A Cyclic di-GMP Two-Component System Contributes to *Legionella pneumophila* Differentiation and Resilience

by

Elisa D. Hughes

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
(Microbiology and Immunology)
in the University of Michigan
2019

Doctoral Committee:

Professor Michele S. Swanson, Chair
Professor Matthew R. Chapman
Professor Harry L. T. Mobley
Professor Chuanwu Xi

"The most exciting phrase to hear in science, the one that heralds new discoveries, is not 'Eureka!' but 'That's funny...'"

- Isaac Asimov

Elisa D. Hughes

elisadh@umich.edu

ORCID iD: 0000-0003-4897-8094

© Elisa D. Hughes 2019

DEDICATION

To my sister Teresa Hughes, PhD. You were right.

ACKNOWLEDGMENTS

There are many people who contributed to making this work a reality. First and foremost, I must thank my mentor Michele Swanson. She took me into her lab and gave me free reign to design and develop this dissertation project from scratch and was always available for advice and encouragement. When days, weeks and sometimes months would pass without any success at the bench, she would be the first to remind me “that is why it is called re-search.” She helped me grow into the assured, independent scientist I am today, and for that I will be forever grateful.

I would also like to thank members of my lab, both past and present, who made coming into work every day a pleasure. These include Zack Abbott and Kaitlin Flynn, who I am glad to still keep in touch with. They were always available for advice on my project, or just to chat when things were going rough. Thank you as well to Sebastian Crepin, his expertise in molecular biology was invaluable in helping me get past several technical roadblocks, and he continued to be a great resource even after his departure from the lab. Finally, I have to give the biggest thanks and hugs to Brenda Byrne, who is one of the most gifted and industrious scientists I’ve ever had the pleasure of meeting. She was always happy to discuss my results and gave some incredible insights that helped move my project forward. Brenda is also simply one of the nicest humans on this planet. I count her not only as a co-worker, but as a good friend, and I look forward to future days visiting haunted houses with her.

Thank you to my committee members, Harry Mobley, Matt Chapman and Chuanwu Xi. They guided me to think critically about my data and models, and I always came away from our meetings invigorated to examine new ideas. I would also like to thank the Microbiology & Immunology office staff, including Bonnie Krey, Sheryl Smith, and especially Heidi Thompson, who has helped me maneuver through more administrative issues than I can recall.

Finally, I want to thank my friends and family who have shared this journey with me, including Courtney Luterbach, Matt Foley, Hayley Warsinske, Carla Larios, and Sam Lewis. I am especially grateful to Jay Lubow, who brought fun and humor to my life here. I don't know where I would be without the love and support of Andrea Briski—she's more than a friend, she is like family. I also have to thank the animals that have enriched my life here: my beloved cat Drusilla, a gentle soul who was my constant companion until her passing, my feisty cat Boodle, and of course Meeko, the "cutest puppy that ever was." And last, but certainly not least, thank you to my mom, dad, little brother Jeremy, and sister Terri, who has always been my biggest cheerleader and is my best friend in the world.

This work was supported by the Michigan Predoctoral Genetics Training Program (NIH T32-GM-007544; E.D.H.), a University of Michigan Rackham Graduate Student Research Grant (E.D.H.), and the Endowment for Basic Sciences at the University of Michigan Medical School (M.S.S.).

TABLE OF CONTENTS

DEDICATION ii

ACKNOWLEDGMENTS iii

LIST OF FIGURES vii

LIST OF TABLES viii

ABSTRACT ix

CHAPTER 1: Introduction 1

Legionella genetic diversity 1
Legionella intracellular life cycle 2
Biphasic developmental cycle 2
RelA and ppGpp 5
Alternative sigma factors 5
LetA/LetS two-component system – rsmY/Z – *CsrA* 6
MIF cell development in protozoa 7
Cyclic-di-GMP contribution to *Legionella* development 8
Background on cyclic-di-GMP signaling 8
Cyclic-di-GMP signaling in L. pneumophila 12
Extracellular *Legionella* 18
Biofilms 18
Viable But Not Culturable (VBNC) 19
Public health concerns 20
Legionnaires' Disease 20
Remediation challenges 21
Economic impact 23

CHAPTER 2: A Two-Component System that Modulates Cyclic-di-GMP Metabolism Promotes *Legionella pneumophila* Differentiation and Viability in Low-Nutrient Conditions 25

Abstract 25
Importance 26
Introduction 27
Results 32
Discussion 60
Materials and Methods 66

CHAPTER 3: Discussion and Future Directions 78

Introduction 78
MIF cell development 79
RpoS regulation 83
Planktonic survival and/or VBNC state 84
Final considerations... 86

APPENDIX: How *Legionella* Defend Their Turf..89

REFERENCES 93

LIST OF FIGURES

Figure 1.1. Life cycle of *L. pneumophila* ... 4

Figure 1.2. Cyclic-di-GMP metabolizing proteins in *L. pneumophila* ... 14

Figure 2.1. Conservation of the RR among *L. pneumophila* ... 31

Figure 2.2. The genes *lpg0279*, *lpg0278* and *lpg0277* constitute an operon ... 33

Figure 2.3. Construction of p*lpg0279-gfp* transcriptional reporter plasmid ... 35

Figure 2.4. Promoter activity for the *lpg0279-77* operon increases upon entry into PE phase ... 36

Figure 2.5. Expression of *lpg0279-77* is RpoS-dependent ... 38

Figure 2.6. Nutrient limitation induces expression of *lpg0279-77* ... 42

Figure 2.7. *L. pneumophila* lacking a complete TCS or ectopically expressing *lpg0279* resemble WT E phase cells ... 46

Figure 2.8. The TCS promotes PHB production and long-term viability ... 50

Figure 2.9. The DCG activity of RR Lpg0277 promotes transition to PE phase ... 53

Figure 2.10. The GGDEF domain and phosphoacceptor site of the RR contribute to PHB production and promote survival during prolonged nutrient deprivation ... 55

Figure 2.11. Constitutively expressed *lpg0279* is epistatic to RR^{E521A} PDE mutation 58

Figure 2.12. Disruption of the Lpg0277 RR^{E521A} PDE domain is not sufficient to suppress the differentiation defect of PE phase *L. pneumophila* constitutively expressing *lpg0279* ... 59

Figure 2.13. Model for *lpg0279-77* regulation of *L. pneumophila* differentiation ... 65

Figure 3.1 Candidate IHF-binding sites in the *lpg0279* promoter region ... 82

Figure A.1 High-density colonies of *Legionella* protect themselves by secreting pulses of HGA ... 92

LIST OF TABLES

Table 2.1. Strains used in this study 73

Table 2.2. Primers and plasmids used in this study 75

ABSTRACT

Legionella pneumophila is a waterborne pathogen and the causative agent of the severe pneumonia Legionnaires' Disease. This bacterium is an intracellular parasite of both human lung macrophages, as well as amoeba and ciliated protists, its natural host. Within the environment, *L. pneumophila* exists as either an intracellular parasite of protozoa, within biofilms, or as free-living, planktonic cells. Unfortunately, remediation of contaminated water systems has proven an ongoing challenge, mostly due to the ability of *L. pneumophila* to differentiate into an environmentally resistant, highly infectious but spore-like cell type called a Mature Infectious Form (MIF). The genetic pathways and regulatory mechanisms that promote MIF cell development remain largely unknown. A few proteins have been identified as abundant in MIF cells, however, including Lpg0279. In this dissertation, I explore the impact of Lpg0279 on *L. pneumophila* cellular differentiation and its interaction with a two-component system encoded by *lpg0278-lpg0277*. I show that *lpg0279* is co-transcribed with *lpg0278* and *lpg0277*, expression is controlled by the stationary phase sigma factor RpoS, and that transcription is enhanced in nutrient-limited conditions.

The two-component system encoded by *lpg0278-lpg0277* modulates the turnover of the second messenger molecule cyclic-di-GMP. This ubiquitous molecule directs lifestyle changes in multiple bacterial species. The *L. pneumophila* genome codes for multiple proteins that make and break cyclic-di-GMP, but few studies have been

conducted examining the impact of this second messenger molecule on the *L. pneumophila* life cycle. Lpg0277 is a bifunctional enzyme and the response regulator of the two-component system. Using isogenic mutants and a series of phenotypic assays, I show that the diguanylate cyclase activity of Lpg0277 (and therefore an increase in c-di-GMP) promotes transition of *L. pneumophila* to the stationary phase of growth, the generation of pyomelanin pigment and poly-3-hydroxybutyrate granules, and contributes to long-term survival in low-nutrient conditions. In addition, I provide evidence to support a model in which Lpg0279 initially acts as a negative regulator of this enzymatic activity. This research provides important insight into how c-di-GMP contributes both to *L. pneumophila* differentiation, and to persistence of this bacterium in low-nutrient environments. This information can inform future surveillance methods, detection of MIF cells, and remediation efforts to control spread of this pathogen in the built environment.

CHAPTER 1

Introduction

Legionella genetic diversity

Legionella, a gram-negative bacterium, is a ubiquitous inhabitant of both freshwater and engineered water systems. Although *L. pneumophila* is just one of over 50 species within the *Legionella* genus, it accounts for roughly 90% of human infections (1). Of the 16 serogroups within *L. pneumophila*, serogroup 1 is the most prevalent and accounts for most clinical cases of Legionnaires' Disease (2, 3). Serogroup 1 includes the Philadelphia-1 strain isolated from the initial outbreak in 1976 and its laboratory derivatives, including the thymidine auxotroph Lp02 that is used in this study (4).

In addition to the Philadelphia-1 strain, the genomes of four other strains of *L. pneumophila* serogroup 1 have been fully sequenced: Paris, Lens, Alcoy, and Corby (5). Although these strains are phylogenetically closely related, only 66.9% of coding regions (including genes that code for hypothetical proteins) comprise the "core" genome that is common in all strains (5). This diversity is in part due to the ability of *L. pneumophila* to acquire new DNA elements through horizontal gene transfer (3). The remarkable plasticity of the *L. pneumophila* genome suggests that conserved genes comprising the core genome are important to the *L. pneumophila* life cycle.

Legionella intracellular life cycle

A facultative intracellular pathogen, *L. pneumophila* has adapted to survive both intracellularly as well as in the extracellular milieu. In the environment, it has co-evolved to survive grazing by predatory amoeba and other protozoans, its natural hosts (6). When aerosolized water droplets carrying *L. pneumophila* are inhaled, the bacteria can be transported to the lungs where they infect and replicate within alveolar macrophages, using many of the same regulatory mechanisms that contribute to successful colonization of amoeba (7).

Biphasic developmental cycle

Adaption to the intracellular environment requires not only the ability to circumvent host cell defenses, but the capacity to rapidly respond to changes in pH, temperature, osmolarity, and nutrient availability. In the course of its intracellular life cycle, *L. pneumophila* responds to these fluctuating conditions by differentiating into morphologically distinct cell types (8). At the most fundamental level, intracellular *L. pneumophila* possesses a biphasic life cycle, shifting between a non-infectious replicative state and a virulent transmissive state (9) (Fig. 1.1A).

Following phagocytosis, *Legionella* avoids degradation in the endosomal pathway by blocking both vesicle acidification and fusion with the lysosome (10). Bacteria then recruit host cell proteins to the vesicle, transforming it into a protective replicative niche referred to as the *Legionella*-containing vesicle (LCV) (11, 12). This activity is dependent on the Dot/Icm (Defect in Organelle Trafficking; Intracellular Multiplication) Type IV secretion system (T4SS), which translocates over 300 proteins into the host cell (13). This T4SS is not only required for efficient remodeling of the

LCV, but is essential for both intracellular replication and later egress from spent host cells (14).

Sequestered within the LCV, *L. pneumophila* switches to a replicative form, which is neither infectious nor motile. In addition to fatty acids, *Legionella* utilizes amino acids as a carbon source during replication, which the bacteria acquire from the host cell using a variety of amino acid transporters (15-17). During replication, the post-transcriptional regulator protein CsrA inhibits expression of transmissive genes by binding to and destabilizing mRNA transcripts (18-21). In these conditions, *L. pneumophila* replicates to high numbers. Eventually, the nutrient supply becomes exhausted, and this triggers a complex regulatory cascade dubbed the stringent response, which suppresses replication and promotes expression of virulence traits including cytotoxicity, motility, and the ability to avoid phagolysosome degradation (9, 22)—traits designed to promote transmission to a new host cell or support survival in the extracellular space.

This replicative/transmissive dichotomy is mirrored in the switch between the exponential (E) phase and stationary or post-exponential (PE) growth phases in broth cultures (12) (Fig. 1.1B). Microarray experiments analyzing the life cycle *in vitro* and in amoebae confirm a different transcriptional profile associated with E and PE phase *L. pneumophila*, and that gene expression in each case is tightly regulated (23). In fact, examination of this biphasic switch *in vitro* has aided in identifying key regulatory mechanisms that drive this transition process within macrophages or a protozoan host (23).

Figure 1.1

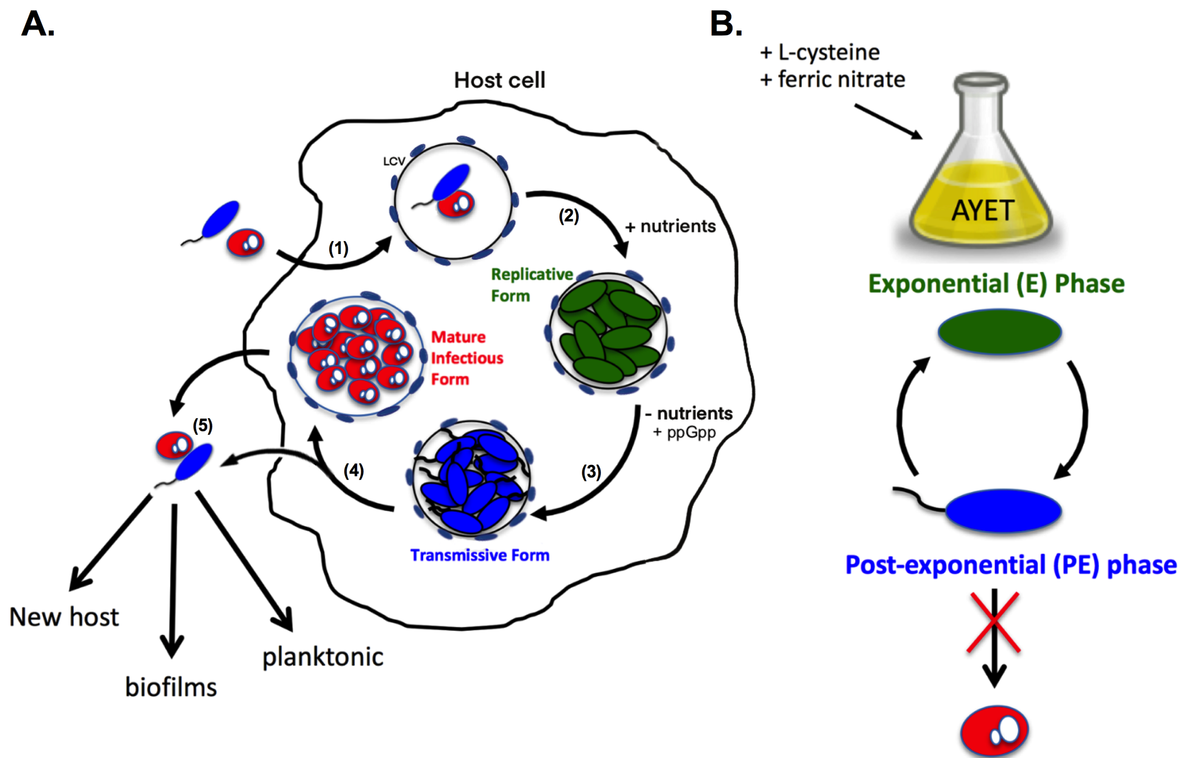


Fig. 1.1 Life cycle of *L. pneumophila*.

