.-Y. and Kwaik, Y.A. (2000) From Protozoa to Mammalian Cells: A New Paradigm in the Life Cycle of Intracellular Bacterial Pathogens. Environ Microbiol 2(3), 251-265.

Harb, O.S., Venkataraman, C., Haack, B.J., Gao, L.Y. and Abu Kwaik, Y. (1998) Heterogeneity in the attachment and uptake mechanisms of the Legionnaires' disease bacterium, *Legionella pneumophila*, by protozoan hosts. Appl Environm Microbiol 64(1), 126-132.

Hardiman, C.A., McDonough, Justin A., Newton, Hayley J. and Roy, Craig R. (2012) The role of Rab GTPases in the transport of vacuoles containing *Legionella pneumophila* and *Coxiella burnetii*. Biochem Soc Trans 40(6), 1353-1359.

Harrison, T.G., Doshi, N., Fry, N.K. and Joseph, C.A. (2007) Comparison of clinical and environmental isolates of *Legionella pneumophila* obtained in the UK over 19 years. Clin Microbiol Infect 13(1), 78-85.

Haseltine, W.A. and Block, R. (1973) Synthesis of Guanosine Tetra- and Pentaphosphate Requires the Presence of a Codon-Specific, Uncharged Transfer Ribonucleic Acid in the Acceptor Site of Ribosomes. Proc Natl Acad Sci 70(5), 1564-1568.

Hassett, D.J., Alsabbagh, E., Parvatiyar, K., Howell, M.L., Wilmott, R.W. and Ochsner, U.A. (2000) A Protease-Resistant Catalase, KatA, Released upon Cell Lysis during Stationary Phase Is Essential for Aerobic Survival of a *Pseudomonas aeruginosa oxyR* Mutant at Low Cell Densities. J Bacteriol 182(16), 4557-4563.

Haupt, T.E., Heffernan, R.T., Kazmierczak, J.J., Nehls-Lowe, H., Rheineck, B., Powell, C., Leonhardt, K.K., Chitnis, A.S. and Davis, J.P. (2012) An Outbreak of Legionnaires' Disease Associated with a Decorative Water Wall Fountain in a Hospital. Infect Control Hosp Epidemiol 33(2), 185-191.

Hauryliuk, V., Atkinson, G.C., Murakami, K.S., Tenson, T. and Gerdes, K. (2015) Recent functional insights into the role of (p)ppGpp in bacterial physiology. Nat Rev Microbiol 13(5), 298-309.

Häuslein, I., Manske, C., Goebel, W., Eisenreich, W. and Hilbi, H. (2016) Pathway analysis using ^{13}C-glycerol and other carbon tracers reveals a bipartite metabolism of *Legionella pneumophila*. Mol Microbiol 100(2), 229-246.

Hayashi, T., Nakamichi, M., Naitou, H., Ohashi, N., Imai, Y. and Miyake, M. (2010) Proteomic Analysis of Growth Phase-Dependent Expression of *Legionella pneumophila* Proteins Which Involves Regulation of Bacterial Virulence Traits. PLoS ONE 5(7), e11718.

Hayden, R.T., Uhl, J.R., Qian, X., Hopkins, M.K., Aubry, M.C., Limper, A.H., Lloyd, R.V. and Cockerill, F.R. (2001) Direct Detection of *Legionella* Species from Bronchoalveolar Lavage and Open Lung Biopsy Specimens: Comparison of LightCycler PCR, In Situ Hybridization, Direct Fluorescence Antigen Detection, and Culture. J Clin Microbiol 39(7), 2618-2626.

Heffelfinger, J.D., Kool, J.L., Fridkin, S., Fraser, V.J., Hageman, J., Carpenter, J. and Whitney, C.G. (2003) Risk of Hospital-Acquired Legionnaires' Disease in Cities Using

Monochloramine Versus Other Water Disinfectants. Infect Control Hosp Epidemiol 24(8), 569-574.

Hengge-Aronis, R. (1993) Survival of hunger and stress: The role of *rpoS* in early stationary phase gene regulation in *E. coli*. Cell 72(2), 165-168.

Henne, K., Kahlisch, L., Höfle, M.G. and Brettar, I. (2013) Seasonal dynamics of bacterial community structure and composition in cold and hot drinking water derived from surface water reservoirs. Water Res 47(15), 5614-5630.

Heo, Y.-J., Chung, I.-Y., Cho, W.-J., Lee, B.-Y., Kim, J.-H., Choi, K.-H., Lee, J.-W., Hassett, D.J. and Cho, Y.-H. (2010) The Major Catalase Gene (katA) of *Pseudomonas aeruginosa* PA14 Is under both Positive and Negative Control of the Global Transactivator OxyR in Response to Hydrogen Peroxide. J Bacteriol 192(2), 381-390.

Herren, C.D., Mitra, A., Palaniyandi, S.K., Coleman, A., Elankumaran, S. and Mukhopadhyay, S. (2006) The BarA-UvrY Two-Component System Regulates Virulence in Avian Pathogenic *Escherichia coli* O78:K80:H9. Infect Immun 74(8), 4900-4909.

Heuner, K., Bender-Beck, L., Brand, B.C., Lück, P.C., Mann, K.H., Marre, R., Ott, M. and Hacker, J. (1995) Cloning and genetic characterization of the flagellum subunit gene (*flaA*) of *Legionella pneumophila* serogroup 1. Infect Immun 63(7), 2499-2507.

Heuner, K., Brand, B.C. and Hacker, J. (1999) The expression of the flagellum of *Legionella pneumophila* is modulated by different environmental factors. FEMS Microbiol Lett 175(1), 69-77.

Heuner, K., Dietrich, C., Skriwan, C., Steinert, M. and Hacker, J. (2002) Influence of the Alternative σ^{28} Factor on Virulence and Flagellum Expression of *Legionella pneumophila*. Infect Immun 70(3), 1604-1608.

Heuner, K., Hacker, J. and Brand, B.C. (1997) The Alternative Sigma Factor σ^{28} of *Legionella pneumophila* Restores Flagellation adn Motility to an *Escherichia coli fliA* Mutant. J Bacteriol 179(1), 17-23.

Heuner, K. and Steinert, M. (2003) The flagellum of *Legionella pneumophila* and its link to the expression of the virulent phenotype. Int J Med Microbiol 293(2), 133-143.

Heurlier, K., Williams, F., Heeb, S., Dormond, C., Pessi, G., Singer, D., Cámara, M., Williams, P. and Haas, D. (2004) Positive Control of Swarming, Rhamnolipid Synthesis, and

Lipase Production by the Posttranscriptional RsmA/RsmZ System in *Pseudomonas aeruginosa* PAO1. J Bacteriol 186(10), 2936-2945.

Hilbi, H., Jarraud, S., Hartland, E. and Buchrieser, C. (2010) Update on Legionnaires' disease: pathogenesis, epidemiology, detection and control. Mol Microbiol 76(1), 1-11.

Hilbi, H., Segal, G. and Shuman, H.A. (2001) Icm/Dot-dependent upregulation of phagocytosis by *Legionella pneumophila*. Mol Microbiol 42(3), 603-617.

Hiltz, M.F., Sisson, G.R., Brassinga, A.K.C., Garduno, E., Garduno, R.A. and Hoffman, P.S. (2004) Expression of *magA* in *Legionella pneumophila* Philadelphia-1 Is Developmentally Regulated and a Marker of Formation of Mature Intracellular Forms. J Bacteriol 186(10), 3038-3045.

Hindré, T., Brüggemann, H., Buchrieser, C. and Héchard, Y. (2008) Transcriptional profiling of *Legionella pneumophila* biofilm cells and the influence of iron on biofilm formation. Microbiology 154(1), 30-41.

Hoffman, P.S., Pine, L. and Bell, S. (1983) Production of superoxide and hydrogen peroxide in medium used to culture *Legionella pneumophila*: catalytic decomposition by charcoal. Appl Environ Microbiol 45(3), 784-791.

Hoffmann, C., Finsel, I., Otto, A., Pfaffinger, G., Rothmeier, E., Hecker, M., Becher, D. and Hilbi, H. (2014) Functional analysis of novel Rab GTPases identified in the proteome of purified *Legionella*-containing vacuoles from macrophages. Cell Microbiol 16(7), 1034-1052.

Horwitz, M.A. (1983a) Formation of a novel phagosome by the Legionnaires' disease bacterium (*Legionella pneumophila*) in human monocytes. J Exp Med 158(4), 1319-1331.

Horwitz, M.A. (1983b) The Legionnaires' disease bacterium (*Legionella pneumophila*) inhibits phagosome-lysosome fusion in human monocytes. J Exp Med 158(6), 2108-2126.

Horwitz, M.A. (1984) Phagocytosis of the Legionnaires' Disease Bacterium (*Legionella pneumophila*) Occurs by a Novel Mechanism: Engulfment within a Pseudopod Coil. Cell 36(1), 27-33.

Horwitz, M.A. and Silverstein, S.C. (1980) Legionnaires' Disease Bacterium (*Legionella pneumophila*) Multiplies Intracellularly in Human Monocytes. J Clin Invest 66(3), 441-450.

Horwitz, M.A. and Silverstein, S.C. (1981) Interaction of the Legionnaires' Disease Bacterium (*Legionella pneumophila*) with Human Phagocytes. II. Antibody Promotes Binding of *L. pneumophila* to Monocytes but Does Not Inhibit Intracellular Multiplication. J Exp Med 153(2), 398-406.

Hovel-Miner, G., Faucher, S.P., Charpentier, X. and Shuman, H.A. (2010) ArgR-Regulated Genes Are Derepressed in the *Legionella*-Containing Vacuole. J Bacteriol 192(17), 4504-4516.

Hovel-Miner, G., Pampou, S., Faucher, S.P., Clarke, M., Morozova, I., Morozov, P., Russo, J.J., Shuman, H.A. and Kalachikov, S. (2009) σ^S Controls Multiple Pathways Associated with Intracellular Multiplication of *Legionella pneumophila*. J Bacteriol 191(8), 2461-2473.

Hruba, L. (2009) The colonization of hot water systems by *Legionella*. Ann Agric Environ Med 16(1), 115-119.

Hsu, B.-M., Huang, C.-C., Chen, J.-S., Chen, N.-H. and Huang, J.-T. (2011) Comparison of potentially pathogenic free-living amoeba hosts by *Legionella* spp. in substrate-associated biofilms and floating biofilms from spring environments. Water Res 45(16), 5171-5183.

Husmann, L.K. and Johnson, W. (1992) Adherence of *Legionella pneumophila* to guinea pig peritoneal macrophages, J774 mouse macrophages, and undifferentiated U937 human monocytes: role of Fc and complement receptors. Infect Immun 60(12), 5212-5218.

Hussong, D., Colwell, R.R., O'Brien, M., Weiss, E., Pearson, A.D., Weiner, R.M. and Burge, W.D. (1987) Viable *Legionella pneumophila* Not Detectable by Culture on Agar Media. Nat Biotech 5(9), 947-950.

Ikedo, M. and Yabuuchi, E. (1986) Ecological Studies of *Legionella* Species. Microbiol Immunol 30(5), 413-423.

Imazaki, I. and Kobori, Y. (2010) Improving the culturability of freshwater bacteria using FW70, a low-nutrient solid medium amended with sodium pyruvate. Can J Microbiol 56, 333-341.

Imlay, J.A. (2003) Pathways of Oxidative Damage. Annu Rev Microbiol 57(1), 395-418.

Imlay, J.A. (2015) Transcription Factors That Defend Bacteria Against Reactive Oxygen Species. Annu Rev Microbiol 69, 93-108.

Isaac, D.T. and Isberg, R. (2014) Master manipulators: an update on *Legionella pneumophila* Icm/Dot translocated substrates and their host targets. Future Microbiol 9(3), 343-359.

Isenman, H.L., Chambers, S.T., Pithie, A.D., MacDonald, S.L.S., Hegarty, J.M., Fenwick, J.L., Maze, M.J., Metcalf, S.C.L. and Murdoch, D.R. (2016) Legionnaires' disease caused by *Legionella longbeachae*: Clinical features and outcomes of 107 cases from an endemic area. Respirology 21(7), 1292-1299.

Iuchi, S. and Weiner, L. (1996) Cellular and Molecular Physiology of *Escherichia coli* in the Adaptation to Aerobic Environments. J Biochem 120(6), 1055-1063.

Jacobi, S. and Heuner, K. (2003) Description of a putative type I secretion system in *Legionella pneumophila*. Int J Med Microbiol 293(5), 349-358.

Jacobi, S., Schade, R. and Heuner, K. (2004) Characterization of the Alternative Sigma Factor σ^{54} and the Transcriptional Regulator FleQ of *Legionella pneumophila*, Which Are Both Involved in the Regulation Cascade of Flagellar Gene Expression. J Bacteriol 186(9), 2540-2547.

Jacobs, R.F., Locksley, R.M., Wilson, C.B., Haas, J.E. and Klebanoff, S.J. (1984) Interaction of primate alveolar macrophages and *Legionella pneumophila*. J Clin Invest 73(6), 1515-1523.

James, B.W., Mauchline, W.S., Dennis, P.J., Keevil, C.W. and Wait, R. (1999) Poly-3-Hydroxybutyrate in *Legionella pneumophila*, an Energy Source for Survival in Low-Nutrient Environments. Appl Environ Microbiol 65(2), 822-827.

Ji, P., Parks, J., Edwards, M.A. and Pruden, A. (2015) Impact of Water Chemistry, Pipe Material and Stagnation on the Building Plumbing Microbiome. PLoS ONE 10(10), e0141087.

Johnson, J.R., Russo, T.A., Drawz, S.M., Clabots, C., Olson, R., Kuskowski, M.A. and Rosen, H. (2013) OxyR contributes to the virulence of a Clonal Group A *Escherichia coli strain* (O17:K+:H18) in animal models of urinary tract infection, subcutaneous infection, and systemic sepsis. Microb Pathog 64, 1-5.

Joly, J. (1985) *Legionella* and domestic water heaters in the Quebec City area. Can Med Assoc J 132(2), 160-160.

Joly, J.R., Boissinot, M., Duchaine, J., Duval, M., Rafrafi, J., Ramsay, D. and Letarte, R. (1983) Ecological Distribution of *Legionellaceae* in the Quebec City Area. Can J Microbiol 30, 63-67.

Joshi, A.D., Sturgill-Koszycki, S. and Swanson, M.S. (2001) Evidence that Dot-dependent and -independent factors isolate the *Legionella pneumophila* phagosome from the endocytic network in mouse macrophages. Cell Microbiol 3(2), 99-114.

Kajfasz, J.K., Mendoza, J.E., Gaca, A.O., Miller, J.H., Koselny, K.A., Giambiagi-deMarval, M., Wellington, M., Abranches, J. and Lemos, J.A. (2012) The Spx Regulator Modulates Stress Responses and Virulence in *Enterococcus faecalis*. Infect Immun 80, 2265-2275.

Kao, P.-M., Hsu, B.-M., Chang, T.-Y., Hsu, T.-K., Tzeng, K.-J. and Huang, Y.-L. (2015) Seasonal variation of *Legionella* in Taiwan's reservoir and its relationships with environmental factors. Environ Sci Pollut Res 22(8), 6104-6111.

Kao, P.-M., Tung, M.-C., Hsu, B.-M., Chiu, Y.-C., She, C.-Y., Shen, S.-M., Huang, Y.-L. and Huang, W.-C. (2013) Identification and quantitative detection of *Legionella* spp. in various aquatic environments by real-time PCR assay. Environ Sci Pollut Res 20(9), 6128-6137.

Katashkina, J.I., Kuvaeva, T.M., Andreeva, I.G., Skorokhodova, A.Y., Biryukova, I.V., Tokmakova, I.L., Golubeva, L.I. and Mashko, S.V. (2007) Construction of stably maintained non-mobilizable derivatives of RSF1010 lacking all known elements essential for mobilization. BMC Biotechnol 7, 80-80.

Kay, E., Dubuis, C. and Haas, D. (2005) Three small RNAs jointly ensure secondary metabolism and biocontrol in *Pseudomonas fluorescens* CHA0. Proc Natl Acad Sci U S A 102(47), 17136-17141.

Kay, E., Humair, B., Denervaud, V., Riedel, K., Spahr, S., Eberl, L., Valverde, C. and Haas, D. (2006) Two GacA-Dependent Small RNAs Modulate the Quorum-Sensing Response in *Pseudomonas aeruginosa*. J Bacteriol 188(16), 6026-6033.

Keen, M.G. and Hoffman, P.S. (1984) Metabolic pathways and nitrogen metabolism in *Legionella pneumophila*. Curr Microbiol 11(2), 81-88.