A. Schematic of the intracellular life cycle of *L. pneumophila*. (1) Following uptake of the virulent form of *L. pneumophila* (either the transmissive form (in blue) or MIF cell form (in red)) into an amoeba or macrophage host cell, the bacteria establish the protective *Legionella*-containing vesicle (LCV) surrounded by host proteins (grey dots). (2) When nutrients are plentiful and conditions support replication, *L. pneumophila* within the LCV transition to a replicative form (in green) and multiply to high numbers. (3) Depletion of host-cell nutrients triggers the stringent response and production of ppGpp, resulting in conversion back to the transmissive form of the bacteria which are motile, cytotoxic and infectious. In macrophages, the differentiation process typically terminates at this stage. (4) Within a protozoan host such as amoeba, *L. pneumophila* further differentiates into the resilient, cyst-like MIF cell type. (5) Following host-cell lysis, *L. pneumophila* can infect a new host if one is available, associate with a biofilm, or exist as free-living planktonic cells.

B. The biphasic switch between the intracellular replicative and transmissive form can be replicated *in vitro* using a rich medium (AYET) supplemented with excess iron and L-cysteine. These conditions do not support generation of MIF cells.

RelA and ppGpp

A hallmark of the stringent response is the generation of the alarmone guanosine penta- and tetraphosphate (collectively ppGpp) from molecules of ATP and GTP (24). In *L. pneumophila*, this second messenger molecule is primarily synthesized by the enzyme RelA, which is activated by the accumulation of uncharged tRNA at the ribosome resulting from amino acid shortage (25). The dual synthetase/hydrolase enzyme SpoT also contributes to cellular ppGpp concentrations in response to fatty acid perturbations, which is another developmental signal that drives *L. pneumophila* differentiation (SpoT's hydrolase activity also enables a necessary reduction in ppGpp levels when conditions for replication become favorable again (26)). Increased cellular concentrations of ppGpp results in a shift away from the replicative state by reducing DNA and protein synthesis, and triggering a regulatory cascade that promotes conversion to the transmissive state (27, 28).

Alternative sigma factors

Associated with the stringent response is an increase in the activity of multiple alternative sigma factors, including RpoS (σ^S/σ^{38}) (29). First identified in *E. coli* (30), the stationary phase sigma factor RpoS is widespread in proteobacteria (31). In *L. pneumophila*, *rpoS* is maximally transcribed in E phase, but protein levels of RpoS are highest in the stationary phase (32). By a mechanism that remains to be determined (12), accumulation of ppGpp results in RpoS-mediated expression of multiple virulence genes (27, 33). Transmission related phenotypes under the control of RpoS include full flagella production (Δ*rpoS* mutants are roughly half as motile as WT), sodium sensitivity, evasion of lysosomal degradation upon entry into a new phagocytic host, and

intracellular replication in amoeba (27, 34). The RpoS regulon is significant; in fact, microarray data suggest that RpoS either directly or indirectly affects the transcription of up to ¼ of the genes in the *L. pneumophila* genome (in both a positive and negative manner) (33). This includes expression of the flagellar sigma factor FliA (σ^{28}) (33).

The FliA sigma factor also contributes to the transition from the replicative to transmissive stage in *L. pneumophila* (29). Target genes of FliA are mainly components of the flagellar regulon, including *flaA* which encodes the flagellin subunit (35, 36). Despite its principal role in regulating flagella production, FliA also controls expression of other virulence factors, including cytotoxicity and infectivity (29, 32). For example, Δ*fliA* mutants are more deficient for intracellular replication in the amoeba *Dictyostelium discoideum* than a Δ*flaA* mutant, even though both are non-flagellated and non-motile (37). FliA is required for *L. pneumophila* to efficiently avoid lysosomal degradation, an activity independent of motility (38).

LetA/LetS two-component system – rsmY/Z – *CsrA*

Also coinciding with an increase in ppGpp is expression of a two-component system comprised of LetA and LetS (*Legionella* transmission activator and sensor) or simply LetA/S (29). Two-component systems are a common mechanism used by bacteria to rapidly sense and respond to fluctuating environments. The paradigm of bacterial two-component systems include a histidine kinase (HK) and a cognate response regulator (RR) (39). Initiation of signal transduction involves HK autophosphorylation on a conserved histidine residue, then transfer of this phosphate to a conserved aspartate in the receiver (REC) domain of the RR (39). Although the majority of HKs are integral membrane proteins that monitor changes in the

extracellular space, there are soluble HKs that sense fluctuations in cytoplasmic conditions (40). In the *L. pneumophila* LetA/S two-component system, LetS is the membrane-bound HK and LetA functions as the cytoplasmic RR. This system is homologous to the well-studied BvgA/S system in the *Bordetella* species, which deviates slightly from the orthodox two-component system in that an additional phosphorelay occurs within LetS prior to transfer to LetA (41). Similar two-component systems have been identified in other gram-negative bacterial species such as *Escherichia coli* (UvrY/BarA), *Salmonella enterica* (BarA/SirA), *Pseudomonas aeruginosa* (GacA/GacS), and *Vibrio cholerae* (VarA/VarS) (20), suggesting that such two-component systems are widespread regulators of bacterial life cycles.

Although the precise signal that triggers the LetA/S phosphorelay is unknown, the targets of activated LetA are better understood. Most importantly, LetA induces the expression of two small non-coding RNAs, RsmY and RsmZ (20). These RNAs possess a repeated GGA motif that is bound by the post-transcriptional repressor protein CsrA (42, 43). Thus, RsmY/Z alleviate CsrA repression by sequestering it from its mRNA targets (20, 44). As CsrA represses every transmissive phenotype in *L. pneumophila* during replication (19) and has been shown to inhibit the expression of stationary-phase factors such as FliA, RpoS and even RelA (21), alleviation of CsrA repression is a critical prerequisite to *L. pneumophila* differentiation from the replicative to transmissive phase.

MIF cell development in protozoa

The mechanisms that govern the *L. pneumophila* intracellular life cycle in macrophages and its natural protozoan hosts are very similar and involve a biphasic

switch between a replicative and transmissive state (22, 45). However, there are some distinctions, as the cells that emerge from amoeba and ciliated protists such as *Tetrahymena* are more infectious and environmentally resistant than the progeny from macrophages (46). This is because within a protozoan host, *L. pneumophila* can further differentiate from the transmissive form into a resilient, metabolically dormant cell type known as a MIF (Mature Infectious Form) (46, 47) (Fig. 1.1).

In addition to a distinct protein profile (46), electron microscopy work shows that MIF cells sport a tough outer cell membrane and large poly-3-hydroxybutyrate (PHB) storage granules, which are thought to equip *L. pneumophila* to persist for extended periods outside a host cell in low-nutrient aqueous environments (47, 48). MIF cells may represent the predominant form of *L. pneumophila* in the environment and thus the form that survives aerosolization to infect humans (47), although this remains to be verified given the absence of a genetic marker than facilitates detection of this cell type. It is unknown why MIF cells do not readily develop in macrophages. One hypothesis is that a signal required to promote MIF-cell differentiation is lacking in macrophages (47). Another possibility, and the more promising explanation, is that *L. pneumophila* cells cannot complete the differentiation process prior to macrophage lysis (47). However, the failure of *L. pneumophila* to form MIF cells within macrophages may explain the lack of human-to-human transmission of this aquatic pathogen.

Cyclic-di-GMP contribution to *Legionella* development

Background on cyclic-di-GMP signaling

While the contribution of the alarmone ppGpp to the *L. pneumophila* life cycle has been extensively studied, other signaling molecules likely play an equally important

role in *L. pneumophila* cellular processes. Most notably is the second messenger molecule bis-(3'-5')-cyclic dimeric guanosine (cyclic-di-GMP or c-di-GMP). This cyclic dinucleotide is synthesized by diguanylate cyclase (DCG) enzymes from two molecules of GTP (49). DCG enzymes possess a conserved GG(D/E)EF amino acid motif, which is essential for its catalytic activity (50). The hydrolysis of c-di-GMP is carried out by phosphodiesterase (PDE) enzymes, which have a conserved EAL or HD-GYP domain (51, 52). While dimerization of DCG proteins is essential for its catalytic activity, PDEs can function as monomeric proteins (53, 54).

Despite their antagonistic activities, compound GGDEF and EAL proteins are relatively common (55). Often one of these domains is simply catalytically inactive (51). However, some of these proteins are bifunctional and possess both DCG and PDE activities. Similar to the manner in which SpoT alternates between being a ppGpp synthetase and hydrolase, the dominant activity of these hybrid proteins depends largely on the environmental or cellular conditions at the time (26, 55-57). Many c-di-GMP modulating proteins contain N-terminal sensory domains, and the presence of an activating signal can result in enhanced or repressed enzymatic activity. Such signals include (but are not limited to) oxidative stress, nutrient deprivation, UV light, quorum sensing, nitric oxide, and antibiotic exposure (54, 58-60). Likewise, GGDEF and/or EAL-domain containing proteins can comprise the output domain of two-component regulatory systems (39). A model example of this is PleD from *Caulobacter crescentus*, which controls asymmetrical cell division and contains a REC-REC-GGDEF domain arrangement (61).

Without a doubt, c-di-GMP plays a major role in bacterial signal transduction systems. During the 30 years of research on c-di-GMP since its initial discovery (62), c-

di-GMP has been found to contribute to multiple cellular processes including cell-cycle progression and virulence gene expression (59). The predominant role of c-di-GMP in many species, however, is regulation of the switch between motility and sessility (63). The canonical view is that low c-di-GMP levels promote the planktonic state, whereas high levels promote biofilm formation (64). However, this is not always the case; for instance, high c-di-GMP levels inhibit biofilm formation in the important human pathogen *Staphylococcus aureus* (65).

One ongoing challenge in c-di-GMP research is the identification of downstream effectors. This endeavor is complicated by the incredible diversity of proteins that bind c-di-GMP, and (with a few exceptions) the absence of a reliable consensus binding sequence. As several comprehensive reviews of c-di-GMP signaling have been published (54, 59, 66, 67), just a few examples illustrating the prominent types of c-di-GMP receptors is presented here. The most well-known class of c-di-GMP receptors are proteins containing a PilZ domain (55). This domain was first identified in *Pseudomonas aeruginosa* as part of an operon involved in pili formation (68). In *Escherichia coli*, c-di-GMP binding to the PilZ domain of BcsA activates its cellulose biosynthesis activity, which contributes to *E. coli* biofilm formation (69). Although PilZ proteins are widespread in bacteria, many are degenerate and do not actively bind c-di-GMP (59). Transcription factors are another class of protein that bind c-di-GMP. Two that were initially identified in *P. aeruginosa* in connection with biofilm formation are FleQ and PelD (55). Binding of c-di-GMP to these proteins shifts gene expression, culminating in reduced flagella synthesis and increased exopolysaccharide production (70-72).

Another way c-di-GMP controls gene expression is through binding riboswitches in the 5' UTR of nascent mRNAs (73). RNA sequences that serve as aptamers for c-di-GMP possess a conserved GEMM (Genes for the Environment, Membranes and Motility) motif (74). Two types of GEMM motifs (based on stem loop architecture) have been identified, and both of them bind c-di-GMP with very high affinity (75). These riboswitches have been identified in several pathogenic bacteria, including *Clostridium difficile* (76) and *Vibrio cholerae* (77). Binding of the c-di-GMP ligand alters the tertiary structure of the riboswitch, resulting in an "off" or "on" confirmation that promotes or blocks expression of downstream genes (78).

A final binding partner of c-di-GMP that has been identified includes the DCG proteins themselves. Most (but not all) DCG enzymes possess an allosteric inhibitory site (I-site) with a conserved RXXD motif (50). In *Caulobacter crescentus*, the DCG activity of PleD is negatively regulated by I-site binding, which prevents the dimerization that is required for DCG catalytic activity (61). This type of product inhibition acts as a negative feedback that limits cellular c-di-GMP concentrations.

Cyclic-di-GMP signaling is nearly ubiquitous throughout the bacterial kingdom. A comparative analysis in 2018 of 813 bacterial genomes identified at least one GGDEF or EAL protein-encoding gene in 618 of them, representing over 75% of the genomes surveyed (79). Furthermore, species that utilize c-di-GMP generally produce multiple DCG and PDE enzymes. For example, the genome of *E. coli* codes for 29 different GGDEF and/or EAL-containing proteins (80), *P. aeruginosa* has 37 (14 of which are composite GGDEF-EAL proteins) (81), and the *Vibrio cholerea* genome codes for over 50 c-di-GMP metabolizing proteins (59). This raises the obvious challenge of determining specificity in the c-di-GMP signaling network, particularly given the broad

range of cellular processes controlled by this molecule. Arguably proteins involved in c-di-GMP turnover do not contribute to a single diffusible pool of c-di-GMP, but instead are temporally or spatially sequestered. The challenges of experimentally quantifying c-di-GMP supports this, as the physiological concentration of c-di-GMP in whole cells is relatively low (59). One way specificity can be achieved is by limiting expression of GGDEF/EAL proteins to certain stages of the life cycle. For example, in *E. coli* expression of multiple PDE and DCG enzymes that promote biofilm formation are regulated by the stringent response sigma factor RpoS, thus consigning their activity to the stationary phase (80, 82). Asymmetrical cell division in *C. crescentus* is a model of physical sequestration, in which the DCG PleD and the PDE TipF are positioned at opposite ends of the dividing cell; this assembly enables different local c-di-GMP concentrations and the generation of motile swarmer cell and stationary stalker cell, respectively (59, 83).

Cyclic-di-GMP signaling in Legionella

Despite the presence of 22 genes in the *L. pneumophila* (Philadelphia-1) genome that code for proteins involved in c-di-GMP metabolism (84), only a few studies have examined the contribution of this second messenger molecule to the *L. pneumophila* life cycle. In 2011, Levi *et al.* (44) published a survey of all 22 genes in the JR32 strain (a derivative of Philadelphia-1) (Fig. 1.2). Eleven of the identified genes coded for composite proteins with both GGDEF and EAL domains, although many were degenerate suggesting loss of catalytic activity. Almost all of the GGDEF-containing proteins possessed the canonical I-site (RXXD motif), indicating the importance of allosteric inhibition as a means of controlling c-di-GMP levels. One gene, *lpg1401*, was

found to encode a PilZ domain, one of the most prevalent and well-studied c-di-GMP-binding domains (55); however, based on the results of a sequestration assay, the authors concluded that this protein does not actually bind c-di-GMP.

Figure 1.2

Paris	Lens	Locus tag	Active domain	I-site	Domain organization	DGC activity
lpp0029	*lpl0030*	*lpg0029*	GGDEF EAL	RQTD		No
lpp0087	*lpl0075*	*lpg0073*	QSDEF ESS	none		No
lpp0219	*lpl0219*	*lpg0155*	GGDEF	RKTD		Yes
lpp0220	*lpl0220*	*lpg0156*	ECL	none		No
lpp0299	*lpl0283*	*lpg0230*	GGEEF	RLGD		Yes
lpp0352-51	*lpl0329*	*lpg0277*	GGDEF EAL	RKED		Yes
		lpg0373	GGDEY	RSSD		Yes
lpp0809	*lpl0780*	*lpg0744*	GGEEF	RTVD		Yes
lpp0891	*lpl0860*	*lpg0829*	RINGT EVF	none		No
lpp0942	*lpl0912*	*lpg0879*	GGDEF	RSND		Yes
lpp0952	*lpl0922*	*lpg0891*	GGDEF EAL	EDFD		Yes
lpp2355		*lpg1025*	GGDEF	RKDD		Yes
lpp2324	*lpl1054*	*lpg1057*	GGDEF EVL	REED		Yes
lpp1114	*lpl1118*	*lpg1114*	GLGEF EAL	none		No
lpp1170	*lpl1176*	*lpg1168*	GGDEF EIL	RKGD		Yes
lpp1311	*lpl1308*	*lpg1357*	GGDEF EAL	RHSD		Yes
lpl1352	*lpp1356*	*lpg1401*	PilZ	none		No
lpp1425	*lpl1559*	*lpg1469*	EVL	none		No
lpp1475	*lpl1508*	*lpg1518*	SGDQF EAL	none		No
lpp2071	*lpl2061*	*lpg2132*	GGDEF	RSTD		Yes
lpp2528	*lpl2381*	*lpg2642*	GGDEF EAL	RAND		No
lpp2708	*lpl2581*	*lpg2655*	GGDEF	RKND		Yes

Fig. 1.2. Cyclic-di-GMP metabolizing proteins in *L. pneumophila*.

Presented is a list of the genes in the *L. pneumophila* Philadelphia-1 genome that code for GGDEF/EAL proteins, along with schematics on domain organization, the presence of an allosteric I-site and the corresponding RXXD sequence (conserved R and D amino acids are in bold), and whether the proteins likely possess DCG activity based on the experimental details outlined in Levi *et al.* (84). Also listed are the corresponding gene designations in the Paris and Lens strains of *L. pneumophila*. Figure is modified from (84).

To investigate the contribution of c-di-GMP metabolizing proteins to *L. pneumophila* intracellular replication, Levi *et al*. utilized a genetic approach and constructed deletion and overexpression mutants for each of the 22 genes. Interestingly, the overexpression of two genes that code for proteins with opposing enzymatic functions, *lpg0891* (DCG) and *lpg1114* (PDE), resulted in similar phenotypes (84). Namely, both overexpressing strains were defective at replicating in either *Acanthamoeba castellanii* or the THP-1 macrophage-like cell line, and both were significantly less cytotoxic than WT bacteria (84). Loss of the homologs of these genes in the *L. pneumophila* Lens strain (*lpp0922* and *lpp1118*, respectively) impaired intracellular growth due to an inability to escape phagolysosomal degradation (85). That conflicting enzymatic functions can yield the same outcome supports the view that c-di-GMP signaling pathways are likely segregated within an individual cell.

Using reverse-phase HPLC, Levi *et al*. also quantified total intracellular levels of c-di-GMP in *L. pneumophila* strains either lacking or overexpressing one of the 22 c-di-GMP protein-encoding genes (84). In all cases, the amounts of c-di-GMP detected were low, even for the WT control (in the low pmol range), which is unsurprising given the typically low levels of c-di-GMP generated *in vivo* (86). However, this method did provide some insight into the predominant activity of composite GGDEF-EAL proteins. For example, the Δ*lpg0029* mutant generated more c-di-GMP than the WT control, whereas in the Δ*lpg0277* strain c-di-GMP was undetectable (84). These results imply that Lpg0029 is predominantly a PDE, whereas Lpg0277 (which is the subject of Chapter Two) is principally a DCG. This conclusion is supported by a series of phenotypic assays examining classical DCG-related phenotypes such as Congo Red

binding (conducted by ectopically expressing these genes in a strain of *Salmonella enterica* lacking endogenous DCG activity) (84).

In 2011, a series of *in vitro* biochemical experiments was conducted examining the homolog of *lpg0277* in the *L. pneumophila* Lens strain, *lpl0330* (56). Not only does this gene encode a protein with both GGDEF and EAL domains, it is chromosomally adjacent to a gene that encodes a histidine kinase (*lpl0330*). Thus, the focus of the study was to determine if Lpl0330 is truly a bifunctional enzyme having both DCG and PDE functions and whether it serves as the response regulator in a two-component system with Lpl0330. Using purified proteins, the authors demonstrated that Lpl0330 autophosphorylates and is able to transfer this phosphate to a conserved residue (Asp-87) on Lpl0329, although only temporarily as no phosphorylated Lpl0329 (P~Lpl0329) could be detected after 2 minutes of co-incubation (56). In addition, incubation of purified Lpl0329 with radiolabeled GTP demonstrated that the enzyme possesses both DCG and PDE activity (56). However, establishing whether phosphorylation of Lpl0329 altered its enzymatic activity proved a challenge due to the transient existence of P~Lpl0329. To circumvent this problem, Lpl0329 was incubated with acetyl phosphate, which can more stably phosphorylate proteins *in vitro* (81). Under these conditions, less GTP was consumed, leading the authors to conclude that phosphorylation of Lpl0329 *in vitro* reduces DCG function (56). Whether a similar outcome occurs *in vivo*, however, was not determined nor addressed.

As with most bacterial species that utilize c-di-GMP, this second messenger molecule appears to contribute to the switch from a motile to sessile lifestyle in *L. pneumophila*. In the Lens strain, five different GGDEF/EAL proteins were found to contribute to biofilm formation and/or dispersal (60), including *lpl0329* (the *lpg0277*

homolog). Specifically, three genes were determined to be essential for biofilm formation (*lpl1054*, *lpl0075*, and *lpl1559*), while two genes (*lpl10329* and *lpl1176*) were required for biofilm dispersal (60).

The contribution of *lpl1054* (*lpg1057)* to *L. pneumophila* biofilm formation was previously established in 2010 by Carlson *et al.* (87). The authors demonstrated that Lpg1057 is an active DCG (87). Directly upstream of this gene is *lpg1056*, which codes for a protein with an H-NOX (Haem Nitric oxide/OXygen) domain. H-NOX proteins are widespread in prokaryotes, stably bind diatomic gases including O_2 and NO, and are often co-expressed with DCG/PDE enzymes (88). As both the Δ*lpg1056* (Δ*hnox1*) mutant and the *lpg1057* overexpression strain yielded a hyper-biofilm phenotype, the authors concluded that Hnox1 functions as a NO-sensitive regulatory switch suppressing Lpg1057 DCG activity, lowering c-di-GMP levels, and reducing biofilms (87). Although this model is in accord with the paradigm that high c-di-GMP levels stimulate biofilm formation, of the three proteins identified by Pecastaings *et al.* (59) as promoting biofilm formation, only Lpg1057 has an intact GGDEF domain and displays DCG activity (84, 87). Of the other two, Lpl0075 possesses a degenerate GGDEF domain (QSDEF), Lpl1559 has no GGDEF domain at all, and neither appear to be functional DCG enzymes (60, 84) (Fig. 1.2). Further complicating matters is that the two proteins identified as important for biofilm dispersal, Lpl0329 (the Lpg0277 homolog) and Lpl1176, appear to be predominantly DCG enzymes (60, 84). In fact, the Δ*lpl0329* mutant has significantly lower c-di-GMP levels compared to WT, yet confocal microscopy revealed biofilms twice as thick (60). Thus, biofilm regulation in *L. pneumophila* might be disconnected from c-di-GMP signaling, or *L. pneumophila* may be a rare case in which biofilm formation requires a reduction in c-di-GMP levels.

Extracellular *Legionella*

Biofilms

Association with biofilms is a key strategy for long-term survival of *L. pneumophila* in the built environment. Biofilms are defined as microbial aggregates adhered to a biotic or abiotic surface and enclosed in a self-generated extracellular matrix (89). Biofilms are noted for being highly resistant to chemical treatment, antibiotics, and other biocides (90). Association with biofilms may also shield *L. pneumophila* from chlorine and monochloramine, disinfectants commonly used to decontaminate potable water systems (91).

Unlike some other biofilm-forming pathogens such as *Vibrio cholerae* and *Pseudomonas aeruginosa*, *Legionella spp.* do not appear to form monospecies biofilms in the natural environment (89). Rather, it is likely that planktonic *L. pneumophila* colonize pre-existing biofilms formed by other aquatic bacteria. Biofilms established by species such as *Klebsiella pneumoniae* and *P. fluorescens* appear to be more permissive to *L. pneumophila* colonization, while others, such as those formed by *P. aeruginosa*, are not (92). Thus, the composition of multispecies biofilm communities in the environment may be a predictor of *L. pneumophila* occurrence.

The presence of predatory amoeba, which graze on biofilms (93), is generally considered a prerequisite to growth of biofilm-associated *L. pneumophila* (94). In fact, there is no concrete evidence that *Legionella spp*. reproduce in oligotrophic conditions, whether as planktonic cells or within biofilm communities, outside a host cell (95). Thus, the presence of amoeba can lead to an enrichment of *L. pneumophila* in contaminated biofilms (96). Engineered water systems, including potable water mains, are frequently coated with biofilms (91, 97). Given their treatment-resistant nature,

biofilms therefore present a significant environmental reservoir for *L. pneumophila* and an ongoing source of legionellosis outbreaks.

Viable But Not Culturable (VBNC)

One factor confounding environmental surveillance of *L. pneumophila* populations is the ability of starved cells to enter into a reversible “viable but not culturable” (VBNC) state. Since it was first characterized in *V. cholerae* and *E. coli* in 1982 (98), several bacterial species have shown to enter the VBNC state during periods of cell stress. The defining characteristic of VBNC cells is the lack of culturability on routine growth media. As the name implies, VBNC cells are still alive, but have reduced metabolic activity, a unique gene expression profile, and are more environmentally resistant compared to their culturable counterparts (reviewed by (99)).

Conditions that promote VBNC development in *L. pneumophila* include extreme heat, oxidative stress, and nutrient starvation. Thermal disinfection of water distribution systems, which involves superheating the water to 70°C and flushing the system for 30 minutes, is one method of *Legionella* control recommended by the US Environmental Protection Agency (100). While these conditions appear to partially reduce *L. pneumophila* loads, they are also conducive to generating VBNC cells (101, 102). Disinfecting water distribution systems with oxidative chemicals such as monochloramine is another common practice (103). A shift to monochloramine treatment of the San Francisco, CA municipal water supply significantly reduced *L. pneumophila* colonization of hot water systems (from 60% to 4% of those tested), as determined by CFU counts (103). However, as monochloramine exposure has also been linked to VBNC generation in *L. pneumophila* (104), this reduction may not be entirely

due to cell death. Mere long-term exposure to nutrient-limited conditions is sufficient to drive cells into a VBNC state. For example, Schrammel *et al*. subjected four strains of *L. pneumophila* serogroup 1 to starvation in ultrapure water and found that all of them had completely lost culturability within 14 days (105).

In general, transitioning to a VBNC state may be a common mechanism used by *L. pneumophila* to persevere in both natural and engineered water systems. Given that the most common method used to quantify *L. pneumophila* in the environment is CFU enumeration, lack of culturability can lead to faulty conclusions about water contamination levels. This is concerning because VBNC cells were recently shown to remain infectious to both amoebae and human macrophages (albeit at a much lower efficiency than fresh *in vitro* stationary phase cells) (106).

Public health concerns

Legionnaires' Disease

In July of 1976, members of the American Legion attended the 58th annual convention at the Bellevue-Stratford Hotel Philadelphia, Pennsylvania. The occasion, marking the 200-year anniversary of the signing of the Declaration of Independence, drew a gathering into the thousands. At the end of the three-day meeting, several of the attendees began to experience flu-like symptoms, including high fever and severe respiratory distress. Of the 182 Legionnaires who fell ill, 29 succumbed to their symptoms and died (107). The cause of the outbreak was eventually traced to a gram-negative, water-borne bacterium that had contaminated the hotel's water supply and was most likely circulated through the air-conditioning system (3). This bacterium now bears the moniker of this group of men, *Legionella pneumophila*, and the resultant

pneumonia is known as Legionnaires' Disease. A self-limiting syndrome, termed Pontiac Fever, is also attributable to *Legionella*. The term legionellosis encompasses both illnesses.

Remediation challenges

Since that fateful time in 1976, there continue to be periodic outbreaks of *L. pneumophila*, such as the one at a Veterans' Home in Quincy, Illinois in 2015, which resulted in the deaths of 12 residents (108). Despite the millions spent in remediation efforts by the State of Illinois (almost $10M as of 2018), subsequent outbreaks at the facility occurred in 2016, 2017, and 2018, involving 17 people and resulting in one additional death. The probable source of contamination in this case was a faulty water heater (108). Improperly maintained engineered systems such as cooling towers and heating, ventilation and air conditioning (HVAC) units are a principal source of *L. pneumophila* exposure. In fact, any contaminated water supplies that generate aerosols are a potential source of infection, such as hot tubs or decorative fountains (109). Residential water distribution systems are not immune from contamination, as evidenced in Flint, Michigan where *L. pneumophila* was isolated from showers and kitchen faucets (110). The risk of contamination has prompted several state and local governments to require periodic sampling of public water systems to reduce the risk of contamination. The state of New York now mandates testing cooling towers every 90 days of use, after a 2015 outbreak traced to a contaminated water tower in the Bronx hospitalized 128 persons and left 16 dead (111). The prevalence of cooling towers as a source of *Legionella* (109) inspired the city of Garland, Texas, to be the first in the nation to require periodic testing of multifamily housing water coolers (112).