Kessler, A., Schell, U., Sahr, T., Tiaden, A., Harrison, C., Buchrieser, C. and Hilbi, H. (2013) The *Legionella pneumophila* orphan sensor kinase LqsT regulates competence and pathogen–host interactions as a component of the LAI-1 circuit. Environ Microbiol 15(2), 646-662.

Kilvington, S. and Price, J. (1990) Survival of *Legionella pneumophila* within cysts of *Acanthamoeba polyphaga* following chlorine exposure. J Appl Bacteriol 68(5), 519-525.

Kim, B.R., Anderson, J.E., Mueller, S.A., Gaines, W.A. and Kendall, A.M. (2002) Literature review—efficacy of various disinfectants against *Legionella* in water systems. Water Res 36(18), 4433-4444.

Kim, J.-S. and Holmes, R.K. (2012) Characterization of OxyR as a Negative Transcriptional Regulator That Represses Catalase Production in *Corynebacterium diphtheriae*. PLoS ONE 7(3), e31709.

King, C.H., Shotts, E.B., Wooley, R.E. and Porter, K.G. (1988) Survival of coliforms and bacterial pathogens within protozoa during chlorination. Appl Environ Microbiol 54(12), 3023-3033.

Kirby, J.E., Vogel, J.P., Andrews, H.L. and Isberg, R.R. (1998) Evidence for pore-forming ability by *Legionella pneumophila*. Mol Microbiol 27(2), 323-336.

Kirschner, A.K.T., Rameder, A., Schrammel, B., Indra, A., Farnleitner, A.H. and Sommer, R. (2012) Development of a new CARD-FISH protocol for quantification of *Legionella pneumophila* and its application in two hospital cooling towers. J Appl Microbiol 112(6), 1244-1256.

Kishimoto, R.A., Kastello, M.D., White, J.D., Shirey, F.G., McGann, V.G., Larson, E.W. and Hedlund, K.W. (1979) *In vitro* interaction between normal cynolmolgus monkey alveolar macrophages and Legionnaires disease bacteria. Infect Immun 25(2), 761-763.

Kishimoto, R.A., White, J.D., Shirey, F.G., McGann, V.G., Berendt, R.F., Larson, E.W. and Hedlund, K.W. (1981) *In vitro* responses of guinea pig peritoneal macrophages to *Legionella pneumophila*. Infect Immun 31(3), 1209-1213.

Klepeis, N.E., Nelson, W.C., Ott, W.R., Robinson, J.P., Tsang, A.M., Switzer, P., Behar, J.V., Hern, S.C. and Engelmann, W.H. (2001) The National Human Activity Pattern Survey (NHAPS): a resource for assessing exposure to environmental pollutants. J Expo Anal Environ Epidemiol 11(3), 231-252.

Koide, M., Saito, A., Okazaki, M., Umeda, B. and Benson, R.F. (1999) Isolation of *Legionella longbeachae* Serogroup 1 from Potting Soils in Japan. Clin Infect Dis 29(4), 943-944.

Kong, I.-S., Bates, T.C., Hülsmann, A., Hassan, H., Smith, B.E. and Oliver, J.D. (2004) Role of catalase and *oxyR* in the viable but nonculturable state of *Vibrio vulnificus*. FEMS Microbiol Ecol 50, 133-142.

Koubar, M., Rodier, M.-H., Garduño, R.A. and Frère, J. (2011) Passage through *Tetrahymena tropicalis* enhances the resistance to stress and the infectivity of *Legionella pneumophila*. FEMS Microbiol Lett 325(1), 10-15.

Kovach, M.E., Elzer, P.H., Steven Hill, D., Robertson, G.T., Farris, M.A., Roop Ii, R.M. and Peterson, K.M. (1995) Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. Gene 166(1), 175-176.

Krell, T., Lacal, J., Busch, A., Silva-Jimenez, H., Guazzaroni, M.-E. and Ramos, J.L. (2010) Bacterial Sensor Kinases: Diversity in the Recognition of Environmental Signals. Annu Rev Microbiol 64, 539-559.

Krogh, A., Larsson, G., von Heijne, G. and Sonnhammer, L.L. (2001) Predicting transmembrane protein topology with a hidden Markov model: Application to complete genomes. J Mol Biol 305(3), 567-580.

Kruse, E.-B., Wehner, A. and Wisplinghoff, H. (2016) Prevalence and distribution of *Legionella* spp in potable water systems in Germany, risk factors associated with contamination, and effectiveness of thermal disinfection. Am J Infect Control 44(4), 470-474.

Kuchta, J.M., States, S.J., McGlaughlin, J.E., Overmeyer, J.H., Wadowsky, R.M., McNamara, A.M., Wolford, R.S. and Yee, R.B. (1985) Enhanced chlorine resistance of tap water-adapted *Legionella pneumophila* as compared with agar medium-passaged strains. Appl Environ Microbiol 50(1), 21-26.

Kuroki, T., Amemura-Maekawa, J., Ohya, H., Furukawa, I., Suzuki, M., Masaoka, T., Aikawa, K., Hibi, K., Morita, M., Lee, K.I., Ohnishi, M. and Kura, F. (2017) Outbreak of Legionnaires' Disease Caused by *Legionella pneumophila* Serogroups 1 and 13. Emerg Infect Dis 23(2), 349-351.

Kuroki, T., Ishihara, T., Ito, K. and Kura, F. (2009) Bathwater-associated cases of legionellosis in Japan, with a special focus on *Legionella* concentrations in water. Jpn J Infect Dis 62(3), 201-205.

Kusnetsov, J., Iivanainen, E., Elomaa, N., Zacheus, O. and Martikainen, P.J. (2001) Copper and silver ions more effective against Legionellae than against mycobacteria in a hospital warm water system. Water Res 35(17), 4217-4225.

Kusnetsov, J., Torvinen, E., Perola, O., Nousiainen, T. and Katila, M.L. (2003) Colonization of hospital water systems by legionellae, mycobacteria and other heterotrophic bacteria potentially hazardous to risk group patients. APMIS 111(5), 546-556.

Kusnetsov, J.M., Ottoila, E. and Martikainen, P.J. (1996) Growth, respiration and survival of *Legionella pneumophila* at high temperatures. J Appl Bacteriol 81(4), 341-347.

Kwaik, Y.A., Gao, L.-Y., Harb, O.S. and Stone, B.J. (1997) Transcriptional regulation of the macrophage-induced gene (*gspA*) of *Legionella pneumophila* and phenotypic characterization of a null mutant. Mol Microbiol 24(3), 629-642.

Laguna, R.K., Creasey, E.A., Li, Z., Valtz, N. and Isberg, R.R. (2006) A *Legionella pneumophila*-translocated substrate that is required for growth within macrophages and protection from host cell death. Proc Natl Acad Sci 103(49), 18745-18750.

Lammertyn, E. and Anné, J. (2004) Protein secretion in *Legionella pneumophila* and its relation to virulence. FEMS Microbiol Lett 238(2), 273-279.

Landeen, L.K., Yahya, M.T. and Gerba, C.P. (1989) Efficacy of copper and silver ions and reduced levels of free chlorine in inactivation of *Legionella pneumophila*. Appl Environ Microbiol 55(12), 3045-3050.

Landini, P., Egli, T., Wolf, J. and Lacour, S. (2014) sigmaS, a major player in the response to environmental stresses in *Escherichia coli*: role, regulation and mechanisms of promoter recognition. Environ Microbiol Rep 6(1), 1-13.

Lange, R., Fischer, D. and Hengge-Aronis, R. (1995) Identification of transcriptional start sites and the role of ppGpp in the expression of *rpoS*, the structural gene for the sigma S subunit of RNA polymerase in *Escherichia coli*. J Bacteriol 177(16), 4676-4380.

Lau, H.Y. and Ashbolt, N.J. (2009) The role of biofilms and protozoa in *Legionella* pathogenesis: implications for drinking water. J Appl Microbiol 107(2), 368-378.

LeBlanc, J.J., Brassinga, A.K.C., Ewann, F., Davidson, R.J. and Hoffman, P.S. (2008) An Ortholog of OxyR in *Legionella pneumophila* Is Expressed Postexponentially and Negatively Regulates the Alkyl Hydroperoxide Reductase (ahpC2D) Operon. J Bacteriol 190(10), 3444-3455.

LeBlanc, J.J., Davidson, R.J. and Hoffman, P.S. (2006) Compensatory Functions of Two Alkyl Hydroperoxide Reductases in the Oxidative Defense System of *Legionella pneumophila*. J Bacteriol 188(17), 6235-6244.

Lee, G., Bigham, J.M. and Faure, G. (2002) Removal of trace metals by coprecipitation with Fe, Al and Mn from natural waters contaminated with acid mine drainage in the Ducktown Mining District, Tennessee. Appl Geochem 17(5), 569-581.

Lee, T.C., Stout, J.E. and Yu, V.L. (1988) Factors Predisposing to *Legionella pneumophila* Colonization in Residential Water Systems. Arch Environ Health 43(1), 59-62.

Lenz, D.H., Miller, M.B., Zhu, J., Kulkarni, R.V. and Bassler, B.L. (2005) CsrA and three redundant small RNAs regulate quorum sensing in *Vibrio cholerae*. Mol Microbiol 58(4), 1186-1202.

Leoni, E., De Luca, G., Legnani, P.P., Sacchetti, R., Stampi, S. and Zanetti, F. (2005) *Legionella* waterline colonization: detection of Legionella species in domestic, hotel and hospital hot water systems. J Appl Microbiol 98(2), 373-379.

Lesnik, R., Brettar, I. and Hofle, M.G. (2016) *Legionella* species diversity and dynamics from surface reservoir to tap water: from cold adaptation to thermophily. ISME J 10(5), 1064-1080.

Lesser, M.P. (2006) Oxidative Stress in Marine Environments: Biochemistry and Physiological Ecology. Annu Rev Physiol 68, 253-278.

Lévesque, S., Lalancette, C., Bernard, K., Pacheco, A.L., Dion, R., Longtin, J. and Tremblay, C. (2016) Molecular Typing of *Legionella pneumophila* Isolates in the Province of Quebec from 2005 to 2015. PLoS ONE 11(10), e0163818.

Levi, A., Folcher, M., Jenal, U. and Shuman, H.A. (2011) Cyclic Diguanylate Signaling Proteins Control Intracellular Growth of *Legionella pneumophila*. mBio 2(1), e00316-00310.

Ley, H.L.J. and Mueller, J.H. (1946) On the Isolation from Agar of an Inhibitor *Neisseria gonorrhoeae*. J Bacteriol 52(4), 453-460.

Li, J.S., O'Brien, E.D. and Guest, C. (2002) A Review of National Legionellosis Surveillance in Autralia, 1991 to 2000. Commun Dis Intell Q Rep 26(3), 461-468.

Li, L. and Faucher, S.P. (2016) The Membrane Protein LasM Promotes the Culturability of *Legionella pneumophila* in Water. Front Cell Infect Microbiol 6, 113.

Li, L. and Faucher, S.P. (2017) Role of the LuxR family transcriptional regulator Lpg2524 in the survival of *Legionella pneumophila* in water. Can J Microbiol.

Li, L., Mendis, N., Trigui, H. and Faucher, S. (2015) Transcriptomic changes of *Legionella pneumophila* in water. BMC Genomics 16(1), 637.

Li, L., Mendis, N., Trigui, H., Oliver, J.D. and Faucher, S.P. (2014) The importance of the viable but non-culturable state in human bacterial pathogens. Front Microbiol 5, 258.

Liles, M.R., Viswanathan, V.K. and Cianciotto, N.P. (1998) Identification and Temperature Regulation of *Legionella pneumophila* Genes Involved in Type IV Pilus Biogenesis and Type II Protein Secretion. Infect Immun 66(4), 1776-1782.

Lin, Y.-S.E., Vidic, R.D., Stout, J.E. and Yu, V.L. (2002) Negative Effect of High pH on Biocidal Efficacy of Copper and Silver Ions in Controlling *Legionella pneumophila*. Appl Environ Microbiol 68(6), 2711-2715.

Lin, Y.E., Lu, W.M., Huang, H.I. and Huang, W.K. (2007) Environmental survey of *Legionella pneumophila* in hot springs in Taiwan. J Toxicol Environ Health A 70(1), 84-87.

Lin, Y.E., Stout, J.E. and Yu, V.L. (2011) Controlling *Legionella* in Hospital Drinking Water: An Evidence-Based Review of Disinfection Methods. Infect Control Hosp Epidemiol 32(2), 166-173.

Liu, Y., Bauer, S.C. and Imlay, J.A. (2011) The YaaA Protein of the *Escherichia coli* OxyR Regulon Lessens Hydrogen Peroxide Toxicity by Diminishing the Amount of Intracellular Unincorporated Iron. J Bacteriol 193(9), 2186-2196.

Liu, Y. and Luo, Z.Q. (2007) The *Legionella pneumophila* effector SidJ is required for efficient recruitment of endoplasmic reticulum proteins to the bacterial phagosome. Infect Immun 75(2), 592-603.

Lo Presti, F., Riffard, S., Meugnier, H., Reyrolle, M., Lasne, Y., Grimont, P.A., Grimont, F., Benson, R.F., Brenner, D.J., Steigerwalt, A.G., Etienne, J. and Freney, J. (2001) *Legionella gresilensis* sp. nov. and *Legionella beliardensis* sp. nov., isolated from water in France. Int J Syst Evol Microbiol 51(6), 1949-1957.

Loret, J.F., Robert, S., Thomas, V., Cooper, A.J., McCoy, W.F. and Levi, Y. (2005) Comparison of disinfectants for biofilm, protozoa and Legionella control. J Water Health 3(4), 424-433.

Lowewen, P.C., Hu, B., Strutinsky, J. and Sparling, R. (1998) Regulation in the *rpoS* regulon of *Escherichia coli*. Can J Microbiol 44(8), 707-717.

Lück, P.C., Jacobs, E., Röske, I., Schröter-Bobsin, U., Dumke, R. and Gronow, S. (2010) *Legionella dresdenensis* sp. nov., isolated from river water. Int J Syst Evol Microbiol 60(11), 2557-2562.

Lück, P.C., Leupold, I., Hlawitschka, M., Helbig, J.H., Carmienke, I., Jatzwauk, L. and Guderitz, T. (1993) Prevalence of *Legionella* species, serogroups, and monoclonal subgroups in hot water systems in south-eastern Germany. Zentralbl Hyg Umweltmed 193(5), 450-460.

Lynch, D., Fieser, N., Glöggler, K., Forsbach-Birk, V. and Marre, R. (2003) The response regulator LetA regulates the stationary-phase stress response in *Legionella pneumophila* and is required for efficient infection of *Acanthamoeba castellanii*. FEMS Microbiol Lett 219(2), 241-248.

Machner, M.P. and Isberg, R.R. (2006) Targeting of Host Rab GTPase Function by the Intravacuolar Pathogen *Legionella pneumophila*. Dev Cell 11(1), 47-56.

Mahoney, F.J., Hoge, C.W., Farley, T.A., Barbaree, J.M., Breiman, R.F., Benson, R.F. and McFarland, L.M. (1992) Communitywide Outbreak of Legionnaires' Disease Associated with a Grocery Store Mist Machine. J Infect Dis 165(4), 736-739.

Maisa, A., Brockmann, A., Renken, F., Lück, C., Pleischl, S., Exner, M., Daniels-Haardt, I. and Jurke, A. (2015) Epidemiological investigation and case-control study: a Legionnaires' disease outbreak associated with cooling towers in Warstein, Germany, August-September 2013. Euro Surveill 20(46).

Mallama, C.A., McCoy-Simandle, K. and Cianciotto, N.P. (2017) The Type II Secretion System of *Legionella pneumophila* Dampens the MyD88 and TLR2 Signaling Pathway in Infected Human Macrophages. Infect Immun, pii: IAI.00897-00816.

Mampel, J., Spirig, T., Weber, S.S., Haagensen, J.A.J., Molin, S. and Hilbi, H. (2006) Planktonic Replication Is Essential for Biofilm Formation by *Legionella pneumophila* in a Complex Medium under Static and Dynamic Flow Conditions. Appl Environ Microbiol 72(4), 2885-2895.

Manske, C. and Hilbi, H. (2014) Metabolism of the vacuolar pathogen *Legionella* and implications for virulence. Front Cell Infect Microbiol 4(125).

Marchesi, I., Ferranti, G., Bargellini, A., Marchegiano, P., Predieri, G., Stout, J.E. and Borella, P. (2013) Monochloramine and chlorine dioxide for controlling *Legionella pneumophila* contamination: biocide levels and disinfection by-product formation in hospital water networks. J Water Health 11(4), 738-747.

Marcus, S.L., Brumell, J.H., Pfeifer, C.G. and Finlay, B.B. (2000) *Salmonella* pathogenicity islands: big virulence in small packages. Microbes Infect 2(2), 145-156.