Yet despite increased monitoring and prevention efforts, both outbreaks and isolated incidences of *L. pneumophila* infection continue to occur. Due to the difficulty in distinguishing Legionnaires' Disease from other forms of pneumonia, the disease is likely underreported; nonetheless, the Centers for Disease Control note a 249% increase in the number of reported cases from 2001 to 2011 (113). However, humans are a dead-end host, and there has only been one recorded case of human-to-human transmission (114). Thus, the growth in disease incidence can be attributed to a rise in *L. pneumophila* colonization of the built environment, which itself is likely due to the lack of effective prevention and remediation methods, and/or emergence and spread of more resilient strains. Techniques currently used range from heat treatment (raising the water temperature above 80°C), filtration, chloride disinfection, and eradiation with UV light (2). However, none of these methods are 100% successful, and once a water system has been compromised with *Legionella*, full eradication is nearly impossible (2). One study examining the occurrence of travel-associated legionellosis concluded that previous incidences of disease in hotels is predictive of future recurrences (115). Indeed, a hotel in the U.S. Virgin Islands that was the source of several illnesses was suspected of being colonized by *Legionella* for more than two decades (116). Eradication of the bacterium from hospital water systems appears especially challenging. A study that examined 106 water samples acquired from ten hospitals in France found that 67% were positive for *Legionella* contamination (117). One hospital in Italy experienced recurrent cases of Legionnaires' Disease over a 15-year period, despite multiple decontamination efforts including heat treatment and installation of a continuous chlorination system (118).

Economic impact

The inability to successfully control *Legionella* in the environment has a costly public health and economic toll. Roughly 10% of those diagnosed with Legionnaires' Disease will succumb to the pneumonia. This number rises substantially in the context of health care settings such as long-term nursing facilities and hospitals, as the elderly and immunocompromised are a particularly vulnerable populace (119, 120). Among illnesses derived from waterborne pathogens, including Legionnaires' Disease, giardiasis, cryptosporidiosis, otitis externa, and non-tuberculous mycobacterial infection, Legionnaires' Disease has the highest treatment cost, averaging over $30,000 per episode and incurring over $400M annually in national hospitalization costs (as of 2012) (121).

Clearly there is a need for improved prevention and remediation methods to reduce the risk of *L. pneumophila* colonization of public water systems. The irony is that *Legionella* is a fastidious organism, and growth in a laboratory setting requires a rich media supplemented with both iron and L-cysteine (15). Yet the ability of this bacterium to withstand long periods in low-nutrient environments is well-documented. In fact, *L. pneumophila* remains viable and infectious even after six months in the nutrient-limited water media Fraquil (122). To date, *L. pneumophila* research has predominantly focused on pathogenesis, such as the virulence factors that promote invasion of and replication within a host cell. However, we need a better understanding of the genetic determinants that enable both *L. pneumophila* adaption to oligotrophic environments and progression to the MIF cell type, which likely involves alternate regulatory pathways to those that predominate within a host cell or in rich media.

In this dissertation, I examine a locus consisting of *lpg0279* through *lpg0277*, which encodes a protein abundant in MIF cells (Lpg0279) and a two-component system (Lpg0278-77) that modulates the turnover of c-di-GMP and asses its contribution both to cellular differentiation and resilience of *L. pneumophila*. This knowledge, in concert with our understanding of the *L. pneumophila* intracellular lifecycle, will aid in the development of effective strategies to control the transmission and spread of this pathogen.

CHAPTER 2

A Two-Component System that Modulates Cyclic-di-GMP Metabolism Promotes *Legionella pneumophila* Differentiation and Viability in Low-Nutrient Conditions

Elisa D. Hughes, Brenda G. Byrne, Michele S. Swanson

J. Bacteriol. 2019, doi:10.1128/JB.00253-19

ABSTRACT

During its life cycle, the environmental pathogen *Legionella pneumophila* alternates between a replicative and transmissive cell type when cultured in broth, macrophages, or amoebae. Within a protozoan host, *L. pneumophila* further differentiates into the hardy cell type known as the Mature Infectious Form (MIF). The second messenger cyclic-di-GMP coordinates lifestyle changes in many bacterial species, but its role in the *L. pneumophila* life cycle is less understood. Using an *in vitro* broth culture model that approximates the intracellular transition from the replicative to transmissive form, here we investigate the contribution to *L. pneumophila* differentiation of a two-component system (TCS) that regulates cyclic-di-GMP metabolism. The TCS is encoded by *lpg0278-lpg0277* and is co-transcribed with *lpg0279*, which encodes a protein upregulated in MIF cells. The promoter for this

operon is RpoS-dependent and induced in nutrient-limiting conditions that do not support replication, as demonstrated using a *gfp*-reporter and quantitative PCR (qPCR). The response regulator of the TCS (Lpg0277) is a bifunctional enzyme that both synthesizes and degrades cyclic-di-GMP. Using a panel of site-directed point mutants, we show that cyclic-di-GMP synthesis mediated by a conserved GGDEF domain promotes growth arrest of replicative *L. pneumophila*, accumulation of pigment and poly-3-hydroxybutyrate storage granules, and viability in nutrient-limiting conditions. Genetic epistasis tests predict that the MIF protein Lpg0279 acts upstream of the TCS, possibly as a negative regulator. Thus, *L. pneumophila* is equipped with a regulatory network in which cyclic-di-GMP stimulates the switch from a replicative to a resilient state equipped to survive in low-nutrient environments.

IMPORTANCE

Although an intracellular pathogen, *L. pneumophila* has developed mechanisms to ensure long-term survival in low-nutrient aqueous conditions. Eradication of *L. pneumophila* from contaminated water supplies has proven challenging, as outbreaks have been traced to previously remediated systems. Understanding the genetic determinants that support *L. pneumophila* persistence in low-nutrient environments can inform design and assessment of remediation strategies. Here we characterize a genetic locus that encodes a two-component signaling system (*lpg0278-lpg0277*) and a putative regulator protein (*lpg0279*) that modulates production of the messenger molecule cyclic-di-GMP. We show that this locus promotes both *L. pneumophila* cell differentiation and survival in nutrient-limiting conditions, thus advancing

understanding of the mechanisms that contribute to *L. pneumophila* environmental resilience.

INTRODUCTION

Legionella pneumophila is a gram-negative bacterium commonly found in aquatic environments, where it replicates within protozoan hosts and persists within biofilms (89). When inhaled, contaminated water droplets transmit *L. pneumophila* to the human lung, where this opportunistic pathogen can infect alveolar macrophages. Studies examining the life cycle of *L. pneumophila* cultured in broth, macrophages, and amoebae support a developmental model in which nutrient levels govern cellular differentiation (12, 23). When nutrients are plentiful, the bacteria activate pathways that support growth; when nutrients become limiting, the progeny stop replicating and express multiple factors that promote *L. pneumophila* transmission to a new host, including flagella and the Dot/Icm Type IV secretion system (22). Within protozoan hosts, *L. pneumophila* can differentiate further to generate the resilient, metabolically dormant but highly infectious Mature Infectious Form (MIF), a cell-type believed to be prevalent in the environment (46, 47).

To alternate between replication within phagocytes and persistence within nutrient-poor aquatic environments, *L. pneumophila* relies on multiple regulatory mechanisms that coordinate rapid adaption to changing conditions (12). For example, replication in broth requires amino acids as the primary carbon source (15, 123), and a reduction in amino acid availability activates regulatory pathways that trigger conversion from the exponential (E) phase to the post-exponential (PE) transmissive phase (8, 9). The *L. pneumophila* life cycle is governed by a sophisticated regulatory

network that includes the stringent response enzymes RelA and SpoT, multiple alternative sigma factors including RpoS and FliA, two-component regulatory systems, small regulatory RNAs, and CsrA post-transcriptional repressors (12, 21, 124). Driving the E to PE differentiation is a stringent response pathway coordinated by the alarmone guanosine penta- and tetraphosphate (abbreviated here as ppGpp) (24). Concomitant with ppGpp accumulation, the two-component system LetA/LetS induces transcription of small regulatory RNAs RsmY and RsmZ (29, 41, 44). These non-coding RNAs bind to and relieve repression by CsrA, enabling expression of multiple virulence traits associated with PE phase *L. pneumophila,* including cytotoxicity, motility, and lysosome evasion (reviewed by 12).

Another second messenger molecule that regulates lifestyle switches in *L. pneumophila* and multiple other bacterial species is bis-(3'-5')-cyclic dimeric guanosine (c-di-GMP) (84); reviewed by (54, 55, 59). Diguanylate cyclases (DCG), which possess a conserved GGDEF motif, synthesize c-di-GMP from two molecules of GTP; conversely, phosphodiesterases (PDE) catalyze the hydrolysis of c-di-GMP back to GMP and contain either an EAL or HD-GYP domain (63, 125). Most bacterial species utilizing c-di-GMP produce multiple enzymes that control c-di-GMP levels; for example, *Escherichia coli* encodes 29 proteins with GGDEF and/or EAL domains (52), and *Vibrio cholerae* encodes over 60 such proteins (125).

The *L. pneumophila* genome (Philadelphia-1 and Lens strains) encodes 22 different enzymes involved in c-di-GMP metabolism, including several composite proteins possessing both GGDEF and EAL domains (84, 85). The range of activities influenced by these proteins is diverse and even includes control of opposing biological functions within the same cell. Recently Pecastaings and colleagues (60) identified five

c-di-GMP proteins in the Lens strain involved in biofilm regulation, three of which enhance biofilm formation while the other two inhibit this developmental program. In *L. pneumophila*, some c-di-GMP producing and degrading proteins enhance virulence by altering translocation of multiple Dot/Icm Type IV secretion system effectors, interfering with phagosome/lysosome fusion, and enhancing cytotoxicity—functions that promote replication within and transmission between host cells (84, 85). In general, GGDEF and/or EAL motifs are crucial for the protein's enzymatic activity (85). However, genetic disruption of these domains does not always cause detectable changes in the cellular c-di-GMP concentration, leaving open the possibility that some of these proteins perform regulatory roles independently of c-di-GMP metabolism (60, 84).

Two-component regulatory systems, classically comprised of a histidine kinase and a response regulator, are a widespread signal transduction mechanism in bacteria that enables rapid adaptation to fluctuating conditions (reviewed by (39, 126). Some response regulators contain GGDEF and/or EAL domains; when phosphorylated on a conserved aspartate residue by their cognate histidine kinase, these enzymes can alter their c-di-GMP synthesis or hydrolysis (55). For example, in *Xanthomonas campestris*, the composite GGDEF/EAL protein RavR is the response regulator in a two-component system whose activation by the histidine kinase RavA shifts the enzyme from DCG to PDE activity and ultimately increases virulence (127). In the *L. pneumophila* Lens strain, Levet-Paulo and colleagues (56) characterized a putative two-component system comprised of a histidine kinase Lp0330 and its cognate response regulator Lpl0329, a bifunctional enzyme with both a GGDEF and an EAL domain. Their series of *in vitro* experiments using purified proteins demonstrated that Lpl0329 possesses both DGC and the opposing PDE enzymatic activity, and phosphotransfer from Lpl0330 to

Lpl0329 reduces DGC activity (56). In the *L. pneumophila* Philadelphia-1 strain, the homolog of Lpl0330 is Lpg0278 (hereafter HK), and the homolog of Lpl0329 is Lpg0277 (hereafter RR; HK and RR collectively are hereafter the TCS). On the Philadelphia-1 chromosome located directly 5' of *lpg0278* and *lpg0277* is *lpg0279*, a gene encoding a hypothetical protein that is abundant in MIF cells (46).

Despite the high degree of genomic variability among *L. pneumophila* strains (1, 128, 129), this locus appears to be well conserved. In addition to the Philadelphia-1 and Lens strains, it is also present in the Corby and Alcoy strains. In the Paris strain, a nonsense point mutation between the GGDEF and EAL domains converts a glutamine to a stop codon, splitting the *lpg0277* homolog into two discrete genes (*lpp0352* and *lpp0351*; Fig. 2.1). Whether *lpp0351* encodes a functional EAL protein that is expressed by the Paris strain has not been determined. However, the overall conservation of this locus hints at a contribution to the *L. pneumophila* lifecycle.