Marra, A., Blander, S.J., Horwitz, M.A. and Shuman, H.A. (1992) Identification of a *Legionella pneumophila* locus required for intracellular multiplication in human macrophages. Proc Natl Acad Sci 89(20), 9607-9611.

Marrie, T., Green, P., Burbridge, S., Bezanson, G., Neale, S., Hoffman, P.S. and Haldane, D. (1994) *Legionellaceae* in the potable water of Nova Scotia hospitals and Halifax residences. Epidemiol Infect 112(1), 143-150.

Marrie, T.J., Haldane, D., Bezanson, G. and Peppard, R. (1992) Each water outlet is a unique ecological niche for *Legionella pneumophila*. Epidemiol Infect 108(2), 261-270.

Marston, B.J., Lipman, H.B. and Breiman, R.F. (1994) Surveillance for legionnaires' disease. Risk factors for morbidity and mortality. Arch Intern Med 154(21), 2417-2422.

Martin, M., Keller, N., Martin, A., Twizeyimana, E., Argemi, X., Hansmann, Y. and Martin, T. (2016) A non-fatal pneumonia due to *Legionella longbeachae* in a patient with systemic lupus erythematosus. Lupus 25(13), 1503-1504.

Martin, N.L., Bass, P. and Liss, S.N. (2015) Antibacterial Properties and Mechanism of Activity of a Novel Silver-Stabilized Hydrogen Peroxide. PLoS ONE 10(7), e0131345.

Martinelli, F., Caruso, A., Moschini, L., Turano, A., Scarcella, C. and Speziani, F. (2000) A Comparison of *Legionella pneumophila* Occurrence in Hot Water Tanks and Instantaneous Devices in Domestic, Nosocomial, and Community Environments. Curr Microbiol 41(5), 374-376.

Martínez, L.C., Yakhnin, H., Camacho, M.I., Georgellis, D., Babitzke, P., Puente, J.L. and Bustamante, V.H. (2011) Integration of a complex regulatory cascade involving the SirA/BarA and Csr global regulatory systems that controls expression of the *Salmonella* SPI-1 and SPI-2 virulence regulons through HilD. Mol Microbiol 80(6), 1637-1656.

Marutani, M., Taguchi, F., Ogawa, Y., Hossain, M.M., Inagaki, Y., Toyoda, K., Shiraishi, T. and Ichinose, Y. (2007) Gac two-component system in *Pseudomonas syringae* pv. *tabaci* is required for virulence but not for hypersensitive reaction. Mol Genet Genomics 279(4), 313.

Mascarenhas, D.P.A. and Zamboni, D.S. (2016) Inflammasome biology taught by *Legionella pneumophila*. J Leukoc Biol.

Mascher, T., Helmann, J.D. and Unden, G. (2006) Stimulus Perception in Bacterial Signal-Transducing Histidine Kinases. Microbiol Mol Biol Rev 70(4), 910-938.

Mathys, W., Stanke, J., Harmuth, M. and Junge-Mathys, E. (2008) Occurrence of *Legionella* in hot water systems of single-family residences in suburbs of two German cities with special reference to solar and district heating. Int J Hyg Environ Health 211(1–2), 179-185.

Mauchline, W.S., James, B.W., Fitzgeorge, R.B., Dennis, P.J. and Keevil, C.W. (1994) Growth temperature reversibly modulates the virulence of *Legionella pneumophila*. Infect Immun 62(7), 2995-2997.

Mauchline, W.S. and Keevil, C.W. (1991) Development of the BIOLOG Substrate Utilization System for Identification of *Legionella* spp. Appl Environ Microbiol 57(11), 3345-3349.

Maurelli, A.T., Blackmon, B. and Curtiss, R. (1984) Temperature-dependent expression of virulence genes in *Shigella* species. Infect Immun 43(1), 195-201.

McCarter, L.L. (2006) Regulation of flagella. Curr Opin Microbiol 9(2), 180-186.

McCoy-Simandle, K., Stewart, C.R., Dao, J., DebRoy, S., Rossier, O., Bryce, P.J. and Cianciotto, N.P. (2011) *Legionella pneumophila* Type II Secretion Dampens the Cytokine Response of Infected Macrophages and Epithelia. Infect Immun 79(5), 1984-1997.

McDade, J.E., Brenner, D.J. and Bozeman, F.M. (1979) Legionnaires' Disease Bacterium Isolated in 1947. Ann Intern Med 90(4), 659-661.

McDonald, L., Hackney, C. and Ray, B. (1983) Enhanced recovery of injured *Escherichia coli* by compounds that degrade hydrogen peroxide or block its formation. Appl Environ Microbiol 45, 360-365.

McDougald, D., Gong, L., Srinivasan, S., Hild, E., Thompson, L., Takayama, K., Rice, S. and Kjelleberg, S. (2002) Defences against oxidative stress during starvation in bacteria. Antonie van Leeuwenhoek 81(1-4), 3-13.

McNealy, T.L., Forsbach-Birk, V., Shi, C. and Marre, R. (2005) The Hfq Homolog in *Legionella pneumophila* Demonstrates Regulation by LetA and RpoS and Interacts with the Global Regulator CsrA. J Bacteriol 187(4), 1527-1532.

Mead, P.B., Lawson, J.M. and Patterson, J.W. (1988) Chlorination of water supplies to control *Legionella* may corrode the pipes. JAMA 260(15), 2216-2216.

Mendis, N., McBride, P. and Faucher, S.P. (2015) Short-term and long-term survival and virulence of *Legionella pneumophila* in the defined freshwater medium Fraquil. PLoS ONE 10(9), e0139277.

Mérault, N., Rusniok, C., Jarraud, S., Gomez-Valero, L., Cazalet, C., Marin, M., Brachet, E., Aegerter, P., Gaillard, J.L., Etienne, J., Herrmann, J.L., the, D.-I.S.G., Lawrence, C. and Buchrieser, C. (2011) Specific Real-Time PCR for Simultaneous Detection and Identification of *Legionella pneumophila* Serogroup 1 in Water and Clinical Samples. Appl Environ Microbiol 77(5), 1708-1717.

Mercante, J.W. and Winchell, J.M. (2015) Current and Emerging *Legionella* Diagnostics for Laboratory and Outbreak Investigations. Clin Microbiol Rev 28(1), 95-133.

Merighi, M., Ellermeier, C.D., Slauch, J.M. and Gunn, J.S. (2005) Resolvase-in vivo expression technology analysis of the *Salmonella enterica* serovar Typhimurium PhoP and PmrA regulons in BALB/c mice. J Bacteriol 187(21), 7407-7416.

Messi, P., Anacarso, I., Bargellini, A., Bondi, M., Marchesi, I., de Niederhäusern, S. and Borella, P. (2011) Ecological behaviour of three serogroups of *Legionella pneumophila* within a model plumbing system. Biofouling 27(2), 165-172.

Mey, A.R., Butz, H.A. and Payne, S.M. (2015) *Vibrio cholerae* CsrA Regulates ToxR Levels in Response to Amino Acids and Is Essential for Virulence. mBio 6(4), e01064-01015.

Miller, C.L., Romero, M., Karna, S.L.R., Chen, T., Heeb, S. and Leung, K.P. (2016) RsmW, *Pseudomonas aeruginosa* small non-coding RsmA-binding RNA upregulated in biofilm versus planktonic growth conditions. BMC Microbiol 16, 155.

Milse, J., Petri, K., Rückert, C. and Kalinowski, J. (2014) Transcriptional response of *Corynebacterium glutamicum* ATCC 13032 to hydrogen peroxide stress and characterization of the OxyR regulon. J Biotechnol 190, 40-54.

Mitra, A., Palaniyandi, S., Herren, C.D., Zhu, X. and Mukhopadhyay, S. (2013) Pleiotropic Roles of *uvrY* on Biofilm Formation, Motility and Virulence in Uropathogenic *Escherichia coli* CFT073. PLoS ONE 8(2), e55492.

Miuetzner, S., Schwille, R.C., Farley, A., Wald, E.R., Ge, J.H., States, S.J., Libert, T. and Wadowsky, R.M. (1997) Efficacy of thermal treatment and copper-silver ionization for controlling *Legionella pneumophila* in high-volume hot water plumbing systems in hospitals. Am J Infect Control 25(6), 452-457.

Moffat, J.F., Edelstein, P.H., Regula, D.P., Cirillo, J.D. and Tompkins, L.S. (1994) Effects of an isogenic Zn-metalloprotease-deficien mutant of *Legionella pneumophila* in a guinea-pig pneumonia model. Mol Microbiol 12(5), 693-705.

Moffat, J.F. and Tompkins, L.S. (1992) A quantitative model of intracellular growth of *Legionella pneumophila* in *Acanthamoeba castellanii*. Infect Immun 60(1), 296-301.

Moiraghi, A., Castellani Pastoris, M., Barral, C., Carle, F., Sciacovelli, A., Passarino, G. and Marforio, P. (1987) Nosocomial legionellosis associated with use of oxygen bubble humidifiers and underwater chest drains. J Hosp Infect 10(1), 47-50.

Molmeret, M. and Abu Kwaik, Y. (2002) How does *Legionella pneumophila* exit the host cell? Trends Microbiol 10(6), 258-260.

Molmeret, M., Bitar, D.M., Han, L. and Abu Kwaik, Y. (2004a) Disruption of the Phagosomal Membrane and Egress of *Legionella pneumophila* into the Cytoplasm during the Last Stages of Intracellular Infection of Macrophages and *Acanthamoeba polyphaga*. Infect Immun 72(7), 4040-4051.

Molmeret, M., Zink, S.D., Han, L., Abu-Zant, A., Asari, R., Bitar, D.M. and Abu Kwaik, Y. (2004b) Activation of caspase-3 by the Dot/Icm virulence system is essential for arrested biogenesis of the *Legionella*-containing phagosome. Cell Microbiol 6(1), 33-48.

Molofsky, A.B., Byrne, B.G., Whitfield, N.N., Madigan, C.A., Fuse, E.T., Tateda, K. and Swanson, M.S. (2006) Cytosolic recognition of flagellin by mouse macrophages restricts *Legionella pneumophila* infection. J Exp Med 203(4), 1093-1104.

Molofsky, A.B., Shetron-Rama, L.M. and Swanson, M.S. (2005) Components of the *Legionella pneumophila* Flagellar Regulon Contribute to Multiple Virulence Traits, Including Lysosome Avoidance and Macrophage Death. Infect Imm 73(9), 5720-5734.

Molofsky, A.B. and Swanson, M.S. (2003) *Legionella pneumophila* CsrA is a pivotal repressor of transmission traits and activator of replication. Mol Microbiol 50(2), 445-461.

Molofsky, A.B. and Swanson, M.S. (2004) Differentiate to thrive: lessons from the *Legionella pneumophila* life cycle. Mol Microbiol 53(1), 29-40.

Montagna, M.T., Cristina, M.L., De Giglio, O., Spagnolo, A.M., Napoli, C., Cannova, L., Deriu, M.G., Delia, S.A., Giuliano, A., Guida, M., Laganà, P., Liguori, G., Mura, I., Pennino, F., Rossini, A., Tardivo, S., Torre, I., Torregrossa, M.V., Villafrate, M.R., Albertini, R. and Pasquarella, C. (2016) Serological and molecular identification of *Legionella* spp.

isolated from water and surrounding air samples in Italian healthcare facilities. Environ Res 146, 47-50.

Morales, V.M., Bäckman, A. and Bagdasarian, M. (1991) A series of wide-host-range low-copy-number vectors that allow direct screening for recombinants. Gene 97(1), 39-47.

Moran-Gilad, J., Lazarovitch, T., Mentasti, M., Harrison, T., Weinberger, M., Mordish, Y., Mor, Z., Stocki, T., Anis, E., Sadik, C., Amitai, Z. and Grotto, I. (2012) Humidifier-associated paediatric Legionnaires' disease, Israel, February 2012. Euro Surveill 17(41), 20293.

Morel, F.M.M., Westall, J.C., Ruerer, J.G. and Chaplick, J.P. (1975) Descriptions of algal growth media AQUIL and FRAQUIL. Technology, R.M.P.L.f.W.R.a.H.M.I.o. (ed), Cambridge, MA. .

Moritz, M.M., Flemming, H.-C. and Wingender, J. (2010) Integration of *Pseudomonas aeruginosa* and *Legionella pneumophila* in drinking water biofilms grown on domestic plumbing materials. Int J Hyg Environ Health 213(3), 190-197.

Moss, C.W., Weaver, R.E., Dees, S.B. and Cherry, W.B. (1977) Cellular fatty acid composition of isolates from Legionnaires disease. J Clin Microbiol 6(2), 140-143.

Mouchtouri, V., Velonakis, E., Tsakalof, A., Kapoula, C., Goutziana, G., Vatopoulos, A., Kremastinou, J. and Hadjichristodoulou, C. (2007) Risk Factors for Contamination of Hotel Water Distribution Systems by *Legionella* Species. Appl Environ Microbiol 73(5), 1489-1492.

Mouchtouri, V.A., Goutziana, G., Kremastinou, J. and Hadjichristodoulou, C. (2010) *Legionella* species colonization in cooling towers: Risk factors and assessment of control measures. Am J Infect Control 38(1), 50-55.

Muder, R.R., Yu, V.L. and Woo, A.H. (1986) Mode of transmission of *Legionella pneumophila*: A critical review. Arch Intern Med 146(8), 1607-1612.

Murga, R., Forster, T.S., Brown, E., Pruckler, J.M., Fields, B.S. and Donlan, R.M. (2001) Role of biofilms in the survival of *Legionella pneumophila* in a model potable-water system. Microbiology 147(11), 3121-3126.

Nagai, H., Kagan, J.C., Zhu, X., Kahn, R.A. and Roy, C.R. (2002) A bacterial guanine nucleotide exchange factor activates ARF on *Legionella* phagosomes. Science 295(5555), 679-682.

NCBI Taxonomy Database https://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Tree&id=445&lvl=3&lin=f&keep=1&srchmode=1&unlock.

Nevo, O., Zusman, T., Rasis, M., Lifshitz, Z. and Segal, G. (2014) Identification of *Legionella pneumophila* Effectors Regulated by the LetAS-RsmYZ-CsrA Regulatory Cascade, Many of Which Modulate Vesicular Trafficking. J Bacteriol 196(3), 681-692.

Newton, H.J., Ang, D.K.Y., van Driel, I.R. and Hartland, E. (2010) Molecular Pathogenesis of Infections Caused by *Legionella pneumophila*. Clin Microbiol Rev 23(2), 274-298.

Ng, V., Tang, P., Jamieson, F., Drews, S.J., Brown, S., Low, D.E., Johnson, C.C. and Fisman, D.N. (2008) Going with the Flow: Legionellosis Risk in Toronto, Canada Is Strongly Associated with Local Watershed Hydrology. EcoHealth 5(4), 482-490.

Ng, W.-L. and Bassler, B.L. (2009) Bacterial Quorum-Sensing Network Architectures. Annu Rev Genet 43, 197-222.

Ninio, S. and Roy, C.R. (2007) Effector proteins translocated by *Legionella pneumophila*: strength in numbers. Trends Microbiol 15(8), 372-380.

Nishimori, I., Vullo, D., Minakuchi, T., Scozzafava, A., Capasso, C. and Supuran, C.T. (2014) Sulfonamide inhibition studies of two β-carbonic anhydrases from the bacterial pathogen *Legionella pneumophila*. Bioorg Med Chem 22(11), 2939-2946.

O'Loughlin, R.E., Kightlinger, L., Werpy, M.C., Brown, E., Stevens, V., Hepper, C., Keane, T., Benson, R.F., Fields, B.S. and Moore, M.R. (2007) Restaurant outbreak of Legionnaires' disease associated with a decorative fountain: an environmental and case-control study. BMC Infect Dis 7, 93-93.

Ochsner, U.A., Vasil, M.L., Alsabbagh, E., Parvatiyar, K. and Hassett, D.J. (2000) Role of the *Pseudomonas aeruginosa oxyR-recG* Operon in Oxidative Stress Defense and DNA Repaire: OxyR-Dependent Regulation of *katB-ankB, ahpB,* and *ahpC-ahpF*. J Bacteriol 182(16), 4533-4544.

Ohnishi, K., Kutsukake, K., Suzuki, H. and Iino, T. (1990) Gene *fliA* encodes an alternative sigma factor specific for flagellar operons in *Salmonella typhimurium*. Mol Gen Genet 221(2), 139-147.

Ohno, A., Kato, N., Yamada, K. and Yamaguchi, K. (2003) Factors Influencing Survival of *Legionella pneumophila* Serotype 1 in Hot Spring Water and Tap Water. Appl Environ Microbiol 69(5), 2540-2547.

Oren, I., Zuckerman, T., Avivi, I., Finkelstein, R., Yigla, M. and Rowe, J.M. (2002) Nosocomial Outbreak of *Legionella pneumophila* Serogroup 3 Pneumonia in a New Bone Marrow Transplant Unit: Evaluation, Treatment and Control. Bone Marrow Transplant 30, 175-179.