Figure 2.1

Color Align Conservation results

```
lpg0277            MSTESFRILVIDDNPAIHQDFMKILNVSKNSVLLNKFDAELFDDETEQLGISLPEFEIDVATQGIEGIEKVKRSLEEGRP 80
lpl0329            MSTESFHILVIDDNPAIHQDFMKILNVSKNSALLNKFDAELFGDETEQLGISLPEFKIDVATQGIEGIEKVKRSLEEGRP 80
lpp0352-51         MSTESFRILVIDDNPAIHQDFMKILNVSKNSVLLNKFDVELFDDETEQLGISLPEFEIDVATQGIEGIEKVKRSLEEGRP 80
LPC_0354(Corby)    MSTESFRILVIDDNPAIHQDFMKILNVSKNSALLNKFDAELFDDETEQLGISLPEFEIDVATQGIEGIEKVKRSLEEGRP 80
lpa_00480(Alcoy)   MSTESFRILVIDDNPAIHQDFMKILNVSKNSALLNKFDAELFDDETKQLGISLPEFEIDVATQGIEGIEKVKRSLEEGRP 80

lpg0277            YPLAFVDIRMPPGIDGIETIKRIWAIDPQIQIVICSAYSDYSWESTVNTLGLSDNLLVLKKPFDVIAVRQLASALTQKWV 160
lpl0329            YPLAFVDIRMPPGIDGIETIKRIWAIDPQIQIVICSAYSDYSWESTVNTLGLSDNLLVLKKPFDVIAVRQLASALTQKWV 160
lpp0352-51         YPLAFVDIRMPPGIDGIETIKRIWAIDPQIQIVICSAYSDYSWESTVNTLGLSDNLLVLKKPFDVIAVRQLASALTQKWV 160
LPC_0354(Corby)    YPLAFVDIRMPPGIDGVETIKRIWAIDPQIQIVICSAYSDYSWESTVNTLGLSDNLLVLKKPFDVIAVRQLASALTQKWV 160
lpa_00480(Alcoy)   YPLAFVDIRMPPGIDGVETIKRIWAIDPQIQIVICSAYSDYSWESTVNTLGLSDNLLVLKKPFDVIAVRQLASALTQKWV 160

lpg0277            LARETQKHTEFLNQLVEERTESLQQSFSLLRATIESSSDGILVVDLQGKIVDYNSQFVKLWNIPESIMKK-TSESQLIQY 239
lpl0329            LARETQKHTEFLNQLVEERTESLQQSFSLLRATIESSSDGILVVDLQGKIVDYNSQFVKLWNIPESIMKK-TSESQLIQY 239
lpp0352-51         LARETQKHTKFLNQLVEERTESLQQSFSLLRATIESSSDGILVVDLQGKIVDYNSQFVKLWNIPESIIKKETSEPQLIQY 240
LPC_0354(Corby)    LARETQKHTEFLNQLVEERTESLQQSFSLLRATIESSSDGILVVDLQGKIVDYNSQFVKLWNIPESIIKKETSEPQLIQY 240
lpa_00480(Alcoy)   LARETQKHTEFLNQLVEERTESLQQSFSLLRATIESSSDGILVVDLQGKIVDYNSQFVKLWNIPESIIKKETSEPQLIQY 240

lpg0277            MSTQLLNSEQHAQHVKEFSIHIDDSSIQIYHFRNGKVVECCSKPHRVGPQTVGRVWSFRDITEQTRLKEKLEYQATHDAL 319
lpl0329            MSTQLLNSEQHAQHVKEFSIHIDDSSIQIYHFRNGKVVECCSKPHRVGPQTVGRVWSFRDITEQTRLKEKLEYQATHDAL 319
lpp0352-51         MSTQLLNSEQHAQHVKEFSIHIDDSSMQIYHFRNGKVVECCSKPHRVGLQTVGRVWSFRDITEQTRLKEKLEYQATHDAL 320
LPC_0354(Corby)    MSTQLLNSEQHAQHVKEFSIHIDDSSMQIYHFRNGKVVECCSKPHRVGLQTVGRVWSFRDITEQTRLKEKLEYQATHDAL 320
lpa_00480(Alcoy)   MSTQLLNSEQHAQHVKEFSIHIDDSSMQIYHFRNGKVVECCSKPHRVGLQTVGRVWSFRDITEQTRLKEKLEYQATHDAL 320

lpg0277            TNLPNRLLLIDRIEHAISSYARHKMKFAILFFDLDRFKMINDSLSHEAGDQLLCAVAKRLRSLVRKEDTLARIGGDEFVM 399
lpl0329            TNLPNRLLLIDRIEHAISSYARHKMKFAILFFDLDRFKMINDSLSHEAGDQLLCAVAKRLRSLVRKEDTLARIGGDEFVM 399
lpp0352-51         TNLPNRLLLIDRIEHAISGYARHKMKFAILFFDLDRFKMINDSLSHEAGDQLLCDVAERLRSLVRKEDTLARIGGDEFVM 400
LPC_0354(Corby)    TNLPNRLLLIDRIEHAISGYARHKMKFAILFFDLDRFKMINDSLSHEAGDQLLCAVAERLRSLVRKEDTLARIGGDEFVM 400
lpa_00480(Alcoy)   TNLPNRLLLIDRIEHAISGYARHKMKFAILFFDLDRFKMINDSLSHEAGDQLLCAVAERLRSLVRKEDTLARIGGDEFVM 400

lpg0277            LFQSFNSEEQIAGVAQKILKSFQKPFHIAERDINIHVSIGISVYPTDGKTVNTLLSNADMAMYQAKFRGGNQFSFYTEKL 479
lpl0329            LFQSFNSEEQIAGVAQKILKSFQKPFHIAERDINIHVSIGISVYPTDGKTVNTLLSNADMAMYQAKFRGGNQFSFYTEKL 479
lpp0352-51         LFQSFNSEEQIAGVAQKILKSFQKPFHIAERDINIHVSIGISVYPTDGKTVNTLLSNADMAMYQAKFRGGNQFSFYTEKL 480
LPC_0354(Corby)    LFQSFNSEEQIAGVAQKILKSFQKPFHIAERDINIHVSIGISVYPTDGKTVNTLLSNADMAMYQAKFRGGNQFSFYTEKL 480
lpa_00480(Alcoy)   LFQSFNSEEQIAGVAQKILKSFQKPFHIAERDINIHVSIGISVYPTDGKTVNTLLSNADMAMYQAKFRGGNQFSFYTEKL 480

lpg0277            NKKTNQQFQLELDLQKAIIHHEFFLLYQPQFTVNTHEIRSMEALIRWNHPQKGIILPLDFIPTAESSGLIVPIGEWVLRE 559
lpl0329            NKKTNQQFQLELDLQKAIIHHEFFLLYQPQFTVNTHEIRSMEALIRWNHPQKGIILPLDFIPTAESSGLIVPIGEWVLRE 559
lpp0352-51         NKKTNQQFQLELDLQKAIVHHEFFLLY-------------MEALIRWNHPQKGIILPLDFIPTAESSGLIVPIGEWVLRE 547
LPC_0354(Corby)    NKKTNQQFQLELDLQKAIVHHEFFLLYQPQFTVNTHEIRSMEALIRWNHPQKGIILPLDFIPTAESSGLIVPIGEWVLRE 560
lpa_00480(Alcoy)   NKKTNQQFQLELDLQKAIVHHEFFLLYQPQFTVNTHEIRSMEALIRWNHPQKGIILPLDFIPTAESSGLIVPIGEWVLRE 560

lpg0277            TCKQIGTWRTMGLPEIRVACNVASKQLRQRDFPAMVEDLLKEYSVKPHCLELEITENVIIDKEIQRTIKKLKSLGVLIIL 639
lpl0329            TCKQIGTWRTMGLPEIRVACNVASKQLRQRDFPAMVKDLLKEYSVEPHCLELEITENVIIDKEIQRTIKKLKSLGVLIIL 639
lpp0352-51         TCKQIGAWRTMGLPDIRVACNVASKQLRQRDFPAMVEDLLKEYSVEPHCLELEITENVIIDKEIQRTIKKLKSLGVLIIL 627
LPC_0354(Corby)    TCKQIGAWRTMGLPEIRVACNVASKQLRQRDFPAMVEDLLKEYSVEPHCLELEITENVIIDKEIQRTIKKLKSLGVLIIL 640
lpa_00480(Alcoy)   TCKQIGAWRTMGLPEIRVACNVASKQLRQRDFPAMVEDLLKEYSVEPHCLELEITENVIIDKEIQRTIKKLKSLGVLIIL 640

lpg0277            DDFGTGNSSLNLLKQVAVDSLKIDQSFIQNISKSPGDEAIIDAIIAIAQSMNFNIIAEGVETQNQLKFLKKRRCNDIQGF 719
lpl0329            DDFGTGNSSLNLLKQVAVDSLKIDQSFIQNISKSPGDEAIIDAIIAIAQSMNFNIIAEGVETQNQLKFLKKRRCNDIQGF 719
lpp0352-51         DDFGTGNSSLNLLKQVAVDSLKIDQSFIQNISKSPGDEAIIDAIIAIAQSMNFNIIAEGVETQNQLKFLKKRRCNDIQGF 707
LPC_0354(Corby)    DDFGTGNSSLNLLKQVAVDSLKIDQSFIQNISKSPGDEAIIDAIIAIAQSMNFNIIAEGVETQNQLKFLKKRRCNDIQGF 720
lpa_00480(Alcoy)   DDFGTGNSSLNLLKQVAVDSLKIDQSFIQNISKSPGDEAIIDAIIAIAQSMNFNIIAEGVETQNQLKFLKKRRCNDIQGF 720

lpg0277            LMSKPIPSEEIEKLLQGNRTK 740
lpl0329            LMSKPIPPEEIEKLLQGNRTK 740
lpp0352-51         LMSKPIPPEEIEKLLQGNRTK 728
LPC_0354(Corby)    LMSKPIPPEEIEKLLQGNRTK 741
lpa_00480(Alcoy)   LMSKPIPPEEIEKLLQGNRTK 741
```


Fig 2.1. Conservation of the RR among *L. pneumophila*.

Sequence alignment of the RR-encoding gene in five strains of L. pneumophila: *lpg0277* (Philadelphia-1), *lpg0329* (Lens), *LPC_0354* (Corby), *lpa_00480* (Alcoy), and *lpp0352-lpp0351* (Paris), using DNA and protein sequences obtained from Legiolist (www.legiolist.com). Protein alignment generated with Sequence Manipulation Suite (http://www.bioinformatics.org/sms2/color_align_cons.html); expanded alignment of DNA sequence in the grey region generated with Emboss Needle (EMBL-EBI). Boxes identify the GGDEF and EAL enzymatic domains and the point mutation in the Paris strain converting a glutamine to a stop codon. Presence of this point mutation in the Swanson lab Paris strain (MB545), a gift from C. Buchrieser (Institute Pasteur), was verified by sequencing.

The genetic proximity of the MIF gene *lpg0279* to the TCS-encoding *lpg0277* and *lpg0278* loci suggests potential co-regulation and related functions (130). As MIF cells are resilient forms that develop from PE phase *L. pneumophila* within protozoan hosts (8), here we test the hypothesis that the locus consisting of *lpg0279*, *lpg0278* and *lpg0277* (hereafter referred to as *lpg0279-77*) promotes persistence of *L. pneumophila* in nutrient-poor environments.

RESULTS

lpg0277, *lpg0278* and *lpg0279* are co-transcribed

The TCS-encoding genes *lpg0277* and *lpg0278* are located on the same DNA strand, 22 bp 3' of *lpg0279*. To test the hypothesis that these three genes constitute an operon, we analyzed RNA extracted from wild-type (WT) *L. pneumophila* cultured to PE phase in rich AYET medium. After conversion to cDNA, an endpoint PCR assay was conducted using primer sets designed to span the intergenic regions between the three genes (Fig. 2.2A). As predicted, amplicons of ~ 300 bp were generated by primer sets A/B as well as by primers C/D, indicating that *lpg0279* and *lpg0278* form a single transcriptional unit, as do *lpg0278* and *lpg0277* (Fig. 2.2B). A similar experiment was conducted to determine if this transcriptional unit includes *lpg0280*, which is located 165 bp 5' of *lpg0279* and codes for a putative transcriptional regulator of the LysR family. No product was generated for primer pairs E/F (Fig. 2.2C), indicating that the operon consists solely of *lpg0279*, *lpg0278* and *lpg0277*.

Figure 2.2

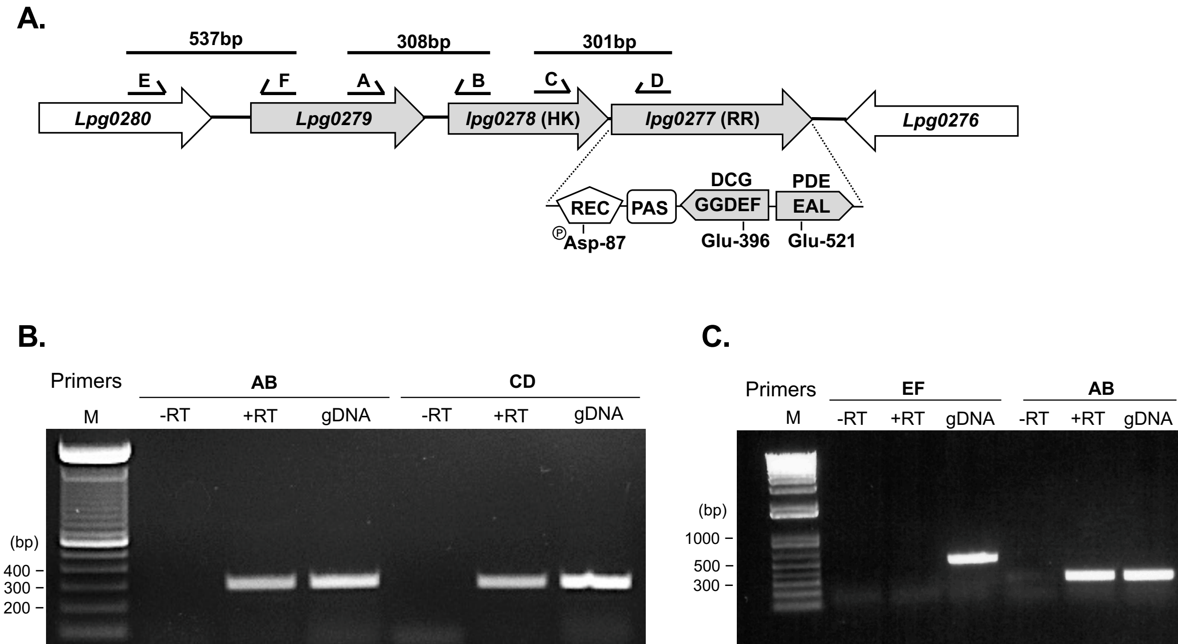


Fig. 2.2. The genes *lpg0279*, *lpg0278* and *lpg0277* constitute an operon.

A. Schematic of the locus containing *lpg0279*, the TCS-encoding genes *lpg0278* and *lpg0277*, and the primer sets used to characterize mRNA by PCR. Also shown are the genes located 5' and 3' of the *lpg0279-77* locus. Co-transcription of **B.** *lpg0279*, *lpg0278* and *lpg0277* and **C.** *lpg0280* and *lpg0279* and, as a positive control, *lpg0279* and *lpg0278* was assessed by end-point PCR assay with or without reverse transcriptase **(RT)** using RNA isolated from PE phase WT L. pneumophila that was converted to cDNA. As a reference for the PCR product length, genomic DNA **(gDNA)** was also used as a template. **M:** DNA size marker.

The *lpg0279-77 locus* is induced at the transition from E to PE phase

To gain insight into the role of *lpg0279-77* in the *L. pneumophila* life cycle, we first analyzed the timing and conditions that induce its promoter activity. To do so, we generated a transcriptional reporter by ligating a DNA fragment corresponding to the 832 bp immediately 5' of the *lpg0279* open reading frame to a promoterless copy of the *gfp-mut3* gene encoded on plasmid pBH6119 (131), generating p*lpg0279-gfp* (Fig. 2.3). This transcriptional reporter was then transferred to WT *L. pneumophila*.

Expression of *lpg0279-gfp* was monitored in E and PE phase broth cultures, which function as proxies for the intracellular replicative and transmissive stages, respectively (22, 23). In each case, E phase cultures were sub-cultured to an OD_{600} of ~ 0.8 in rich AYE medium, incubated at 37°C on an orbital shaker, and then GFP fluorescence and cell density were measured at 2-3 h intervals. As a reference for PE phase expression, we analyzed in parallel a transcriptional reporter strain in which *gfp* expression is driven by the promoter for the flagellin subunit *flaA* (p*flaA-gfp*) (24). Serving as the negative control was a strain carrying the promoterless *gfp* vector pBH6119. All strains grew equally well as measured by OD_{600}, and no fluorescence was observed for the vector control (Fig. 2.4). Throughout E phase, the p*lpg0279-gfp* strain generated only minimal levels of GFP fluorescence, whereas promoter activity increased markedly upon entry into PE phase—kinetics similar to that of the p*flaA-gfp* marker of the PE transmissive phase (Fig. 2.4).