Orsi, G.B., Vitali, M., Marinelli, L., Ciorba, V., Tufi, D., Del Cimmuto, A., Ursillo, P., Fabiani, M., De Santis, S., Protano, C., Marzuillo, C. and De Giusti, M. (2014) *Legionella* control in the water system of antiquated hospital buildings by shock and continuous hyperchlorination: 5 years experience. BMC Infect Dis 14(1), 394.

Ortiz-Roque, C.M. and Hazen, T.C. (1987) Abundance and distribution of Legionellaceae in Puerto Rican waters. Appl Environ Microbiol 53(9), 2231-2236.

Ott, M., Messner, P., Heesemann, J., Marre, R. and Hacker, J. (1991) Temperature-dependent expression of flagella in *Legionella*. J Gen Microbiol 137(8), 1955-1961.

Pagnier, I., Boughalmi, M., Croce, O., Robert, C., Raoult, D. and La Scola, B. (2012) Genome Sequence of *Legionella tunisiensis* Strain LegM(T), a New *Legionella* Species Isolated from Hypersaline Lake Water. J Bacteriol 194(21), 5978-5978.

Palaniyandi, S., Mitra, A., Herren, C.D., Lockatell, C.V., Johnson, D.E., Zhu, X. and Mukhopadhyay, S. (2012) BarA-UvrY Two-Component System Regulates Virulence of Uropathogenic *E. coli* CFT073. PLoS ONE 7(2), e31348.

Palma, M., Zurita, J., Ferreras, J.A., Worgall, S., Larone, D.H., Shi, L., Campagne , F. and Quadri, L.E.N. (2005) *Pseudomonas aeruginosa* SoxR Does Not Conform to the Archetypal Paradigm for SoxR-Dependent Regulation of the Bacterial Oxidative Stress Adaptive Response. Infect Immun 73, 2958-2966.

Palmer, C.J., Tsai, Y.L., Paszko-Kolva, C., Mayer, C. and Sangermano, L.R. (1993) Detection of *Legionella* species in sewage and ocean water by polymerase chain reaction, direct fluorescent-antibody, and plate culture methods. Appl Environ Microbiol 59(11), 3618-3624.

Park, M., Yun, S.T., Hwang, S.-Y., Chun, C.-I. and Ahn, T.I. (2006) The *dps* Gene of Symbiotic "*Candidatus* Legionella jeonii" in *Amoeba proteus* Responds to Hydrogen Peroxide and Phagocytosis. J Bacteriol 188(21), 7572-7580.

Parthuisot, N., West, N.J., Lebaron, P. and Baudart, J. (2010) High Diversity and Abundance of *Legionella* spp. in a Pristine River and Impact of Seasonal and Anthropogenic Effects. Appl Environ Microbiol 76(24), 8201-8210.

Paszko-Kolva, C., Shahamat, M. and Colwell, R.R. (1992) Long-term survival of *Legionella pneumophila* serogroup 1 under low-nutrient conditions and associated morphological changes. FEMS Microbiol Lett 102(1), 45-55.

Paszko-Kolva, C., Yamamoto, H., Shahamat, M., Sawyer, T.K., Morris, G. and Colwell, R.R. (1991) Isolation of Amoebae and *Pseudomonas* and *Legionella* spp. from Eyewash Stations. Appl Environ Microbiol 57(1), 163-167.

Paul, B.J., Barker, M.M., Ross, W., Schneider, D.A., Webb, C., Foster, J.W. and Gourse, R.L. (2004) DksA: A critical component of the transcription initiation machinery that potentiates the regulation of rRNA promoters by ppGpp and the initiating NTP. Cell 118(3), 311-322.

Paul, B.J., Berkmen, M.B. and Gourse, R.L. (2005) DksA potentiates direct activation of amino acid promoters by ppGpp. Proc Natl Acad Sci U S A 102(22), 7823-7828.

Payne, N.R. and Horwitz, M.A. (1987) Phagocytosis of *Legionella pneumophila* is mediated by human monocyte complement receptors. J Exp Med 166(5), 1377-1389.

Pécastaings, S., Bergé, M., Dubourg, K.M. and Roques, C. (2010) Sessile *Legionella pneumophila* is able to grow on surfaces and generate structured monospecies biofilms. Biofouling 26(7), 809-819.

Pécastaings, S. and Roques, C. (2013) Legionella: Methods and Protocols. Buchrieser, C. and Hilbi, H. (eds), pp. 219-224, Humana Press, Totowa, NJ.

Perez, J.C. and Groisman, E.A. (2007) Acid pH activation of the PmrA/PmrB two-component regulatory system of *Salmonella enterica*. Mol Microbiol 63(1), 283-293.

Pernestig, A.-K., Georgellis, D., Romeo, T., Suzuki, K., Tomenius, H., Normark, S. and Melefors, Ö. (2003) The *Escherichia coli* BarA-UvrY Two-Component System Is Needed for Efficient Switching between Glycolytic and Gluconeogenic Carbon Sources. J Bacteriol 185(3), 843-853.

Pernestig, A.K., O., M. and Georgellis, D. (2001) Identification of UvrY as the cognate response regulator for the BarA sensor kinase in *Escherichia coli* J Biol Chem 276(1), 225-231.

Pessi, G., Williams, F., Hindle, Z., Heurlier, K., Holden, M.T.G., Cámara, M., Haas, D. and Williams, P. (2001) The Global Posttranscriptional Regulator RsmA Modulates Production of Virulence Determinants and N-Acylhomoserine Lactones in *Pseudomonas aeruginosa*. J Bacteriol 183(22), 6676-6683.

Phares, C.R., Wangroongsarb, P., Chantra, S., Paveenkitiporn, W., Tondella, M.-L., Benson, R.F., Lanier Thacker, W., Fields, B.S., Moore, M.R., Fischer, J., Dowell, S.F. and Olsen, S.J. (2007) Epidemiology of Severe Pneumonia Caused by *Legionella longbeachae*, *Mycoplasma pneumoniae*, and *Chlamydia pneumoniae*: 1-Year, Population-Based Surveillance for Severe Pneumonia in Thailand. Clin Infect Dis 45(12), e147-e155.

Phin, N., Parry-Ford, F., Harrison, T., Stagg, H.R., Zhang, N., Kumar, K., Lortholary, O., Zumla, A. and Abubakar, I. (2014) Epidemiology and clinical management of Legionnaires' disease. Lancet Infect Dis 14(10), 1011-1021.

Picard-Masson, M., Lajoie, É., Lord, J., Lalancette, C., Marchand, G., Levac, É., Lemieux, M.-A., Hudson, P. and Lajoie, L. (2016) Two Related Occupational Cases of *Legionella longbeachae* Infection, Quebec, Canada. Emerg Infect Diss 22(7), 1289-1291.

Pine, L., George, J.R., Reeves, M.W. and Harrell, W.K. (1979) Development of a chemically defined liquid medium for growth of *Legionella pneumophila*. J Clin Microbiol 9(5), 615-626.

Pine, L., Hoffman, P.S., Malcolm, G.B., Benson, R.F. and Franzus, M.J. (1986) Role of keto acids and reduced-oxygen-scavenging enzymes in the growth of *Legionella* species. J Clin Microbiol 23(1), 33-42.

Plouffe, J.F., Webster, L.R. and Hackman, B. (1983) Relationship between colonization of hospital building with *Legionella pneumophila* and hot water temperatures. Appl Environ Microbiol 46(3), 769-770.

Politi, N., Pasotti, L., Zucca, S., Casanova, M., Micoli, G., Cusella De Angelis, M.G. and Magni, P. (2014) Half-life measurements of chemical inducers for recombinant gene expression. J Biol Eng 8, 5-5.

Pope, D.H., Soracco, R.J., Gill, H.K. and Fliermans, C.B. (1982) Growth of *Legionella pneumophila* in two-membered cultures with green algae and cyanobacteria. Curr Microbiol 7(5), 319-321.

Potrykus, K. and Cashel, M. (2008) (p)ppGpp: still magical? Annu Rev Microbiol 62, 35-51.

Potrykus, K., Murphy, H., Philippe, N. and Cashel, M. (2011) ppGpp is the major source of growth rate control in *E. coli*. Environ Microbiol 13(3), 563-575.

Potts A, D.M., Marley M, Othieno R, Stevenson J, Hyland J, Pollock KG, Lindsay D, Edwards G, Hanson MF, Helgason KO (2013) Cluster of Legionnaires' disease cases caused by Legionella longbeachae serogroup 1, Scotland, August to September 2013 Euro Surveill 18(50), pii=20656.

Prashar, A., Bhatia, S., Tabatabaeiyazdi, Z., Duncan, C., Garduño, R.A., Tang, P., Low, D.E., Guyard, C. and Terebiznik, M.R. (2012) Mechanism of invasion of lung epithelial cells by filamentous *Legionella pneumophila*. Cell Microbiol 14(10), 1632-1655.

Prashar, A. and Terebiznik, M.R. (2015) *Legionella pneumophila*: homeward bound away from the phagosome. Curr Opin Microbiol 23, 86-93.

Price, C.T., Al-Khodor, S., Al-Quadan, T., Santic, M., Habyarimana, F., Kalia, A. and Kwaik, Y.A. (2009) Molecular Mimicry by an F-Box Effector of *Legionella pneumophila* Hijacks a Conserved Polyubiquitination Machinery within Macrophages and Protozoa. PLoS Pathog 5(12), e1000704.

Price, C.T.D., Al-Khodor, S., Al-Quadan, T. and Abu Kwaik, Y. (2010) Indispensable Role for the Eukaryotic-Like Ankyrin Domains of the Ankyrin B Effector of *Legionella pneumophila* within Macrophages and Amoebae. Infect Immun 78(5), 2079-2088.

Price, C.T.D., Al-Quadan, T., Santic, M., Rosenshine, I. and Abu Kwaik, Y. (2011) Host Proteasomal Degradation Generates Amino Acids Essential for Intracellular Bacterial Growth. Science 334(6062), 1553-1557.

Price, C.T.D., Richards, A.M., Von Dwingelo, J.E., Samara, H.A. and Kwaik, Y.A. (2014) Amoeba host-*Legionella* synchronization of amino acid auxotrophy and its role in bacterial adaptation and pathogenic evolution. Environ Microbiol 16(2), 350-358.

Principe, L., Tomao, P. and Visca, P. (2017) Legionellosis in the occupational setting. Environ Res 152, 485-495.

Pruckler, J.M., Benson, R.F., Moyenuddin, M., Martin, W.T. and Fields, B.S. (1995) Association of flagellum expression and intracellular growth of *Legionella pneumophila*. Infect Immun 63(12), 4928-4932.

Pryor, M., Springthorpe, S., Riffard, S., Brooks, T., Huo, Y., Davis, G. and Sattar, S.A. (2004) Investigation of opportunistic pathogens in municipal drinking water under different supply and treatment regimes. Water Sci Technol 50(1), 83-90.

Qin, T., Yan, G., Ren, H., Zhou, H., Wang, H., Xu, Y., Zhao, M., Guan, H., Li, M. and Shao, Z. (2013) High Prevalence, Genetic Diversity and Intracellular Growth Ability of *Legionella* in Hot Spring Environments. PLoS ONE 8(3), e59018.

Rainey, J.J., Phelps, T. and Shi, J. (2016) Mass Gatherings and Respiratory Disease Outbreaks in the United States – Should We Be Worried? Results from a Systematic Literature Review and Analysis of the National Outbreak Reporting System. PLoS ONE 11(8), e0160378.

Rakic, A., Peric, J. and Foglar, L. (2012) Influence of temperature, chlorine residual and heavy metals on the presence of *Legionella pneumophila* in hot water distribution systems. Ann Agric Environ Med 19(3), 431-436.

Rankin, S., Li, Z. and Isberg, R.R. (2002) Macrophage-Induced Genes of *Legionella pneumophila*: Protection from Reactive Intermediates and Solute Imbalance during Intracellular Growth. Infect Immun 70(7), 3637-3648.

Rasis, M. and Segal, G. (2009) The LetA-RsmYZ-CsrA regulatory cascade, together with RpoS and PmrA, post-transcriptionally regulates stationary phase activation of *Legionella pneumophila* Icm/Dot effectors. Mol Microbiol 72(4), 995-1010.

Ratcliff, R.M., Lanser, J.A., Manning, P.A. and Heuzenroeder, M.W. (1998) Sequence-Based Classification Scheme for the Genus *Legionella* Targeting the *mip* Gene. J Clin Microbiol 36(6), 1560-1567.

Ratzow, S., Gaia, V., Helbig, J.H., Fry, N.K. and Lück, P.C. (2007) Addition of *neuA*, the gene encoding N-acylneuraminate cytidylyl transferase, increases the discriminatory ability of the consensus sequence-based scheme for typing *Legionella pneumophila* serogroup 1 strains. J Clin Microbiol 45(6), 1965-1968.

Raychaudhury, S., Farelli, J.D., Montminy, T.P., Matthews, M., Ménétret, J.-F., Duménil, G., Roy, C.R., Head, J.F., Isberg, R.R. and Akey, C.W. (2009) Structure and function of

interacting IcmR-IcmQ domains from a Type IVb secretion system in *Legionella pneumophila*. Structure 17(4), 590-601.

Rebers, P.A., Christianson, G.G., Laird, G.A. and Symanowski, J. (1989) Agarose soy casein digest medium for replacement of blood agar for potency determinations of live *Pasteurella* vaccines. Appl Environ Microbiol 55(1), 106-108.

Rechnitzer, C. and Blom, J. (1989) Engulfment of the Philadelphia strain of *Legionella pneumophila* within pseudopod coils in human phagocytes. APMIS 97(1-6), 105-114.

Reen, F.J., Haynes, J.M., Mooij, M.J. and O'Gara, F. (2013) A Non-Classical LysR-Type Transcriptional Regulator PA2206 Is Required for an Effective Oxidative Stress Response in *Pseudomonas aeruginosa*. PLoS ONE 8(1), e54479.

Reeves, M.W., Pine, L., Hutner, S.H., George, J.R. and Harrell, W.K. (1981) Metal requirements of *Legionella pneumophila*. J Clin Microbiol 13(4), 688-695.

Reimmann, C., Valverde, C., Kay, E. and Haas, D. (2005) Posttranscriptional Repression of GacS/GacA-Controlled Genes by the RNA-Binding Protein RsmE Acting Together with RsmA in the Biocontrol Strain *Pseudomonas fluorescens* CHA0. J Bacteriol 187(1), 276-285.

Restrepo, M.I. and Anzueto, A. (2005) Antimicrobial Treatment of Community-Acquired Pneumonia. Clin Chest Med 26(1), 65-73.

Ridenour, D.A., Cirillo, S.L.G., Feng, S., Samrakandi, M.M. and Cirillo, J.D. (2003) Identification of a Gene That Affects the Efficiency of Host Cell Infection by *Legionella pneumophila* in a Temperature-Dependent Fashion. Infect Immun 71(11), 6256-6263.

Riffard, S., Douglass, S., Brooks, T., Springthorpe, S., Filion, L.G. and Sattar, S.A. (2001) Occurrence of *Legionella* in groundwater: An ecological study. Water Sci Tech 43(12), 99-102.

Ristroph, J.D., Hedlund, K.W. and Gowda, S. (1981) Chemically defined medium for *Legionella pneumophila* growth. J Clin Microbiol 13(1), 115-119.

Robey, M. and Cianciotto, N.P. (2002) *Legionella pneumophila feoAB* Promotes Ferrous Iron Uptake and Intracellular Infection. Infect Immun 70(10), 5659-5669.

Robinson, J.M. (2009) Phagocytic leukocytes and reactive oxygen species. Histochem Cell Biol 131(4), 465-469.

Rogers, J., Dowsett, A.B., Dennis, P.J., Lee, J.V. and Keevil, C.W. (1994) Influence of Plumbing Materials on Biofilm Formation and Growth of *Legionella pneumophila* in Potable Water Systems. Appl Environ Microbiol 60(6), 1842-1851.

Rohr, U., Senger, M., Selenka, F., Turley, R. and Wilhelm, M. (1999) Four Years of Experience with Silver-Copper Ionization for Control of *Legionella* in a German University Hospital Hot Water Plumbing System. Clin Infect Dis 29(6), 1507-1511.

Roig, J., Sabria, M. and Pedro-Botet, M.L. (2003) Legionella spp.: community acquired and nosocomial infections. Curr Opin Infect Dis 16(2), 145-151.

Romeo, T., Vakulskas, C.A. and Babitzke, P. (2013) Posttranscriptional regulation on a global scale: Form and function of Csr/Rsm systems. Environ Microbiol 15(2), 313-324.

Rossier, O. and Cianciotto, N.P. (2005) The *Legionella pneumophila tatB* Gene Facilitates Secretion of Phospholipase C, Growth under Iron-Limiting Conditions, and Intracellular Infection. Infect Immun 73(4), 2020-2032.

Rossier, O., Dao, J. and Cianciotto, N.P. (2008) The Type II Secretion System of *Legionella pneumophila* Elaborates Two Aminopeptidases, as Well as a Metalloprotease That Contributes to Differential Infection among Protozoan Hosts. Appl Environ Microbiol 74(3), 753-761.

Rossier, O., Starkenburg, S.R. and Cianciotto, N.P. (2004) *Legionella pneumophila* Type II Protein Secretion Promotes Virulence in the A/J Mouse Model of Legionnaires' Disease Pneumonia. Infect Immun 72(1), 310-321.

Rowbotham, T.J. (1980) Preliminary report on the pathogenicity of *Legionella pneumophila* for freshwater and soil amoebae. J Clin Pathol 33, 1179-1183.

Roy, C.R., Berger, K.H. and Isberg, R.R. (1998) *Legionella pneumophila* DotA protein is required for early phagosome trafficking decisions that occur within minutes of bacterial uptake. Mol Microbiol 28(3), 663-674.

Rusin, P.A., Rose, J.B., Haas, C.N. and Gerba, C.P. (1997) Risk assessment of opportunistic bacterial pathogens in drinking water. Rev Environ Contam Toxicol 152, 57-83.

Rutala, W.A., Weber, D.J. and Committee, H.I.C.P.A. (2008) Guideline for Disinfection and Sterilization in Healthcare Facilities, 2008. CDC (ed), Centres for Disease Control and Prevention, Atlanta, GA.

Sadosky, A.B., Wiater, L.A. and Shuman, H.A. (1993) Identification of *Legionella pneumophila* genes required for growth within and killing of human macrophages. Infect Immun 61, 5361-5373.

Sadosky, A.B., Wilson, J.W., Steinman, H.M. and Shuman, H.A. (1994) The iron superoxide dismutase of *Legionella pneumophila* is essential for viability. J Bacteriol 176(12), 3790-3799.

Sahr, T., Brüggemann, H., Jules, M., Lomma, M., Albert-Weissenberger, C., Cazalet, C. and Buchrieser, C. (2009) Two small ncRNAs jointly govern virulence and transmission in *Legionella pneumophila*. Mol Microbiol 72(3), 741-762.

Sahr, T., Rusniok, C., Dervins-Ravault, D., Sismeiro, O., Coppee, J.-Y. and Buchrieser, C. (2012) Deep sequencing defines the transcriptional map of *L. pneumophila* and identifies growth phase-dependent regulated ncRNAs implicated in virulence. RNA Biol 9(4), 503-519.

Sahr, T., Rusniok, C., Impens, F., Oliva, G., Sismeiro, O., Coppée, J.-Y. and Buchrieser, C. (2017) The *Legionella pneumophila* genome evolved to accommodate multiple regulatory mechanisms controlled by the CsrA-system. PLOS Gen 13(2), e1006629.

Sánchez-Busó, L., Guiral, S., Crespi, S., Moya, V., Camaró, M.L., Olmos, M.P., Adrián, F., Morera, V., González-Morán, F., Vanaclocha, H. and González-Candelas, F. (2015) Genomic Investigation of a Legionellosis Outbreak in a Persistently Colonized Hotel. Front Microbiol 6, 1556.

Sanderson, S.S. and Stewart, P.S. (1997) Evidence of bacterial adaptation to monochloramine in *Pseudomonas aeruginosa* biofilms and evaluation of biocin action model. Biotechnol Bioeng 56(2), 201-209.

Sands, J.G. and Bennett, E.O. (1966) Effect of Different Commercial Agar Preparations on the Inhibitory Activities of Phenols. Appl Microbiol 14(2), 196-202.

Sauer, J.-D., Bachman, M.A. and Swanson, M.S. (2005) The phagosomal transporter A couples threonine acquisition to differentiation and replication of *Legionella pneumophila* in macrophages. PNAS 102(28), 9924-9929.

Saunders, N.A., Harrison, T.G., Kachwalla, N. and Taylor, A.G. (1988) Identification of Species of the Genus Legionella Using a Cloned rRNA Gene from *Legionella pneumophila*. J Gen Microbiol 134(8), 2363-2374.

Scaturro, M., Fontana, S., Crippa, S., Caporali, M.G., Seyler, T., Veschetti, E., Villa, G., Rota, M.C. and Ricci, M.L. (2015) An unusually long-lasting outbreak of community-acquired Legionnaires' disease, 2005–2008, Italy. Epidemiol Infect 143(11), 2416-2425.

Schalk, J.A.C., Euser, S.M., van Heijnsbergen, E., Bruin, J.P., den Boer, J.W. and de Roda Husman, A.M. (2014) Soil as a source of *Legionella pneumophila* sequence type 47. Int J Infect Dis 27, 18-19.

Schell, U., Kessler, A. and Hilbi, H. (2014) Phosphorylation signalling through the *Legionella quorum* sensing histidine kinases LqsS and LqsT converges on the response regulator LqsR. Mol Microbiol 92(5), 1039-1055.

Schell, U., Simon, S., Sahr, T., Hager, D., Albers, M.F., Kessler, A., Fahrnbauer, F., Trauner, D., Hedberg, C., Buchrieser, C. and Hilbi, H. (2016) The α-hydroxyketone LAI-1 regulates motility, Lqs-dependent phosphorylation signalling and gene expression of *Legionella pneumophila*. Mol Microbiol 99(4), 778-793.

Schellhorn, H.E. (2014) Elucidating the function of the RpoS regulon. Future Microbiolo 9(4), 497-507.

Schindelin, J., Rueden, C.T., Hiner, M.C. and Eliceiri, K.W. (2015) The ImageJ ecosystem: An open platform for biomedical image analysis. Mo Reprod Dev 82(7-8), 518-529.

Schulz, T., Rydzewski, K., Schunder, E., Holland, G., Bannert, N. and Heuner, K. (2012) FliA expression analysis and influence of the regulatory proteins RpoN, FleQ and FliA on virulence and in vivo fitness in *Legionella pneumophila*. Arch Microbiol 194(12), 977-989.

Schumann, W. (2016) Regulation of bacterial heat shock stimulons. Cell Stress Chaperones 21(6), 959-968.

Schunder, E., Gillmaier, N., Kutzner, E., Herrmann, V., Lautner, M., Heuner, K. and Eisenreich, W. (2014) Amino Acid Uptake and Metabolism of *Legionella pneumophila* Hosted by *Acanthamoeba castellanii*. J Biol Chem 289(30), 21040-21054.

Schwake, D.O., Garner, E., Strom, O.R., Pruden, A. and Edwards, M.A. (2016) *Legionella* DNA Markers in Tap Water Coincident with a Spike in Legionnaires' Disease in Flint, MI. Environ Sci Technol Lett 3(9), 311-315.

Seaver, L.C. and Imlay, J.A. (2001) Hydrogen Peroxide Fluxes and Compartmentalization inside Growing *Escherichia coli*. J Bacteriol 183(24), 7182-7189.

Segal, G. (2014) Molecular Mechanisms in Legionella Pathogenesis. Hilbi, H. (ed), pp. 35-52, Springer Berlin Heidelberg, Berlin, Heidelberg.

Segal, G., Feldman, M. and Zusman, T. (2005) The Icm/Dot type-IV secretion systems of *Legionella pneumophila* and *Coxiella burnetii*. FEMS Microbiol Rev 29(1), 65-81.

Segal, G., Purcell, M. and Shuman, H.A. (1998) Host cell killing and bacterial conjugation require overlapping sets of genes within a 22-kb region of the *Legionella pneumophila* genome. Proc Natl Acad Sci 95(4), 1669-1674.

Segal, G., Russo, J.J. and Shuman, H.A. (1999) Relationships between a new type IV secretion system and the *icm/dot* virulence system of *Legionella pneumophila*. Mol Microbiol 34(4), 799-809.

Seo, Sang W., Kim, D., Szubin, R. and Palsson, Bernhard O. (2015) Genome-wide Reconstruction of OxyR and SoxRS Transcriptional Regulatory Networks under Oxidative Stress in *Escherichia coli* K-12 MG1655. Cell Rep 12(8), 1289-1299.

Serrano-Suárez, A., Dellundé, J., Salvadó, H., Cervero-Aragó, S., Méndez, J., Canals, O., Blanco, S., Arcas, A. and Araujo, R. (2013) Microbial and physicochemical parameters associated with *Legionella* contamination in hot water recirculation systems. Environ Sci Pollut Res 20(8), 5534-5544.

Seth, D., Hausladen, A., Wang, Y.-J. and Stamler, J.S. (2012) Endogenous protein S-nitrosylation in *E. coli*: regulation by OxyR. Science 336(6080), 10.1126/science.1215643.

Sexton, J.A. and Vogel, J.P. (2004) Regulation of Hypercompetence in *Legionella pneumophila*. J Bacteriol 186(12), 3814-3825.

Seyfzadeh, M., Keener, J. and Nomura, M. (1993) *spoT*-dependent accumulation of guanosine tetraphosphate in response to fatty acid starvation in *Escherichia coli*. Proc Natl Acad Sci 90(23), 11004-11008.

Shi, C., Forsbach-Birk, V., Marre, R. and McNealy, T.L. (2006) The *Legionella pneumophila* global regulatory protein LetA affects DotA and Mip. Int J Med Microbiol 296(1), 15-24.

Shi, M., Wan, F., Mao, Y. and Gao, H. (2015) Unraveling the Mechanism for the Viability Deficiency of *Shewanella oneidensis oxyR* Null Mutant. J Bacteriol 197(13), 2179-2189.

Shohdy, N., Efe, J.A., Emr, S.D. and Shuman, H.A. (2005) Pathogen effector protein screening in yeast identifies *Legionella* factors that interfere with membrane trafficking. Proc Natl Acad Sci 102(13), 4866-4871.

Shuval, H., Yarom, R. and Shenman, R. (2009) An innovative method for the control of *Legionella* infections in the hospital hot water systems with a stabilized hydrogen peroxide-silver formulation. Int J Infect Control 5(1).

Smith, S.S., Ritger, K., Samala, U., Black, S.R., Okodua, M., Miller, L., Kozak-Muiznieks, N.A., Hicks, L.A., Steinheimer, C., Ewaidah, S., Presser, L. and Siston, A.M. (2015) Legionellosis Outbreak Associated With a Hotel Fountain. Open Forum Infect Dis 2(4), ofv164.

Smoot, L.M., Smoot, J.C., Graham, M.R., Somerville, G.A., Sturdevant, D.E., Migliaccio, C.A.L., Sylva, G.L. and Musser, J.M. (2001) Global differential gene expression in response to growth temperature alteration in group A *Streptococcus*. Proc Natl Acad Sci U S A 98(18), 10416-10421.

Söderberg, M.A., Dao, J., Starkenburg, S.R. and Cianciotto, N.P. (2008) Importance of Type II Secretion for Survival of *Legionella pneumophila* in Tap Water and in Amoebae at Low Temperatures. Appl Environ Microbiol 74(17), 5583-5588.

Söderberg, M.A., Rossier, O. and Cianciotto, N.P. (2004) The Type II Protein Secretion System of *Legionella pneumophila* Promotes Growth at Low Temperatures. J Bacteriol 186(12), 3712-3720.

Solimini, A.G., Cottarelli, A., Marinelli, L. and De Giusti, M. (2014) Factors influencing persistence of *Legionella pneumophila* serogroup 1 in laboratory cocultures. BMC Microbiol 14(1), 249.

Spira, B., Silberstein, N. and Yagil, E. (1995) Guanosine 3',5'-bispyrophosphate (ppGpp) synthesis in cells of *Escherichia coli* starved for Pi. J Bacteriol 177(14), 4053-4058.

Spira, B. and Yagil, E. (1998) The relation between ppGpp and the PHO regulon in *Escherichia coli*. Mol Gen Genet 257(4), 469-477.

Spirig, T., Tiaden, A., Kiefer, P., Buchrieser, C., Vorholt, J.A. and Hilbi, H. (2008) The *Legionella* Autoinducer Synthase LqsA Produces an α-Hydroxyketone Signaling Molecule. J Biol Chem 283(26), 18113-18123.

Srivatsan, A. and Wang, J.D. (2008) Control of bacterial transcription, translation and replication by (p)ppGpp. Curr Opin Microbiol 11(2), 100-105.

St. John, G. and Steinman, H.M. (1996) Periplasmic copper-zinc superoxide dismutase of *Legionella pneumophila*: role in stationary-phase survival. J Bacteriol 178(6), 1578-1584.

Starnbach, M.N. and Lory, S. (1992) The *filA* (*rpoF*) gene of *Pseudomonas aeruginosa* encodes an alternative sigma factor required for flagellin synthesis. Mol Microbiol 6(4), 459-469.

States, S.J., Conley, L.F., Ceraso, M., Stephenson, T.E., Wolford, R.S., Wadowsky, R.M., McNamara, A.M. and Yee, R.B. (1985) Effects of metals on *Legionella pneumophila* growth in drinking water plumbing systems. Appl Environ Microbiol 50(5), 1149-1154.

States, S.J., Conley, L.F., Kuchta, J.M., Wolford, R.S., Wadowsky, R.M. and Yee, R.B. (1989) Chlorine, pH, and control of *Legionella* in hospital plumbing systems. JAMA 261(13), 1882-1883.

States, S.J., Conley, L.F., Towner, S.G., Wolford, R.S., Stephenson, T.E., McNamara, A.M., Wadowsky, R.M. and Yee, R.B. (1987) An alkaline approach to treating cooling towers for control of *Legionella pneumophila*. Appl Environ Microbiol 53(8), 1775-1779.

Steele, T.W., Lanser, J. and Sangster, N. (1990) Isolation of *Legionella longbeachae* serogroup 1 from potting mixes. Appl Environ Microbiol 56(1), 49-53.

Steinchen, W. and Bange, G. (2016) The magic dance of the alarmones (p)ppGpp. Mol Microbiol 101(4), 531-544.

Steinert, M., Emödy, L., Amann, R. and Hacker, J. (1997) Resuscitation of viable but nonculturable *Legionella pneumophila* Philadelphia JR32 by *Acanthamoeba castellanii*. Appl Environ Microbiol 63(5), 2047-2053.

Steinert, M., Hentschel, U. and Hacker, J. (2002) *Legionella pneumophila*: an aquatic microbe goes astray. FEMS Microbiol Rev 26(2), 149.

Stewart, C.R., Muthye, V. and Cianciotto, N.P. (2012) *Legionella pneumophila* Persists within Biofilms Formed by *Klebsiella pneumoniae*, *Flavobacterium* sp., and *Pseudomonas fluorescens* under Dynamic Flow Conditions. PLoS ONE 7(11), e50560.

Stock, A.M., Robinson, V.L. and Goudreay, P.N. (2000) Two-Component Signal Transduction. Annu Rev Biochem 69, 183-215.

Stojek, N.M. and Dutkiewicz, J. (2011) Co-existence of *Legionella* and other Gram-negative bacteria in potable water from various rural and urban sources. Ann Agric Environ Med 18(2), 330-334.

Stone, B.J. and Kwaik, Y.A. (1998) Expression of Multiple Pili by *Legionella pneumophila*: Identification and Characterization of a Type IV Pilin Gene and Its Role in Adherence to Mammalian and Protozoan Cells. Infect Immun 66(4), 1768-1775.

Storey, M.V., Winiecka-krusnell, J., Ashbolt, N.J. and Stenström, T.-a. (2004) The Efficacy of Heat and Chlorine Treatment against Thermotolerant *Acanthamoebae* and *Legionellae*. Scand J Infect Dis 36(9), 656-662.

Storz, G. (2016) New perspectives: Insights into oxidative stress from bacterial studies. Arch Biochem Biophys 595, 25-27.

Storz, G., Jacobson, F.S., Tartaglia, L.A., Morgan, R.W., Silveira, L.A. and Ames, B.N. (1989) An alkyl hydroperoxide reductase induced by oxidative stress in *Salmonella typhimurium* and *Escherichia coli*: genetic characterization and cloning of *ahp*. J Bacteriol 171(4), 2049-2055.

Stout, J.E., Best, M.G., Yu, V.L. and Rihs, J.D. (1986) A note on symbiosis of *Legionella pneumophila* and *Tatlockia micdadei* with human respiratory flora. J Appl Bacteriol 60(4), 297-299.

Stout, J.E., Lin, Y.S.E., Goetz, A.M. and Muder, R.R. (1998) Controlling *Legionella* in hospital water systems: Experience with the superheat-and-flush method and copper-silver ionization. Infect Control Hosp Epidemiol 19(12), 911-914.