Figure 2.3

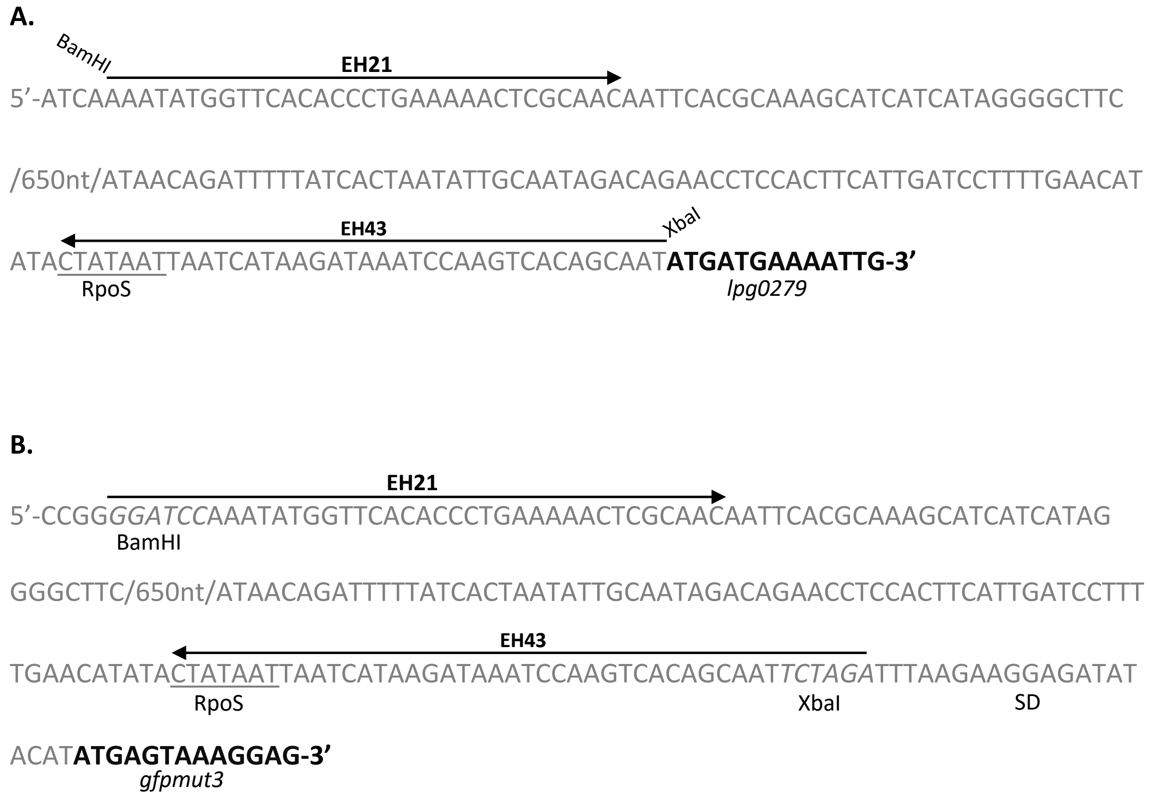


Fig 2.3. Construction of p*lpg0279-gfp* transcriptional reporter plasmid.

A. Promoter region of *lpg0279* amplified from the *L. pneumophila* chromosome using primers EH21 and EH43, encoding BamHI and XbaI restriction sites, respectively.

B. Schematic of region in pBH6119 with the *lpg0279* promoter region inserted at the BamHI and XbaI sites (italics), located 20 bp 5' of *gfpmut3* gene ATG start codon. Putative RpoS -10 recognition sequence is underlined. SD: Shine-Delgarno sequence.

Figure 2.4

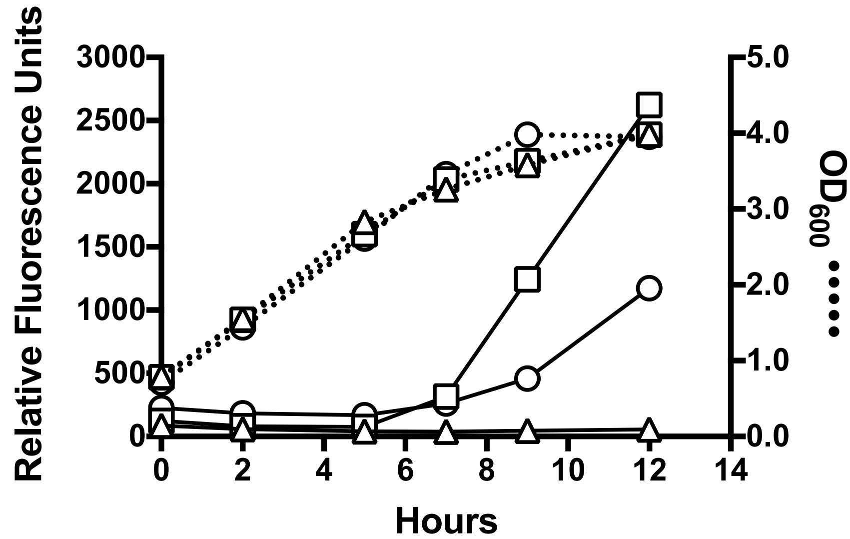


Fig. 2.4. Promoter activity for the *lpg0279-77* operon increases upon entry into PE phase.

GFP fluorescence generated by the transcriptional reporter p*lpg0279-gfp* (○). Negative control strain is Lp02 carrying the empty pBH6119 vector (Δ); PE reference strain carries the flagellin subunit reporter p*flaA-gfp* (□). For all strains, overnight E phase cultures in AYE were diluted to a starting OD_{600} of ~ 0.8, incubated at 37°C, and then fluorescence measured at 2-3 h intervals. Relative Fluorescence Units (solid lines) were calculated as Fluorescence Units/OD_{600} (dotted lines) and represent the means ± SE of triplicate samples. In each case, error < 5%. Data shown are representative of results obtained in at least three independent experiments.

The stationary phase sigma factor RpoS activates *lpg0279-77* expression

Due to the temporal similarity of their promoter activation (Fig. 2.4), we investigated whether transcription of *lpg0279-77* and *flaA* are controlled by the same regulatory proteins. To do so, the p*lpg0279-gfp* and p*flaA-gfp* reporters were transformed into mutants lacking either the alternative sigma factors FliA (σ^{28}) or RpoS (σ^{S}/σ^{38}), the two-component system LetA/S, or the ppGpp synthetase RelA (20, 36, 38, 41, 132, 133). This panel of strains was then cultured on CYET agar at 37°C for 3 days. Visible differences in GFP expression suggest that RpoS is essential for robust transcription of *lpg0279-77*, while the other regulatory factors are not (Fig. 2.5A). In contrast, p*flaA-gfp* expression was only marginally diminished in the Δ*rpoS* mutant; as expected, the FliA sigma factor was its critical regulator (Fig. 2.5B) (27, 36, 38). Thus, although the promoters for *flaA* and *lpg0279-77* are each induced in PE phase, their mechanisms of regulation differ.

To corroborate that RpoS induces *lpg0279-77* expression, we conducted RT-qPCR experiments comparing *lpg0279* transcript levels in WT *L. pneumophila* and a *rpoS* mutant, both cultured to PE phase in AYET medium. As shown in Fig. 2.5C, *lpg0279* expression was significantly reduced in the Δ*rpoS* mutant compared to WT. As it is not uncommon for bacterial genes within operons to be differentially regulated due to the presence of internal promoters (134), we also examined the TCS-encoding genes *lpg0278-77* using a primer set that spans the intergenic region (EH122 & EH123). Similar to *lpg0279*, transcripts of the TCS genes *lpg0278-77* were reduced in the Δ*rpoS* mutant (Fig. 2.5C). Although our experimental design does not rule out a secondary

Figure 2.5

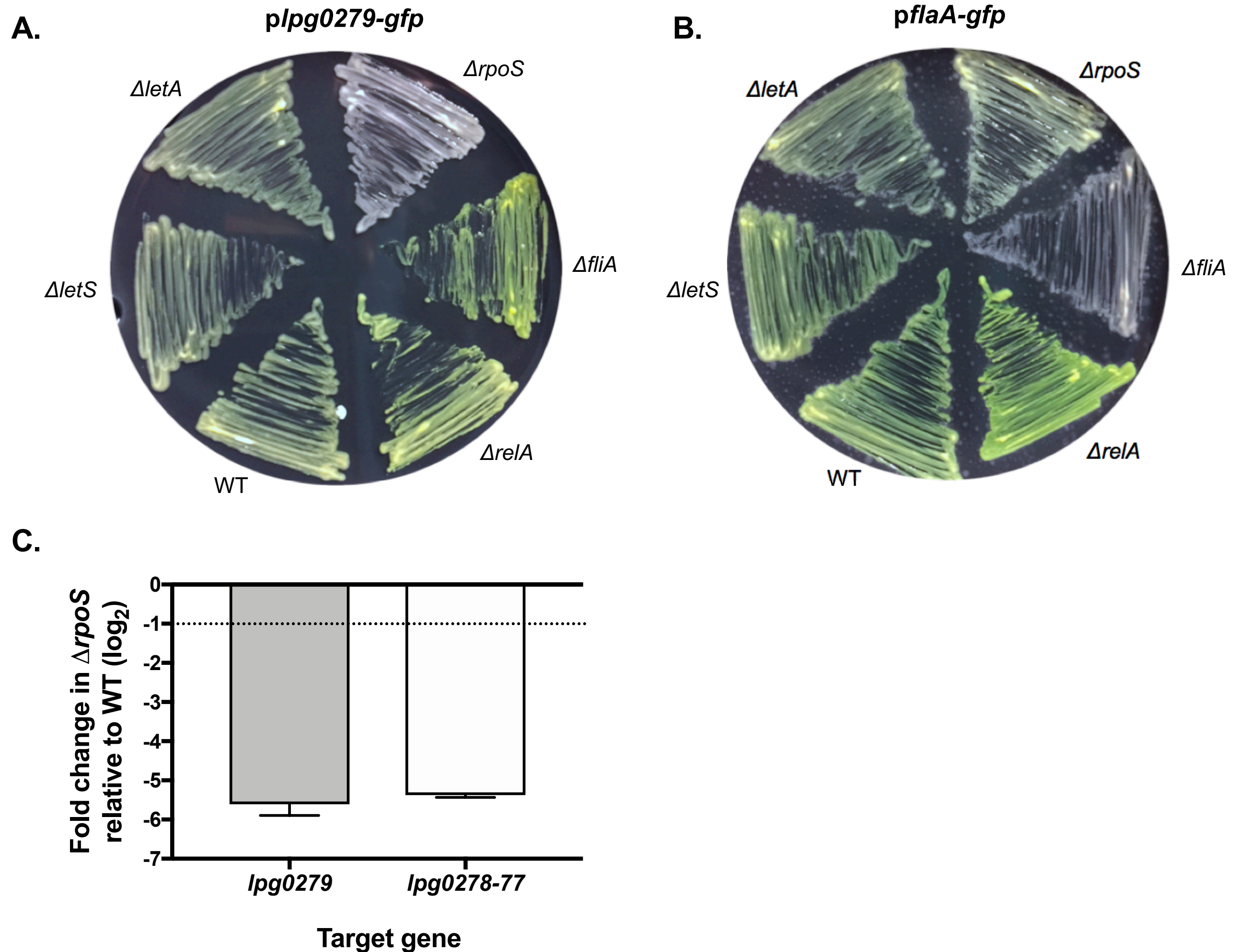


Fig. 2.5. Expression of *lpg0279-77* is RpoS-dependent.

A-B. Images obtained in ambient light of WT Lp02 and *letA*, *letS*, *relA*, *rpoS* and *fliA* mutants harboring the transcriptional reporter plasmids **A.** p*lpg0279-gfp* or **B.** p*flaA-gfp*, which serves as a reference for PE phase gene expression. The strains indicated were cultured on CYET at 37°C for 3 days to allow bacterial growth and GFP accumulation.

C. RT-qPCR analysis of *lpg0279* and *lpg0278-77* gene expression in an isogenic Δ*rpoS* mutant. Values calculated using the $2^{-\Delta\Delta Ct}$ method normalized to 16S ribosomal RNA expression are presented as Log2 fold-change relative to WT Lp02. Data presented are the means ± SE of triplicate samples performed in duplicate; similar results were obtained in at least two additional experiments. Dotted line represents the Log2 fold-change significance threshold of -1.0.

internal promoter controlling *lpg0278* and/or *lpg0277* expression under different culture conditions, this RNA analysis confirms that *lpg0279* and the downstream TCS genes are co-regulated by RpoS.

To evaluate whether RpoS directly regulates transcription of the *lpg0279-77* operon, we examined its 5' region for a RpoS binding site. Indeed, upstream of *lpg0279* is the sequence CTATAAT, which is highly similar to the consensus -10 RpoS binding site identified in *E. coli* (CTATACT), including the four nucleotides (underlined) that are highly conserved among RpoS-dependent promoters (Fig. 2.3)(135-137). The validity of this consensus sequence in *L. pneumophila* was established previously by a genetic and biochemical analysis of RpoS regulation of the *L. pneumophila* Integration Host Factor genes *ifhA* and *ihfB* (138). No similar sequence was identified immediately 5' of the TCS genes. Collectively, these results support the conclusion that the RpoS sigma factor likely activates expression of the *lpg0279-77* operon in a direct manner.

Expression of *lpg0279-77* increases in the absence of amino acids essential for replication

When replicating *L. pneumophila* experience nutrient limitation, bacteria accumulate the stringent response alarmone ppGpp and synthesize RpoS, which activates expression of multiple genes critical for fitness and transmission in the PE phase (12). Because *lpg0279-77* transcription is RpoS-dependent, we next examined whether replicating *L. pneumophila* induce *lpg0279-gfp* expression in response to nutrient limitation. Standard growth media for *L. pneumophila* consists of a rich yeast extract-based medium (AYE) supplemented with both iron and the amino acid L-cysteine, as this bacterium lacks a number of cysteine biosynthesis enzymes (139, 140).

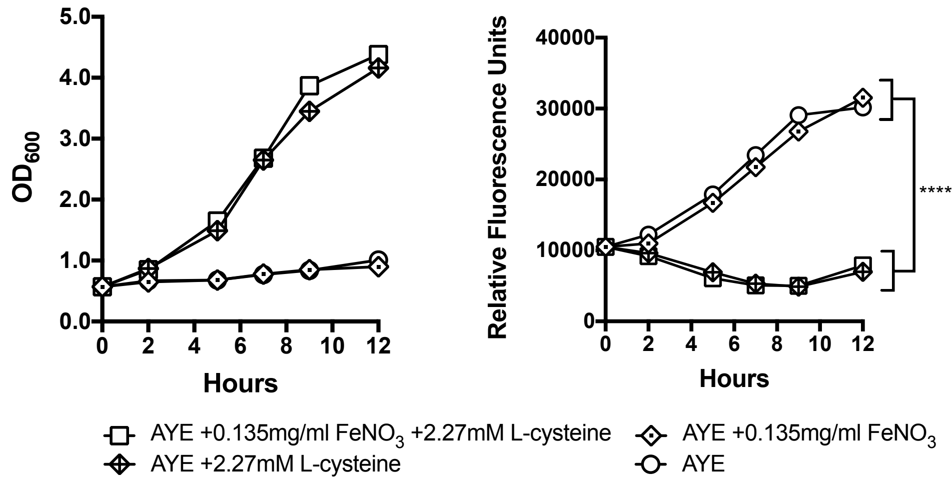
Accordingly, we first quantified *lpg0279-gfp* fluorescence in *L. pneumophila* cultured in AYE containing both L-cysteine and ferric nitrate, either L-cysteine or ferric nitrate alone, or neither supplement. In media supplemented with L-cysteine, either with or without additional iron, *L. pneumophila* continued to replicate for > 9 hours and did not activate the *lpg0279-77* promoter (Fig. 2.6A). In contrast, when the media lacked L-cysteine, bacterial replication stalled and the *lpg0279-77* promoter was induced (Fig. 2.6A).