Stout, J.E., Yu, V.L. and Best, M.G. (1985) Ecology of *Legionella pneumophila* within water distribution systems. Appl Environ Microbiol 49(1), 221-228.

Stout, J.E., Yu, V.L., Yee, Y.C., Vaccarello, S., Diven, W. and Lee, T.C. (1992) *Legionella pneumophila* in residential water supplies: environmental surveillance with clinical assessment for Legionnaires' disease. Epidemiol Inf 109(1), 49-57.

Straus, W.L., Plouffe, J.F., File Jr, T.M., Lipman, H.B., Hackman, B.H., Salstrom, S.J., Benson, R.F. and Breiman, R.F. (1996) Risk factors for domestic acquisition of legionnaires disease. Arch Intern Med 156(15), 1685-1692.

Sturgill-Koszycki, S. and Swanson, M.S. (2000) *Legionella pneumophila* Replication Vacuoles Mature into Acidic, Endocytic Organelles. J Exp Med 192(9), 1261-1272.

Suh, S.-J., Silo-Suh, L., Woods, D.E., Hassett, D.J., West, S.E.H. and Ohman, D.E. (1999) Effect of *rpoS* Mutation on the Stress Response and Expression of Virulence Factors in *Pseudomonas aeruginosa*. J Bacteriol 181(13), 3890-3897.

Surman, S.B., Morton, L.H.G. and Keevil, C.W. (1994) The dependence of *Legionella pneumophila* on other aquatic bacteria for survival on R2A medium. Intl Biodeterior Biodegrad 33(3), 223-236.

Suzuki, K., Babitzke, P., Kushner, S.R. and Romeo, T. (2006) Identification of a novel regulatory protein (CsrD) that targets the global regulatory RNAs CsrB and CsrC for degradation by RNase E. Genes Dev 20(18), 2605-2617.

Suzuki, K., Wang, X., Weilbacher, T., Pernestig, A.-K., Melefors, Ö., Georgellis, D., Babitzke, P. and Romeo, T. (2002) Regulatory Circuitry of the CsrA/CsrB and BarA/UvrY Systems of *Escherichia coli*. J Bacteriol 184(18), 5130-5140.

Svitil, A.L., Cashel, M. and Zyskind, J.W. (1993) Guanosine tetraphosphate inhibits protein-synthesis *in vivo.* A possible protective mechanism for starvation stress in *Escherichia coli*. J Biol Chem 268(4), 2307-2311.

Swanson, M.S. and Hammer, B.K. (2000) *Legionella pneumophila* Pathogenesis: A Fateful Journey from Amoebae to Macrophages. Annu Rev Microbiol 54(1), 567-613.

Swanson, M.S. and Isberg, R.R. (1996) Identification of *Legionella pneumophila* mutants that have aberrant intracellular fates. Infect Immun 64(7), 2585-2594.

Tachibana, M., Nakamoto, M., Kimura, Y., Shimizu, T. and Watarai, M. (2013) Characterization of *Legionella pneumophila* isolated from environmental water and ashiyu foot spa. BioMed Res Intl.

Tamarit, J., Cabiscol, E. and Ros, J. (1998) Identification of the Major Oxidatively Damaged Proteins in *Escherichia coli* Cells Exposed to Oxidative Stress. J Biol Chem 273(5), 3027-3032.

Tanner, J.R., Li, L., Faucher, S.P. and Brassinga, A.K.C. (2016a) The CpxRA two-component system contributes to *Legionella pneumophila* virulence. Mol Microbiol 100(6), 1017-1038.

Tanner, J.R., Patel, P.G., Hellinga, J.R., Donald, L.J., Jimenez, C., LeBlanc, J.J. and Brassinga, A.K.C. (2016b) *Legionella pneumophila* OxyR is a redundant transcriptional regulator that contributes to expression control of the two-component CpxRA system. J Bacteriol.

Tao, K., Makino, K., Yonei, S., Nakata, A. and Shinagawa, H. (1991) Purification and Characterization of the *Escherichia coli* OxyR Protein, the Positive Regulator for a Hydrogen Peroxide-Inducible Regulon. J Biochem 109(2), 262-266.

Tartaglia, L.A., Storz, G. and Ames, B.N. (1989) Identification and molecular analysis of oxyR-regulated promoters important for the bacterial adaptation to oxidative stress. J Mol Biol 210(4), 709-719.

Taylor, M., Ross, K. and Bentham, R. (2009) *Legionella*, Protozoa, and Biofilms: Interactions Within Complex Microbial Systems. Microb Ecol 58(3), 538-547.

Temmerman, R., Vervaeren, H., Noseda, B., Boon, N. and Verstraete, W. (2006) Necrotrophic Growth of *Legionella pneumophila*. Appl Environ Microbiol 72(6), 4323-4328.

Teplitski, M., Goodier, R.I. and Ahmer, B.M.M. (2003) Pathways Leading from BarA/SirA to Motility and Virulence Gene Expression in *Salmonella*. J Bacteriol 185(24), 7257-7265.

Terranova, W., Cohen, M. and Fraser, D. (1978) 1974 Outbreak of Legionnaires' Disease Diagnosed in 1977. Lancet 312(8081), 122-124.

Tesh, M.J., Morse, S.A. and Miller, R.D. (1983) Intermediary Metabolism in *Legionella pneumophila*: Utilization of Amino Acids and Other Compounds as Energy Sources. J Bacteriol 154(3), 1104-1109.

Tessier, A., Campbell, P.G.C. and Bisson, M. (1979) Sequential extraction procedure for the speciation of particulate trace metals. Anal Chem 51(7), 844-851.

Thacker, S.B., Bennett, J.V., Tsai, T.F., Fraser, D.W., McDade, J.E., Shepard, C.C., Williams, K.H., Stuart, W.H., Dull, H.B. and Eickhoff, T.C. (1978) An Outbreak in 1965 of Severe Respiratory Illness Caused by the Legionnaires' Disease Bacterium. J Infect Dis 138(4), 512-519.

Thomas, V., Bouchez, T., Nicolas, V., Robert, S., Loret, J.F. and Lévi, Y. (2004) Amoebae in domestic water systems: resistance to disinfection treatments and implication in *Legionella* persistence. J Appl Microbiol 97(5), 950-963.

Tiaden, A., Spirig, T., Carranza, P., Brüggemann, H., Riedel, K., Eberl, L., Buchrieser, C. and Hilbi, H. (2008) Synergistic Contribution of the *Legionella pneumophila lqs* Genes to Pathogen-Host Interactions. J Bacteriol 190(22), 7532-7547.

Tiaden, A., Spirig, T. and Hilbi, H. (2010a) Bacterial gene regulation by α-hydroxyketone signaling. Trends Microbiol 18(7), 288-297.

Tiaden, A., Spirig, T., Sahr, T., Wälti, M.A., Boucke, K., Buchrieser, C. and Hilbi, H. (2010b) The autoinducer synthase LqsA and putative sensor kinase LqsS regulate phagocyte interactions, extracellular filaments and a genomic island of *Legionella pneumophila*. Environ Microbiol 12(5), 1243-1259.

Tiaden, A., Spirig, T., Weber, S.S., Brüggemann, H., Bosshard, R., Buchrieser, C. and Hilbi, H. (2007) The *Legionella pneumophila* response regulator LqsR promotes host cell interactions as an element of the virulence regulatory network controlled by RpoS and LetA. Cell Microbiol 9(12), 2903-2920.

Tilney, L.G., Harb, O.S., Connelly, P.S., Robinson, C.G. and Roy, C.R. (2001) How the parasitic bacterium *Legionella pneumophila* modifies its phagosome and transforms it into rough ER: implications for conversion of plasma membrane to the ER membrane. J Cell Sci 114(24), 4637-4650.

Tison, D.L., Pope, D.H., Cherry, W.B. and Fliermans, C.B. (1980) Growth of *Legionella pneumophila* in association with blue-green algae (cyanobacteria). Appl Environ Microbiol 39(2), 456-459.

Torres, A., Peetermans, W.E., Viegi, G. and Blasi, F. (2013) Risk factors for community-acquired pneumonia in adults in Europe: a literature review. Thorax 68(11), 1057-1065.

Tossa, P., Deloge-Abarkan, M., Zmirou-Navier, D., Hartemann, P. and Mathieu, L. (2006) Pontiac fever: an operational definition for epidemiological studies. BMC Public Health 6, 112-112.

Toulokhonov, II, Shulgina, I. and Hernandez, V.J. (2001) Binding of the transcription effector ppGpp to *Escherichia coli* RNA polymerase is allosteric, modular, and occurs near the N terminus of the β'-subunit. J Biol Chem 276(2), 1220-1225.

Toze, S., Sly, L.I., MacRae, I.C. and Fuerst, J.A. (1990) Inhibition of Growth of *Legionella* Species by Heterotrophic Plate Count Bacteria Isolated from Chlorinated Drinking Water. Curr Microbiol 21, 139-143.

Travis, T.C., Brown, E.W., Peruski, L.F., Siludjai, D., Jorakate, P., Salika, P., Yang, G., Kozak, N.A., Kodani, M., Warner, A.K., Lucas, C.E., Thurman, K.A., Winchell, J.M., Thamthitiwat, S. and Fields, B.S. (2012) Survey of *Legionella* Species Found in Thai Soil. Int J Microbiol 2012, 218791.

Trigui, H., Dudyk, P., Oh, J., Hong, J.-I. and Faucher, S.P. (2014) A regulatory feedback loop between RpoS and SpoT supports the survival of *Legionella pneumophila* in water. Appl Environ Microbiol 81(3), 918-928.

Tsirigos, K.D., Peters, C., Shu, N., Käll, L. and Elofsson, A. (2015) The TOPCONS web server for consensus prediction of membrane protein topology and signal peptides. Nucleic Acids Res 43(Web Server issue), W401-W407.

Turetgen, I. (2008) Induction of Viable but Nonculturable (VBNC) state and the effect of multiple subculturing on the survival of *Legionella pneumophila* strains in the presence of monochloramine. Ann Microbiol 58(1), 153-156.

Tyson, J.Y., Pearce, M.M., Vargas, P., Bagchi, S., Mulhern, B.J. and Cianciotto, N.P. (2013) Multiple *Legionella pneumophila* Type II Secretion Substrates, Including a Novel Protein, Contribute to Differential Infection of the Amoebae *Acanthamoeba castellanii*, *Hartmannella vermiformis*, and *Naegleria lovaniensis*. Infect Immun 81(5), 1399-1410.

Vakulskas, C.A., Leng, Y., Abe, H., Amaki, T., Okayama, A., Babitzke, P., Suzuki, K. and Romeo, T. (2016) Antagonistic control of the turnover pathway for the global regulatory sRNA CsrB by the CsrA and CsrD proteins. Nucleic Acids Res 44(16), 7896-7910.

Valster, R.M., Wullings, B.A. and van der Kooij, D. (2010) Detection of Protozoan Hosts for *Legionella pneumophila* in Engineered Water Systems by Using a Biofilm Batch Test. Appl Environ Microbiol 76(21), 7144-7153.

Valverde, C., Heeb, S., Keel, C. and Haas, D. (2003) RsmY, a small regulatory RNA, is required in concert with RsmZ for GacA-dependent expression of biocontrol traits in *Pseudomonas fluorescens* CHA0. Mol Microbiol 50(4), 1361-1379.

van der Kooij, D., Brouwer-Hanzens, A.J., Veenendaal, H.R. and Wullings, B.A. (2016) Multiplication fo *Legionella pneumophila* Sequence Types 1, 47, and 62 in Buffered Yeast Extract Broth and Biofilms Exposed to Flowing Tap Water at Temperatures of 38^{o}C to 42^{o}C. Appl Environ Microbiol 82(22), 6691-6700.

van der Kooij, D., Veenendaal, H.R. and Scheffer, W.J.H. (2005) Biofilm formation and multiplication of *Legionella* in a model warm water system with pipes of copper, stainless steel and cross-linked polyethylene. Water Res 39(13), 2789-2798.

van Heijnsbergen, E., de Roda Husman, A.M., Lodder, W.J., Bouwknegt, M., Docters van Leeuwen, A.E., Bruin, J.P., Euser, S.M., den Boer, J.W. and Schalk, J.A.C. (2014) Viable

Legionella pneumophila bacteria in natural soil and rainwater puddles. J Appl Microbiol 117(3), 882-890.

van Heijnsbergen, E., Schalk, J.A.C., Euser, S.M., Brandsema, P.S., den Boer, J.W. and de Roda Husman, A.M. (2015) Confirmed and Potential Sources of *Legionella* Reviewed. Environ Sci Technol 49(8), 4797-4815.

van Heijnsbergen, E., van Deursen, A., Bouwknegt, M., Bruin, J.P., de Roda Husman, A.M. and Schalk, J.A.C. (2016) Presence and Persistence of Viable, Clinically Relevant *Legionella pneumophila* Bacteria in Garden Soil in the Netherlands. Appl Environ Microbiol 82(17), 5125-5131.

Vanrheenen, S.M., Luo, Z.Q., O'Connor, T. and Isberg, R.R. (2006) Members of a *Legionella pneumophila* family of proteins with ExoU (Phospholipase A) active sites are translocated to target cells. Infect Immun 74(6), 3597-3606.

Venkataraman, C., Haack, B.J., Bondada, S. and Abu Kwaik, Y. (1997) Identification of a Gal/GalNAc lectin in the protozoan *Hartmannella vermiformis* as a potential receptor for attachment and invasion by the Legionnaires' disease bacterium. J Exp Med 186(4), 537-547.

Verdon, J., Labanowski, J., Sahr, T., Ferreira, T., Lacombe, C., Buchrieser, C., Berjeaud, J.-M. and Héchard, Y. (2011) Fatty acid composition modulates sensitivity of *Legionella pneumophila* to warnericin RK, an antimicrobial peptide. Biochim Biophys Acta 1808(4), 1146-1153.

Vervaeren, H., Temmerman, R., Devos, L., Boon, N. and Verstraete, W. (2006) Introduction of a boost of *Legionella pneumophila i*nto a stagnant-water model by heat treatment. FEMS Microbiol Ecol 58(3), 583.

Vickers, R.M., Yu, V.L., Hanna, S.S., Muraca, P., Diven, W., Carmen, N. and Taylor, F.B. (1987) Determinants of *Legionella pneumophila* Contamination of Water Distribution Systems: 15-Hospital Prospective Study. Infect Control 8(9), 357-363.

Vincent, C.D., Buscher, B.A., Friedman, J.R., Williams, L.A., Bardill, P. and Vogel, J.P. (2006) Identification of Non-*dot/icm* Suppressors of the *Legionella pneumophila* Δ*dotL* Lethality Phenotype. J Bacteriol 188(23), 8231-8243.

Vinella, D., Albrecht, C., Cashel, M. and D'Ari, R. (2005) Iron limitation induces SpoT-dependent accumulation of ppGpp in *Escherichia coli*. Mol Microbiol 56(4), 958-970.

Viswanathan, V.K., Edelstein, P.H., Pope, C.D. and Cianciotto, N.P. (2000) The *Legionella pneumophila iraAB* Locus Is Required for Iron Assimilation, Intracellular Infection, and Virulence. Infect Immun 68(3), 1069-1079.

Vogel, J.P., Andrews, H.L., Wong, S.K. and Isberg, R.R. (1998) Conjugative Transfer by the Virulence System of *Legionella pneumophila*. Science 279(5352), 873-876.

Vogel, J.P. and Isberg, R.R. (1999) Cell biology of *Legionella pneumophila*. Curr Opin Microbiol 2(1), 30-34.

Vogel, J.P., Roy, C. and Isberg, R.R. (1996) Use of Salt to Isolate *Legionella pneumophila* Mutants Unable to Replicate in Macrophages. Ann N Y Acad Sci 797(1), 271-272.

Wadowsky, R.M., Butler, L.J., Cook, M.K., Verma, S.M., Paul, M.A., Fields, B.S., Keleti, G., Sykora, J.L. and Yee, R.B. (1988) Growth-supporting activity for *Legionella pneumophila* in tap water cultures and implication of hartmannellid amoebae as growth factors. Appl Environ Microbiol 54(11), 2677-2682.

Wadowsky, R.M., Wolford, R., McNamara, A.M. and Yee, R.B. (1985) Effect of temperature, pH, and oxygen level on the multiplication of naturally occurring *Legionella pneumophila* in potable water. Appl Environ Microbiol 49(5), 1197-1205.

Wadowsky, R.M. and Yee, R.B. (1983) Satellite growth of *Legionella pneumophila* with an environmental isolate of *Flavobacterium breve*. Appl Environ Microbiol 46(6), 1447-1449.

Wadowsky, R.M., Yee, R.B., Mezmar, L., Wing, E.J. and Dowling, J.N. (1982) Hot water systems as sources of *Legionella pneumophila* in hospital and nonhospital plumbing fixtures. Appl Environ Microbiol 43(5), 1104-1110.

Walczak, M., Kletkiewicz, H. and Burkowska, A. (2013a) Occurrence of *Legionella pneumophila* in lakes serving as a cooling system of a power plant. Environ Sci Processes Impacts 15(12), 2273-2278.

Walczak, M., Krawiec, A. and Lalke-Porczyk, E. (2013b) *Legionella pneumophila* bacteria in a thermal saline bath. Ann Agric Environ Med 20(4), 649-652.

Wang, C., Saito, M., Tanaka, T., Amako, K. and Yoshida, S.-i. (2015) Comparative analysis of virulence traits between a *Legionella feeleii* strain implicated in Pontiac fever and a strain that caused Legionnaires' disease. Microb Pathog 89, 79-86.

Wang, W., Blaser, M.J., Cravens, J. and Johnson, M.A. (1979) The Microorganism: Growth, Survival, and Resistance of the Legionnaires' Disease Bacterium. Ann Intern Med 90(4), 614-618.

Wang, X., Li, M., Liu, L., Mou, R., Zhang, X., Bai, Y., Xu, H. and Qiao, M. (2012) DsbM, a novel disulfide oxidoreductase affects aminoglycoside resistance in *Pseudomonas aeruginosa* by OxyR-regulated response. J Microbiol 50(6), 932-938.

Watanabe, K., Nakao, R., Fujishima, M., Tachibana, M., Shimizu, T. and Watarai, M. (2016) Ciliate *Paramecium* is a natural reservoir of *Legionella pneumophila*. Sci Rep 6, 24322.

Wayne, L.G. and Diaz, G.A. (1986) A Double Staining Method for Differentiating between Two Classes of Mycobacterial Catalase in Polyacrylamide Electrophoresis Gels. Anal Biochem 157, 89-92.

Wei, Q., Le Minh, P.N., Dötsch, A., Hildebrand, F., Panmanee, W., Elfarash, A., Schulz, S., Plaisance, S., Charlier, D., Hassett, D., Häussler, S. and Cornelis, P. (2012) Global regulation of gene expression by OxyR in an important human opportunistic pathogen. Nucleic Acids Res 40(10), 4320-4333.

Weissenmayer, B.A., Prendergast, J.G.D., Lohan, A.J. and Loftus, B.J. (2011) Sequencing Illustrates the Transcriptional Response of *Legionella pneumophila* during Infection and Identifies Seventy Novel Small Non-Coding RNAs. PLoS ONE 6(3), e17570.

Weissgerber, P., Faigle, M., Northoff, H. and Neumeister, B. (2003) Investigation of mechanisms involved in phagocytosis of *Legionella pneumophila* by human cells. FEMS Microbiol Lett 219(2), 173-179.

Wery, N., Bru-Adan, V., Minervini, C., Delgenes, J.-P., Garrelly, L. and Godon, J.-J. (2008) Dynamics of *Legionella* spp. and Bacterial Populations during the Proliferation of *L. pneumophila* in a Cooling Tower Facility. Appl Environ Microbiol 74(10), 3030-3037.

Whiley, H. and Bentham, R. (2011) *Legionella longbeachae* and Legionellosis. Emerg Infect Dis 17(4), 579-583.

Whitby, P.W., Morton, D.J., VanWagoner, T.M., Seale, T.W., Cole, B.K., Mussa, H.J., McGhee, P.A., Bauer, C.Y.S., Springer, J.M. and Stull, T.L. (2012) *Haemophilus influenzae* OxyR: Characterization of Its Regulation, Regulon and Role in Fitness. PLoS ONE 7(11), e50588.

WHO (2007) *Legionella* and the prevention of legionellosis. Bartram, J., Chartier, Y., Lee, J., Pond, K. and Surman-Lee, S. (eds), World Health Organization.

WHO (2014) Water Safety in Distribution Systems, World Health Organization.

Wiater, L.A., Dunn, K., Maxfield, F.R. and Shuman, H.A. (1998) Early events in phagosome establishment are required for intracellular survival of *Legionella pneumophila*. Infect Immun 66(9), 4450-4460.

Wieland, H., Ullrich, S., Lang, F. and Neumeister, B. (2005) Intracellular multiplication of *Legionella pneumophila* depends on host cell amino acid transporter SLC1A5. Mol Microbiol 55(5), 1528-1537.

Wigneshweraraj, S., Bose, D., Burrows, P.C., Joly, N., Schumacher, J., Rappas, M., Pape, T., Zhang, X., Stockley, P., Severinov, K. and Buck, M. (2008) *Modus operandi* of the bacterial RNA polymerase containing the σ^{54} promoter-specificity factor. Mol Microbiol 68(3), 538-546.

Wilkinson, I.J., Sangster, N., Ratcliff, R.M., Mugg, P.A., Davos, D.E. and Lanser, J.A. (1990) Problems associated with identification of *Legionella* species from the environment and isolation of six possible new species. Appl Environ Microbiol 56(3), 796-802.

Winiecka-Krusnell, J. and Linder, E. (1999) Free-Living Amoeba Protecting *Legionella* in Water: The Tip of an Iceberg? Scand J Infect Dis 31, 383-385.

Winn Jr, W.C. (1988) Legionnaires disease: historical perspective. Clin Microbiol Rev 1(1), 60-81.

Wösten, M.M.S.M. and Groisman, E.A. (1999) Molecular Characterization of the PmrA Regulon. J Biol Chem 274(38), 27185-27190.

Wullings, B.A. and van der Kooij, D. (2006) Occurrence and Genetic Diversity of Uncultured *Legionella* spp. in Drinking Water Treated at Temperatures below 15°C. Appl Environ Microbiol 72(1), 157-166.

Xiao, H., Kalman, M., Ikehara, K., Zemel, S., Glaser, G. and Cashel, M. (1991) Residual guanosine 3',5'-bispyrophosphate synthetic activity of *relA* null mutants can be eliminated by *spoT* null mutations. J Biol Chem 266(9), 5980-5990.

Xu, L., Shen, X., Bryan, A., Banga, S., Swanson, M.S. and Luo, Z.-Q. (2010) Inhibition of Host Vacuolar H^+-ATPase Activity by a *Legionella pneumophila* Effector. PLoS Pathog 6(3), e1000822.

Yamamoto, H., Sugiura, M., Kusunoki, S., Ezaki, T., Ikedo, M. and Yabuuchi, E. (1992) Factors Stimulating Propagation of *Legionellae* in Cooling Tower Water. Appl Environ Microbiol 58(4), 1394-1397.

Yamamoto, Y., Klein, T.W., Newton, C.A., Widen, R. and Friedman, H. (1988) Growth of *Legionella pneumophila* in thioglycolate-elicited peritoneal macrophages from A/J mice. Infect Immun 56(2), 370-375.

Yang, G., Benson, R., Pelish, T., Brown, E., Winchell, J.M. and Fields, B. (2010) Dual detection of *Legionella pneumophila* and *Legionella* species by real-time PCR targeting the 23S-5S rRNA gene spacer region. Clin Microbiol Infect 16(3), 255-261.

Yaradou, D.F., Raze, D., Ginevra, C., Ader, F., Doléans-Jordheim, A., Vandenesch, F., Menozzi, F.D., Etienne, J. and Jarraud, S. (2007) Zinc-dependent cytoadherence of *Legionella pneumophila* to human alveolar epithelial cells in vitro. Microb Pathog 43(5–6), 234-242.

Yee, R.B. and Wadowsky, R.M. (1982) Multiplication of *Legionella pneumophila* in unsterilized tap water. Appl Environ Microbiol 43(6), 1330-1334.

Yesilkaya, H., Andisi, V.F., Andrew, P.W. and Bijlsma, J.J.E. (2013) *Streptococcus pneumoniae* and reactive oxygen species: an unusual approach to living with radicals. Trends Microbiol 21(4), 187-195.

Yiallouros, P.K., Papadouri, T., Karaoli, C., Papamichael, E., Zeniou, M., Pieridou-Bagatzouni, D., Papageorgiou, G.T., Pissarides, N., Harrison, T.G. and Hadjidemetriou, A. (2013) First Outbreak of Nosocomial *Legionella* Infection in Term Neonates Caused by a Cold Mist Ultrasonic Humidifier. Clin Infect Dis 57(1), 48-56.

Yildiz, F.H. and Schoolnik, G.K. (1998) Role of *rpoS* in stress survival and virulence of *Vibrio cholerae*. J Bacteriol 180(4), 773-784.

Yu, M.-j., Ren, J., Zeng, Y.-l., Zhou, S.-n. and Lu, Y.-j. (2009) The *Legionella pneumophila* Dps homolog is regulated by iron and involved in multiple stress tolerance. J Basic Microbiol 49(S1), S79-S86.

Yu, V.L., Plouffe, J.F., Pastoris, M.C., Stout, J.E., Schousboe, M., Widmer, A., Summersgill, J., File, T., Heath, C.M., Paterson, D.L. and Chereshsky, A. (2002) Distribution of *Legionella* Species and Serogroups Isolated by Culture in Patients with Sporadic Community-Acquired Legionellosis: An International Collaborative Survey. J Infect Dis 186(1), 127-128.

Yura, T., Nagai, H. and Mori, H. (1993) Regulation of the Heat-Shock Response in Bacteria. Annu Rev Microbiol 47, 321-350.

Zere, T.R., Vakulskas, C.A., Leng, Y., Pannuri, A., Potts, A.H., Dias, R., Tang, D., Kolaczkowski, B., Georgellis, D., Ahmer, B.M.M. and Romeo, T. (2015) Genomic Targets and Features of BarA-UvrY (-SirA) Signal Transduction Systems. PLoS ONE 10(12), e0145035.

Zheng, H., Chatfield, C.H., Liles, M.R. and Cianciotto, N.P. (2013) Secreted Pyomelanin of *Legionella pneumophila* Promotes Bacterial Iron Uptake and Growth under Iron-Limiting Conditions. Infect Immun 81(11), 4182-4191.

Zheng, M., Åslund, F. and Storz, G. (1998) Activation of the OxyR Transcription Factor by Reversible Disulfide Bond Formation. Science 279(5357), 1718-1722.

Zimmerman, L. (1979) Aerosol Infectivity of *Legionella pneumophila*. N Engl J Med 301(18), 1004-1004.

Zou, C.H., Knirel, Y.A., Helbig, J.H., Zahringer, U. and Mintz, C.S. (1999) Molecular Cloning and Characterization of a Locus Responsible for O Acetylation of the O Polysaccharide of Legionella pneumophila Serogroup 1 Lipopolysaccharide. J Bacteriol 181(13), 4137-4141.

Zuo, Y., Wang, Y. and Steitz, T.A. (2013) The Mechanism of *E. coli* RNA Polymerase Regulation by ppGpp Is Suggested by the Structure of Their Complex. Mol Cell 50(3), 430-436.

Zusman, T., Aloni, G., Halperin, E., Kotzer, H., Degtyar, E., Feldman, M. and Segal, G. (2007) The response regulator PmrA is a major regulator of the *icm/dot* type IV secretion system in *Legionella pneumophila* and *Coxiella burnetii*. Mol Microbiol 63(5), 1508-1523.

Zusman, T., Gal-Mor, O. and Segal, G. (2002) Characterization of a *Legionella pneumophila relA* Insertion Mutant and Roles of RelA and RpoS in Virulence Gene Expression. J Bacteriol 184(1), 67-75.

APPENDIX

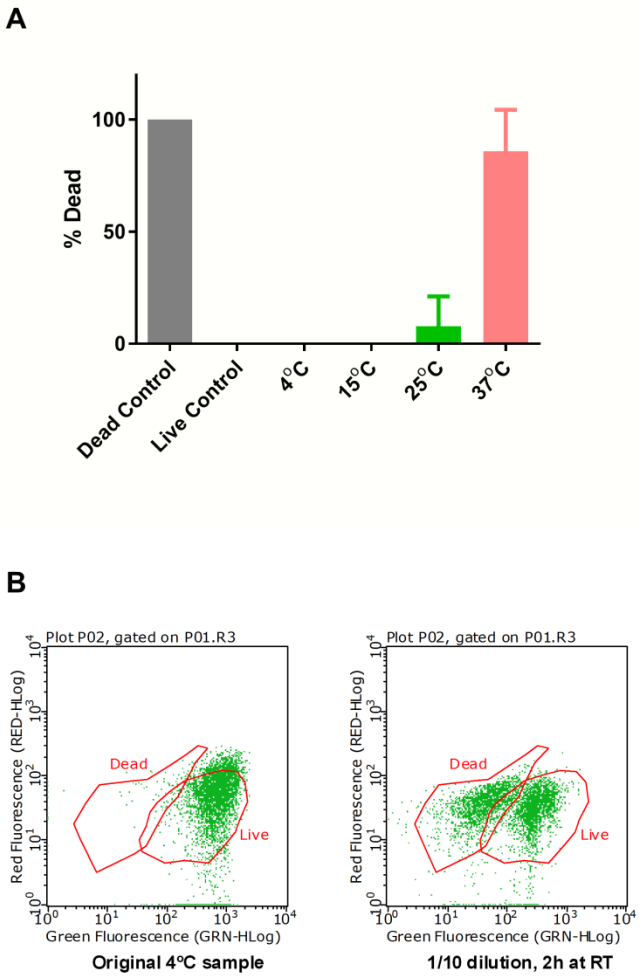

Figure S1: Live/Dead staining of *Lp* incubated in water. A) WT JR32 strain was incubated at the respective temperatures for 91 days. Cells incubated at each temperature were stained using 1.5µl/ml of propidium iodide and SYTO9 for 15 minutes. A GuavaCyte benchtop flow cytometer was used to analyse the fluorescence of 500 cells from each sample. The percentage of dead cells out of the total population is presented in the Y-axis. B) Dot plots of *Lp* incubated at 4^{o}C prior to resuscitation. Left hand panel; staining of undiluted *Lp* cells. Right hand panel; staining of *Lp* after a 1:10 dilution with fresh Fraquil.

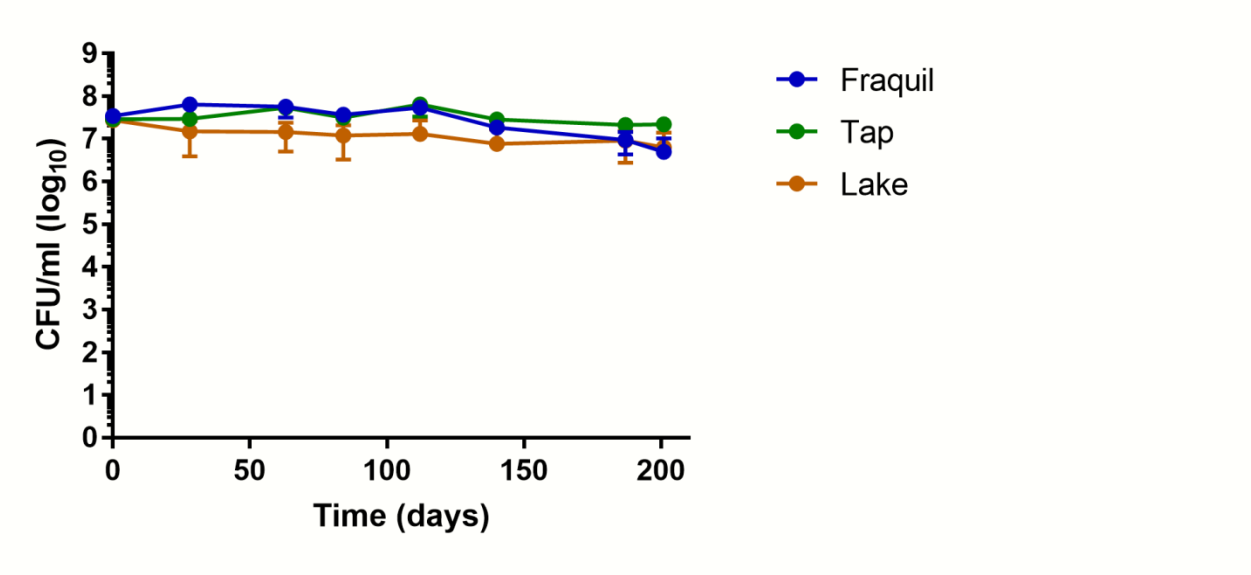

Figure S2: Survival of *Lp* in various water sources. The survival of the WT JR32 strain in Fraquil, filter sterilized tap water and filter sterilized lake water at 25^{o}C. CFU counts were monitored over a period of 201 days.