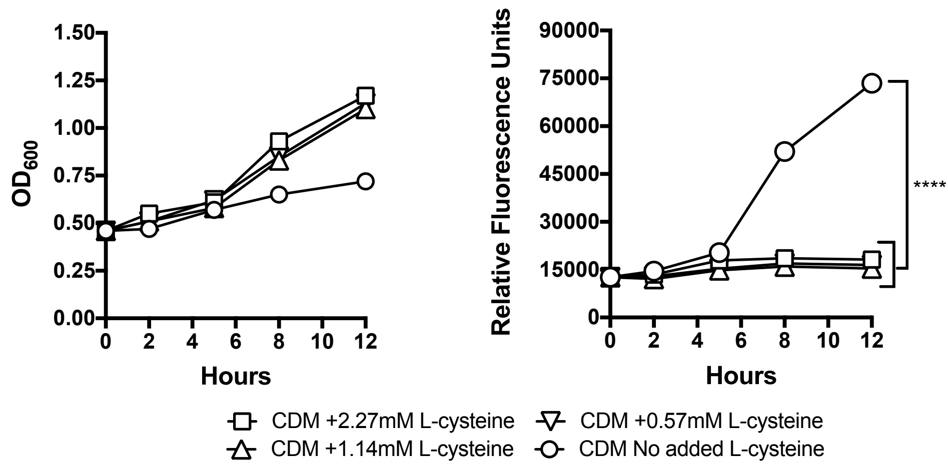
The yeast extract in AYE contains several amino acids including L-cysteine (bdbiosciences.com), so to more accurately address the impact of this amino acid in promoting *lpg0279-77* expression, we repeated the analysis using a chemically defined medium (CDM) (139) in which L-cysteine, L-cystine, and supplemental ferric pyrophosphate were omitted. Initial experiments examined *lpg0279-gfp* fluorescence in this CDM supplemented with L-cysteine, at either 100% (2.27 mM), 50% (1.14 mM), 25% (0.57 mM) or 0% of the standard concentration used to support *in vitro* growth in rich AYE media. In each case, the presence of L-cysteine supported *L. pneumophila* replication and repressed expression of *lpg0279-gfp* (Fig. 2.6B).

We next considered the relationships between L-cysteine availability, *lpg0279-77* promoter activity and bacterial growth (Fig. 2.6A and B; also see Fig. 2.4). The inability of *L. pneumophila* to replicate in the absence of an essential amino acid—in this case L-cysteine—may be a signal that induces *lpg0279-77* expression. An alternative interpretation is that *lpg0279-77* transcription is triggered by L-cysteine deficiency specifically, and this locus in turn suppresses replication. To distinguish between these two possibilities, we examined *lpg0279-gfp* fluorescence in CDM lacking either L-cysteine/L-cystine or a different amino acid that *L. pneumophila* requires for growth in

CDM, namely L-serine or L-methionine (139). Compared to cultures in complete CDM (containing L-cysteine/L-cystine, L-serine and L-methionine), the absence of any of these amino acids prevented replication and stimulated p*lpg0279-gfp* expression (Fig. 2.6C). Thus, *L. pneumophila* induces *lpg0279-77* promoter activity in response to nutrient-limiting conditions that impede bacterial replication.

Figure 2.6





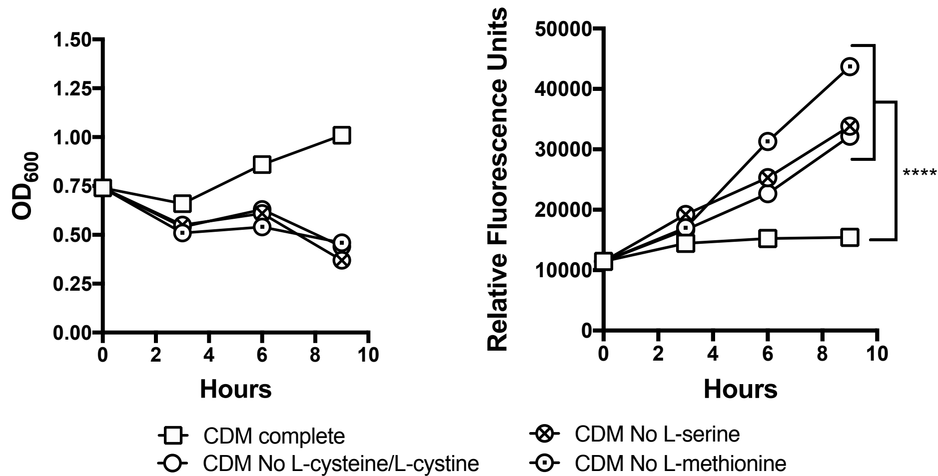


Fig. 2.6. Nutrient limitation induces expression of *lpg0279-77*.

To examine the impact of nutrient limitation on *lpg0279-77* transcription, E phase Lp02 cultures carrying the p*lpg0279-gfp* reporter plasmid were exposed to culture conditions shown, incubated for 9-12 h at 37°C on an orbital shaker, and then GFP fluorescence and cell density (OD_{600}) measured at 2-3 h intervals.

A. OD_{600} measurements and RFU values obtained for cultures exposed to AYE medium with or without 0.135 mg/ml ferric nitrate and/or 2.27 mM L-cysteine.

B. OD_{600} measurements and RFU values obtained for cultures exposed to CDM either without L-cysteine/L-cystine or supplemented with the indicated concentrations of L-cysteine.

C. OD_{600} and RFU values obtained for cultures exposed to CDM lacking either L-cysteine/L-cystine, L-serine or L-methionine, or containing all aforementioned amino acids (CDM complete).

RFU symbols represent the means ± SE of triplicate GFP fluorescence readings, normalized to OD_{600} values obtained by measuring a 1/10 dilution of cell culture in a spectrophotometer (short error bars are masked by symbols). A two-tailed Student's *t*-test was used to determine statistically significant differences between groups at the final time point (****, $p < 0.0001$). Data shown are representative of results obtained in two or more independent experiments.

PE phase *L. pneumophila* lacking either the HK or RR component of the TCS, or constitutively expressing *lpg0279*, exhibit a shortened lag phase and reduced pigmentation

Based on the increase in *lpg0279-77* promoter activity observed in response to conditions that do not support *L. pneumophila* growth (Fig. 2.4 and 2.6), we next examined whether this locus promotes differentiation of replicating *L. pneumophila* to the PE phase. To do so, we generated isogenic mutants containing in-frame deletions in either *lpg0279*, *lpg0278*, or *lpg0277*.

To assess growth of each mutant strain, E phase (OD_{600} < 2.5) or PE phase (OD_{600} < 3.5) cultures were diluted to an OD_{600} of ~ 0.1 in AYET medium, and then cell density was quantified over a 36 h incubation period at 37°C using a Bioscreen growth curve analyzer. For the E phase inocula, growth curves for each mutant resembled the WT strain (data not shown). However, for the PE phase inocula, mutants lacking either the HK (Δ*lpg0278*) or RR (Δ*lpg0277*) component of the TCS mimicked the WT E phase reference culture by exhibiting a minimal lag phase (Fig. 2.7A and B). Also notable after ~ 24 h for the ΔHK and ΔRR mutant cultures was the decline in OD_{600}, which could reflect cell lysis, filamentation, or lack of pigment production (26). Although expression of the HK or RR gene alone was insufficient to remedy the effects of the respective deletion (data not shown), IPTG-induced expression of the full *lpg0278-lpg0277* locus from plasmid pHK/RR restored WT growth kinetics (Fig. 2.7A and B).

In contrast to the TCS genes, growth of mutants lacking *lpg0279* was indistinguishable from WT PE phase cultures (Fig. 2.7C). However, WT *L. pneumophila* constitutively expressing a plasmid-borne allele of *lpg0279* engineered to encode an optimal ribosome binding site (84) exhibited both the initial shortened lag

period and the later decline in OD_{600} observed for the ΔHK and ΔRR mutants (Fig. 2.7C). Therefore, constitutive expression of *lpg0279* inhibits replicating *L. pneumophila* from transitioning to PE phase, whereas the genetically-linked TCS promotes differentiation of replicating *L. pneumophila* to the PE phase.

To test more rigorously the impact of the TCS and Lpg0279 on differentiation of replicating *L. pneumophila* to the PE phase, we quantified the soluble pigment pyomelanin, which accumulates in the late PE phase (141, 142). Derived from secreted homogentisic acid (HGA), this melanin-like substance is not required for intracellular survival; rather it enhances environmental fitness of *L. pneumophila* by protecting bacterial cells from the damaging effects of light and by aiding in iron acquisition through its ferric reductase activity (143, 144). When cultured in rich broth to a cell density typical of PE phase ($OD_{600} > 3.5$), all strains generated minimal pigment. However, when maintained in PE phase for up to three days, WT cultures accumulated pigment, but strains that lacked either of the TCS components did not (Fig. 2.7D). Consistent with their growth phenotypes, deletion of the *lpg0279* gene had no effect on pigmentation, whereas constitutive expression of *lpg0279* by WT *L. pneumophila* inhibited pigment accumulation (Fig. 2.7D). Consistently, the ΔRR mutant harboring the complementing plasmid pHK/RR accumulated higher levels of pigment than did the WT strain, indicating that the TCS in multi-copy may stimulate pigmentation and/or differentiation to PE phase. As *L. pneumophila* cultures require TCS function to transition from E to PE phase (Fig. 2.7), we next examined whether the TCS enhances *L. pneumophila* viability when nutrients are limiting. In TCS signal transduction pathways, RR activity is distal to HK; accordingly, we analyzed the RR mutant as representative of Lpg0278-0277 TCS output.

Figure 2.7

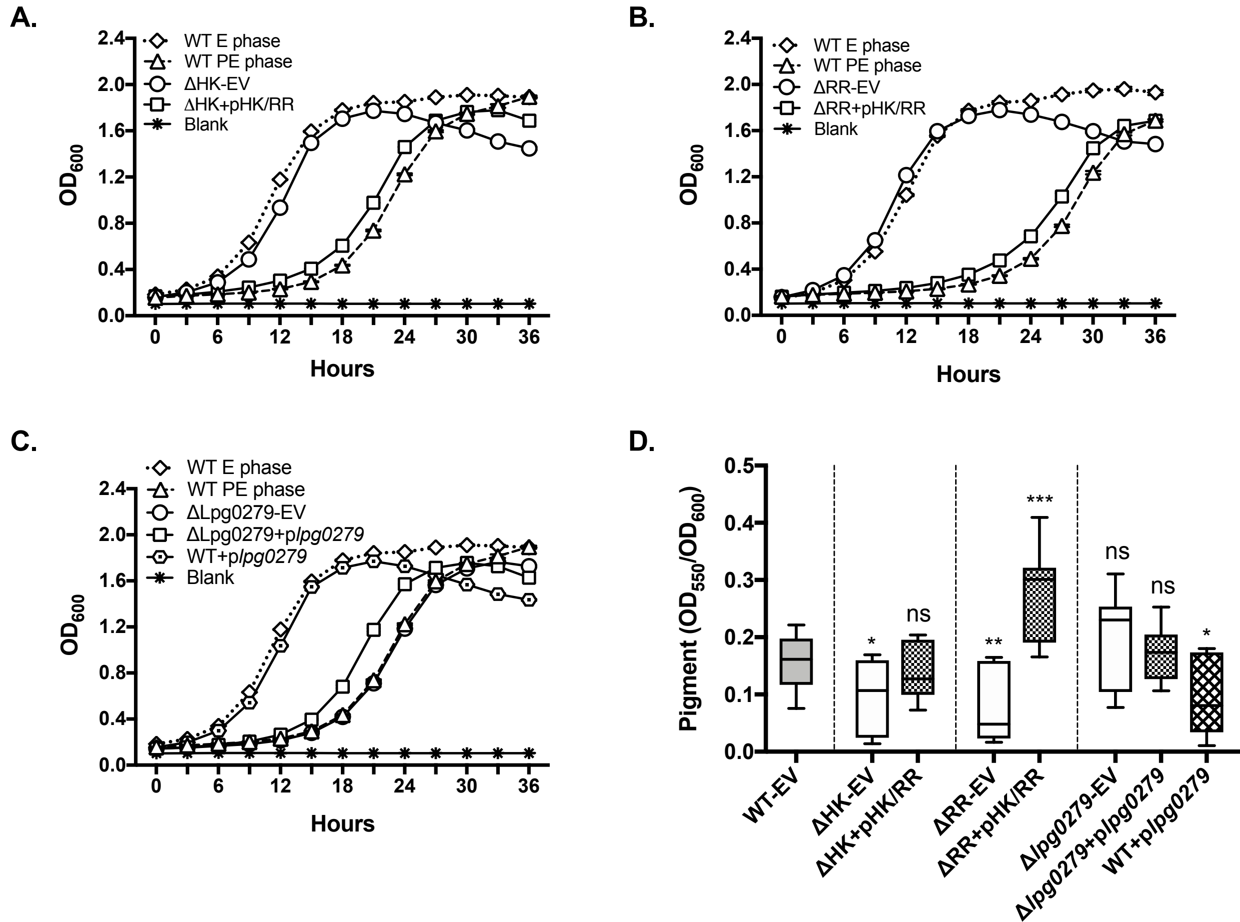


Fig. 2.7. *L. pneumophila* lacking a complete TCS or ectopically expressing *lpg0279* resemble WT E phase cells.

A.-C. The growth kinetics of WT *L. pneumophila* inocula in E phase (dotted lines) or PE phase (dashed lines) was compared to PE phase inocula of **A.** an ΔHK mutant and its complement, **B.** an ΔRR mutant and its complement, and **C.** an Δ*lpg0279* mutant and its complement, together with a WT strain of *L. pneumophila* constitutively expressing *lpg0279*. With the exception of the WT E phase reference culture ($OD_{600} < 2.0$), all strains were cultured overnight in AYET medium to $OD_{600} > 3.5$ (corresponding to WT PE phase cultures), then diluted to a starting OD_{600} of ~ 0.1 in AYET and incubated for 36 h in a Bioscreen growth curve analyzer set at 37°C with continuous shaking; OD_{600} measurements were obtained at 3 h intervals. Shown are means ± SE of triplicate samples, and data shown are representative of three independent experiments.