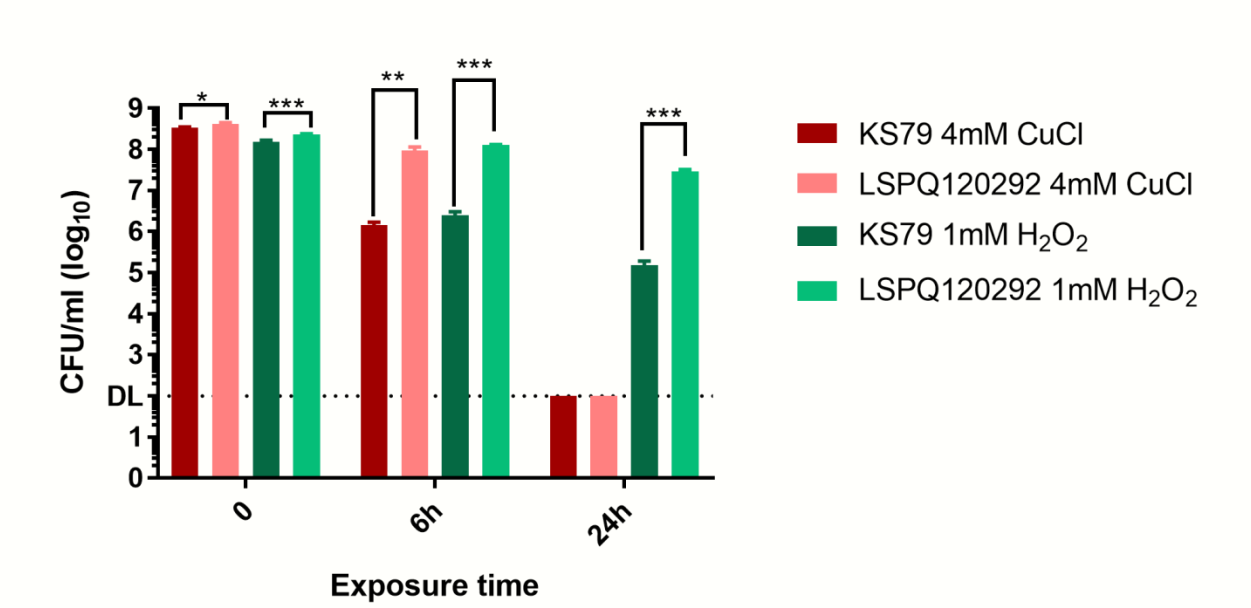

Figure S3: Stress resistance of a lab strain vs. an environmental isolate. Survival of the KS79 WT strain compared to a virulent *Lp* strain isolated from a cooling tower (LSPQ120292). The WT, Δ*letS*, ON and OFF were suspended in water for 2 hours and, then, treated with 4mM of CuCl or 1mM H_2O_2. CFU counts were enumerated 0, 6 or 24 hours post treatment. DL, detection limit. An unpaired Student *t* test was used to assess statistical significance. * $P < 0.05$; ** $P < 0.005$; *** $P < 0.0005$.

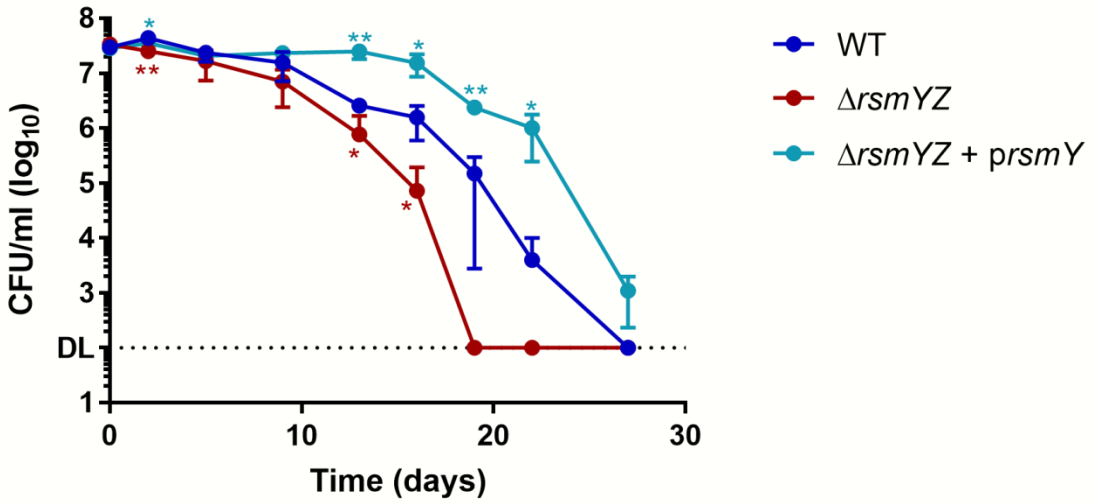

Figure S4: Survival of *rsmYZ* mutant in water. The survival of the WT, Δ*rsmYZ* and Δ*rsmYZ* + p*rsmY* in water at 42°C was monitored using CFU counts. DL, detection limit. Student *t* test was used to assess statistical significance *versus* the WT. * $P < 0.05$; ** $P < 0.005$; *** $P < 0.0005$.

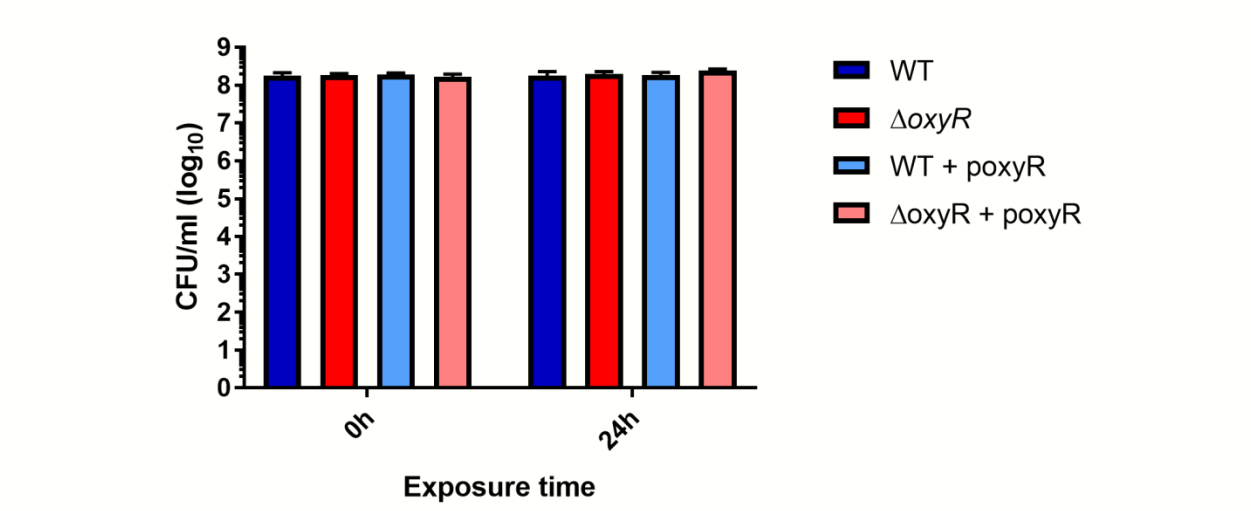

Figure S5: Resistance of the *oxyR* mutant to bleach. Survival of the wild-type (WT), *oxyR* mutant, complemented (Δ*oxyR* + p*oxyR*) and merodiploid (WT + p*oxyR*) strains in Fraquil with bleach. Strains were suspended in water for 2 hours and, then, treated with 1mg/ml of bleach. Bacterial counts were enumerated prior to treatment (0h) and 24 hours post-treatement. CFU counts for Δ*oxyR* were monitored using CYE supplemented with α-ketoglutarate. Data shown represent the mean and SD of three biological replicates.

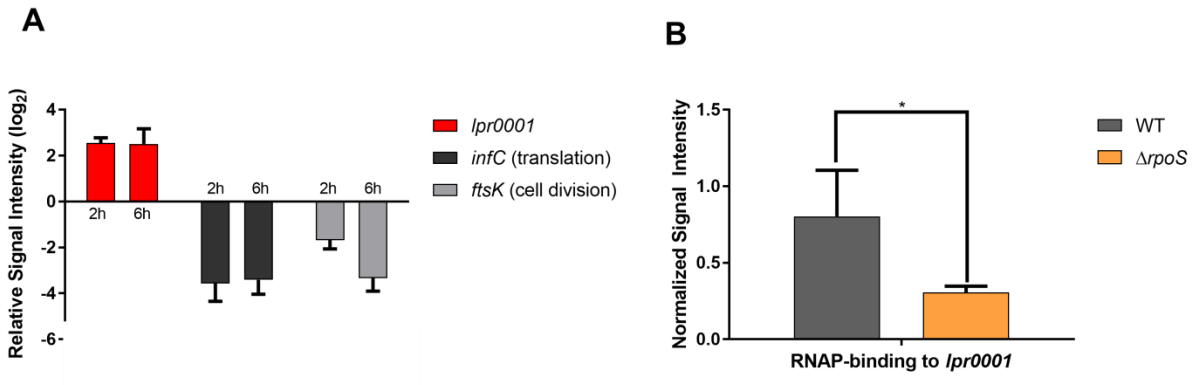

Figure S6: Expression of *lpr0001* in water and control by RpoS. A) The expression of *lpr0001* is significantly ($P < 0.05$) induced 2 and 6 hours after water exposure. In contrast, replication-associated genes such as *infC* or *ftsK* are down-regulated. Expression of genes is relative to an exponential phase culture. B) Frequency of RNAP-binding to the promoter region of *lpr0001* was assayed using Chromatin immunoprecipitaiton and microarray analysis (ChIP-chip). Student *t* test was used to assess statistical significance *versus* the WT. * $P < 0.05$

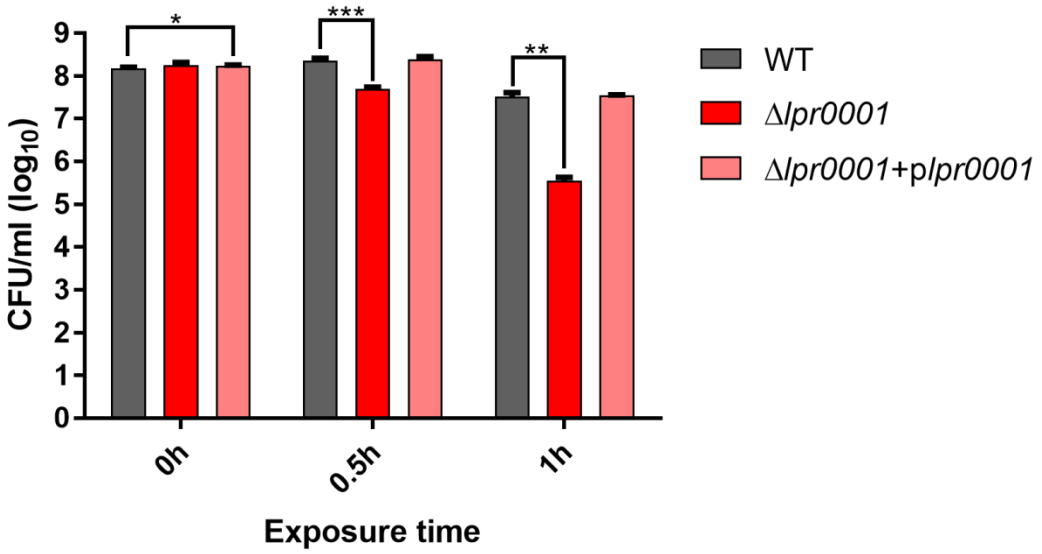

Figure S7: Δ*lpr0001* is impaired for surviving heat shock. The wild-type (WT), *lpr0001* mutant and the complement (Δ*lpr0001* + *plpr0001*) strains were exposed to Fraquil for 2 hours before exposure to a 55^{o}C. Bacterial counts were enumerated prior to treatment (0h) and 30 or 60 minutes post-treatment. Data shown represent the mean and SD of three biological replicates. Student *t* test was used to assess statistical significance *versus* the WT. * $P < 0.05$; ** $P < 0.005$; *** $P < 0.0005$.

Table S1: Expression of selected genes in the *oxyR* mutant *vs.* the WT

lpg number	Product	Gene	Untreated		10 mMH_2O_2	
			P	log 2 (Δ*oxyR*/WT)	*P*	log 2 (Δ*oxyR*/WT)
			Oxidative stress			
lpg2967	superoxide dismutase	sodB	0.490	-0.015	0.479	0.041
lpg2389	catalase-peroxidase	katB	0.438	0.047	0.127	-0.702
lpg0194	catalase-peroxidase KatG	katA	0.251	-0.463	0.235	-0.970
lpg2840	AhpC/TSA family protein	lpg2840	0.206	0.357	0.265	-0.419
lpg2965	alkylhydroperoxidereductase	ahpC2D	0.420	-0.230	0.389	-0.344
lpg2350	alkylhydroperoxidereductase	ahpC1	0.315	0.234	0.117	-1.206
lpg2349	alkylhydroperoxidase	lpg2349	0.461	0.060	0.011	-1.270
lpg2112	alkylhydroperoxidase	lpg2112	0.388	0.371	0.226	-0.997
			Efflux pumps			
lpg0638	major facilitator family transporter	phtD	0.424	0.190	0.005	**-3.073**
lpg1454	multidrug efflux protein		0.118	-1.177	0.003	**-2.598**
lpg0652	major facilitator family transporter		0.152	-0.748	0.000	**-1.835**
lpg0720	multidrug resistance protein		0.195	-0.676	0.001	**-1.768**
lpg2178	probable multidrug-efflux system transmembrane protein	mexF2	0.215	-0.682	0.001	**-1.709**
lpg2512	RND multidrug efflux transporter		0.296	-0.437	0.007	**-1.439**
			Respiratory chain			
lpg2787	NADH dehydrogenase I, C	nuoC	0.255	-0.593	0.007	**-2.352**

	subunit					
lpg0200	cytochrome D ubiquinol oxidase subunit II	qxtB	0.242	-0.569	0.004	**-1.257**
lpg2703	ubiquinol-cytochrome c reductase		0.274	-0.593	0.002	**-1.713**
lpg2704	ubiquinol-cytochrome c reductase	petB	0.283	-0.406	0.000	**-2.196**
lpg1054	ATP synthase F1, beta chain	atpD	0.111	-0.958	0.002	**-2.006**
lpg1048	ATP synthase F1, subunit alpha		0.088	-0.773	0.002	**-1.443**
		TCA cycle				
lpg0530	succinatedehydrogenaseflavoproteinsubunit A	sdhA	0.206	-0.524	0.007	**-1.695**
lpg0531	succinatedehydrogenaseiron-sulfurproteinsubunit B	sdhB	0.117	-1.134	0.001	**-2.108**
lpg1413	glycerol-3-phosphate dehydrogenase	glpD	0.353	-0.385	0.006	**-2.043**
lpg0816	isocitrate dehydrogenase, NADP-dependent	icd	0.244	-0.456	0.007	**-2.022**
lpg2971	malate dehydrogenase	maeA	0.209	-0.372	0.005	**-1.223**
		Metabolism of lipids				
lpg0360	3-hydroxymyristoyl/3-hydroxydecanoyl-(acyl carrier protein) dehydratases	fabA	0.480	-0.034	0.001	**-1.658**
lpg0361	3-oxoacyl-[acyl-carrier protein] synthase II (beta-	fabF	0.331	-0.237	0.001	**-2.216**

	ketoacyl-ACP-synthase)					
lpg0510	(3R)-hydroxymyristoyl-(acyl carrier protein) dehydratase	fabZ	0.210	-0.851	0.004	**-1.898**
lpg1393	3-oxoacyl-(acyl carrier protein) synthase II	fabH	0.448	-0.117	0.000	**-2.084**
lpg1395	3-oxoacyl-(acyl carrier protein) reductase	fabG	0.365	-0.286	0.003	**-1.534**
lpg1396	acyl carrier protein	acpP	0.494	-0.014	0.000	**-1.603**
lpg1397	beta-ketoacyl-acyl carrier protein synthase II	fabF1	0.316	-0.351	0.007	**-1.483**
			Stress response			
lpg0935	universal stress protein A	uspA	0.267	-0.605	0.003	**-2.069**
lpg0123	thiol:disulfide interchange	dsbA	0.123	-1.104	0.008	**-1.817**
lpg0386	heat shock protein	htpX	0.280	-0.552	0.006	**-1.595**
lpg2341	heat shock protein	DnaJ	0.343	0.329	0.000	**-1.339**
lpg0818	ATP binding protease	clpA	0.457	0.083	0.006	**-1.718**
			Miscellaneous			
lpg2836	glucosamine-fructose-6-phosphate aminotransferase	glmS	0.204	-0.787	0.004	**-2.075**
lpg0281	(cationic) amino acid transporter (permease)		0.227	-0.424	0.004	**-1.504**
lpg2905	ubiquinone biosynthesis	AarF	0.294	-0.329	0.006	**-1.725**