D. Pigment accumulation in late PE phase cultures of ΔHK, ΔRR and Δ*lpg0279* mutants and the corresponding complemented strains and of WT L. pneumophila constitutively expressing *lpg0279*. Supernatants of broth cultures maintained in PE phase for 1 or 3

days were collected by centrifugation, their absorbance at OD_{550} quantified and then normalized to cell density (OD_{600}). Results shown are the means ± SE of pooled data from three independent experiments, with duplicate readings obtained for each measurement. Box encompasses the 25th to 75th percentiles, and whiskers range from the maximum to minimum values with the line at the median. A two-tailed Student's *t*-test was used to determine statistically significant differences in pigmentation compared to WT-EV (**ns**: not significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). **EV**: strain harbors the pMMB206cam empty vector.

The TCS facilitates PHB production and long-term survival in low-nutrient conditions

Beginning in the PE phase, *L. pneumophila* generates large poly-3-hydroxybutyrate (PHB) inclusions, a reserve carbon and energy source that is abundant in MIF cells (47, 145) and enhances persistence of *L. pneumophila* in low-nutrient environments (48). Therefore, we next quantified the PHB content of WT and the ∆RR mutant using the lipophilic dye Nile Red, a fluorescent dye that specifically stains intracellular lipids, including PHB (146). To determine the baseline value, the fluorescence of E phase cultures in AYET was quantified. Next, after collecting WT and ∆RR mutant cells by centrifugation, expression of *lpg0279-77* was induced by resuspending each cell sample in CDM medium lacking L-cysteine (Fig. 2.6B) and incubating the bacteria for 24 h at 37°C with aeration before a second PHB quantification. As expected, in E phase the Nile Red PHB signal for both WT and ∆RR was negligible. However, after 24 h of nutrient limitation the WT cells exhibited significantly greater fluorescence, whereas the PHB signal in the ∆RR mutant was diminished (Fig. 2.8A). Expression by the mutant of the TCS from pHK/RR not only fully remedied this defect, but also generated a PHB signal exceeding that of the WT strain. Therefore, the TCS promotes accumulation of PHB storage granules in *L. pneumophila*.

Collectively these results suggest the TCS equips replicating *L. pneumophila* to respond to nutrient limitation by promoting transition to the PE phase, production of pigment, and accumulation of PHB storage granules—all traits that increase resilience in the environment. Moreover, for broth cultures of *L. pneumophila* that lack TCS

function, the OD_{600} values decreased in the PE phase, perhaps indicating cell lysis. Therefore, we next investigated whether this TCS facilitates *L. pneumophila* survival during prolonged exposure to low-nutrient conditions. To do so, we quantified CFUs of WT and ∆RR mutant cells first in E phase and then again after 3 and 7 d incubation in CDM lacking L-cysteine, as described for the Nile Red fluorescence experiments. Indeed, compared to WT, the ∆RR strain suffered a greater loss of viability by day 7, a defect remedied by ectopic expression of the TCS locus (Fig. 2.8B). Therefore, *L. pneumophila* persistence in nutrient-limited conditions is enhanced by the TCS genes, including *lpg0277* which encodes an enzyme equipped to modulate levels of the second messenger c-di-GMP.

Figure 2.8

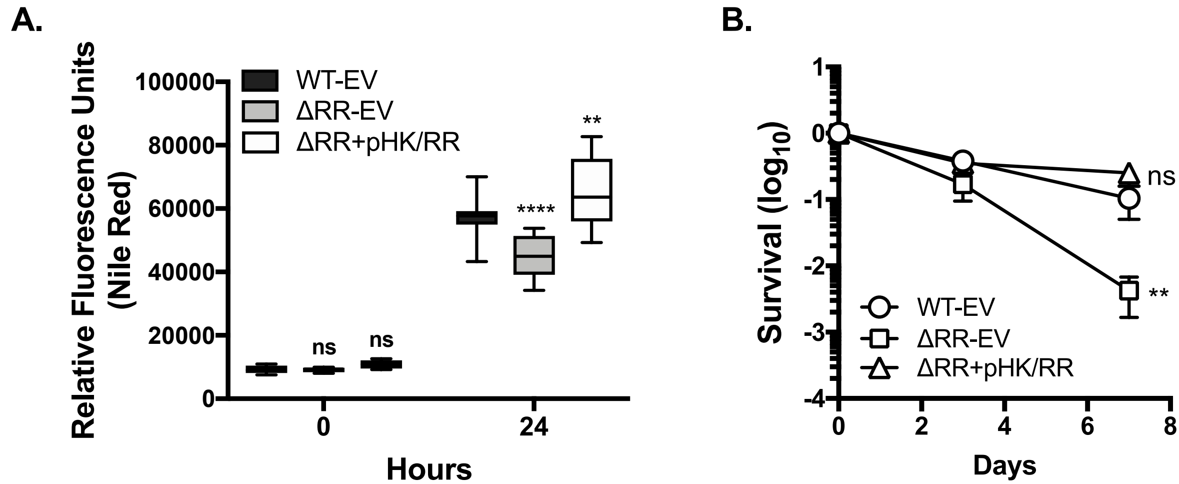


Fig. 2.8. The TCS promotes PHB production and long-term viability.

E phase cultures of WT *L. pneumophila*, the ΔRR mutant, and the ΔRR complemented strain harboring pHK/RR were collected by centrifugation, resuspended in CDM medium lacking L-cysteine, and incubated at 37°C on an orbital shaker for up to 7 d.

A. Quantification of PHB content before and after 24 h CDM exposure. Results shown are the means ± SE of pooled data obtained from triplicate samples in four independent experiments. Box encompasses the 25th to 75th percentiles, and whiskers range from the maximum to minimum values with the line at the median. A two-tailed Student's *t*-test was used to determine statistically significant differences in fluorescence compared to WT-EV (**ns**: no significance; **, $p < 0.01$; ****, $p < 0.0001$).

B. Survival was quantified by plating serial dilutions of the cultures indicated and enumerating CFUs before (titer) and after 3 and 7 d incubation. Shown are ratio of CFU(day)/CFU(titer), with symbols representing the means ± SE of pooled data obtained from duplicate samples in four independent experiments. The Mann-Whitney test was used to determine statistically significant differences in survival compared to WT-EV (**ns**: no significance; **, $p < 0.01$). **EV**: strain harbors the pMMB206cam empty vector.

The GGDEF domain of Lpg0277 promotes transition from E to PE phase

Because the RR is a bifunctional enzyme with both DGC and PDE activity (56), we next examined which of these opposing functions accounts for the ΔRR mutant phenotypes. To do so, we took advantage of the known contributions of the GGDEF and EAL amino acid motifs to DCG and PDE activity, respectively (55, 63). To abrogate DGC function, we engineered a point mutant strain in which the conserved Glu-396 residue was replaced with Lys (56), generating the RR^{E396K} allele. Likewise, to impair PDE activity, the Glu-521 residue in the EAL domain was replaced with Ala, creating the RR^{E521A} allele (52). After confirming the DNA sequence of each *L. pneumophila* chromosomal point mutation, the corresponding mutant strains were transformed with either the complementing plasmid pHK/RR or the empty vector.

We first examined the growth kinetics and pigment production of the RR^{E396K} DCG and RR^{E521A} PDE point mutants after culturing to an OD_{600} > 3.5, correlating with PE phase in WT cells. The RR^{E396K} DCG mutant resembled E phase WT cells, as judged by its minimal lag phase (Fig. 2.9A) and decreased pigmentation (Fig. 2.9C), two defects that were complemented by ectopic expression of the WT TCS. In contrast, the RR^{E521A} PDE mutant mimicked WT PE phase cells in growth kinetics (Fig. 2.9B), and its pigment production exceeded that of the WT strain (Fig. 2.9C). Thus, the transition of replicating *L. pneumophila* to the PE phase is promoted by the DGC activity of the RR, which generates c-di-GMP (27).

To further probe TCS function, we genetically abrogated the ability of the RR to be phosphorylated by its cognate HK, a post-translational modification that induces a change in RR enzymatic activity (56). For this purpose, the conserved Asp-87 residue in the putative phosphoacceptor site of the RR was replaced with Asn, generating the

mutant strain RR^{D87N} (56). This strain exhibited growth and pigmentation defects similar to that observed for both the ΔRR and RR^{E396K} mutants (Fig. 2.9D and E), indicating a functional link between TCS phosphorylation and RR DGC activity.

Given the contribution of DGC activity to differentiation of replicating *L. pneumophila* to the PE phase and concomitant pigmentation, we investigated whether DGC activity also promotes accumulation of PHB and survival of nutrient-deprived bacteria (Fig. 2.8). To do so, we exposed E phase cultures of the RR^{E396K} DGC, RR^{E521A} PDE, and RR^{D87N} phosphoacceptor point mutants to CDM lacking L-cysteine, and then quantified PHB production via Nile Red fluorescence and survival via CFU enumeration. As expected, PHB accumulation by the RR^{E521A} PDE mutant was indistinguishable from WT *L. pneumophila*, and both strains survived well after 7 d exposure to CDM lacking L-cysteine (Fig. 2.10A). However, the RR^{E396K} DCG and RR^{D87N} phosphoacceptor point mutants each had reduced PHB content, as judged by Nile Red fluorescence (Fig. 2.10B and C). Both strains also lost viability after extended CDM exposure, a phenotype mimicking the ΔRR strain (Fig. 2.10B and C, and 2.8B). It is notable that these defects were only partially complemented by ectopic expression of the TCS locus, perhaps due to pleiotropic effects of perturbed cellular c-di-GMP pools. Nevertheless, the phenotypic profile of each point mutant is consistent with a model in which DCG activity and concomitant accumulation of cyclic-di-GMP stimulates replicating *L. pneumophila* to transition to PE phase and generate PHB stores that support bacterial survival in nutrient-limited conditions.

Figure 2.9

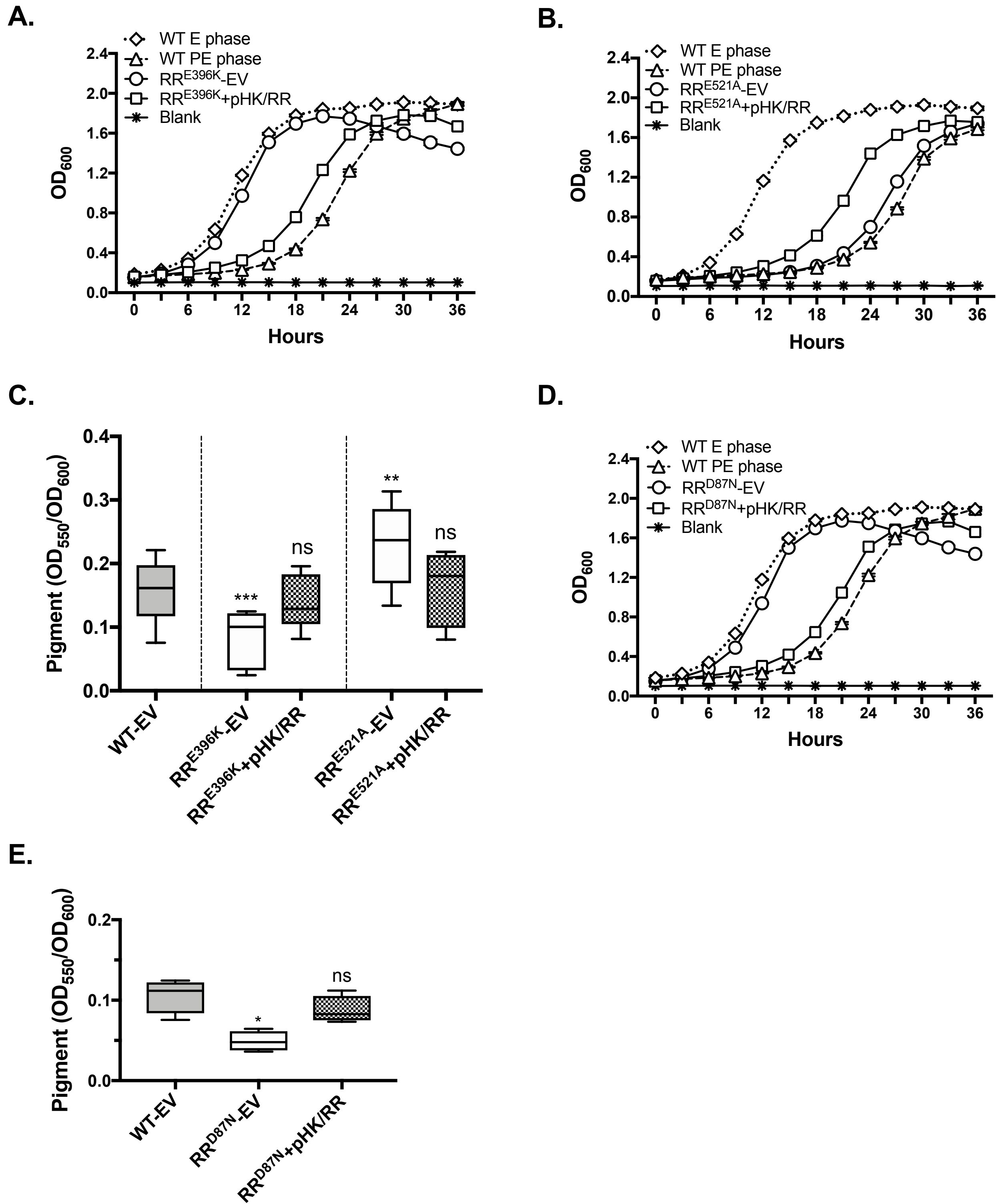


Fig. 2.9. The DCG activity of RR Lpg0277 promotes transition to PE phase.

A.-B. Growth kinetics in AYET of WT L. pneumophila inocula in E (dotted lines) or PE phase (dashed lines) was compared to PE phase inocula of **A.** RR^{E396K} point mutant and **B.** RR^{E521A} point mutant strains. Symbols denote the means ± SE of triplicate samples (short error bars are masked by symbols), and data are representative of three independent experiments.

C. Pigment accumulation by WT L. pneumophila after maintenance in PE phase for 1-3 days, compared with the RR^{E396K} DGC and RR^{E521A} PDE point mutants and their respective complemented strains. Results shown are the means ± SE of pooled data obtained from duplicate samples in three independent experiments. A two-tailed Student's *t*-test was used to determine statistically significant differences in pigmentation compared to WT-EV (ns: not significant; ***p* < 0.01; ***; *p* < 0.001). **EV**: strain harbors the pMMB206cam empty vector.

D.-E. Comparison of **D.** growth kinetics and **E.** pigment production by WT L. pneumophila and a RR^{D87N} phosphoacceptor site point mutant and the complemented strain. Results shown are representative of one other independent experiment. The Mann-Whitney test was used to determine statistically significant differences in pigmentation compared to WT (**ns**: not significant; *, *p* <0.05). Boxes in **C.** and **E.** encompass the 25th to 75th percentiles, and whiskers range from the maximum to minimum values with the line at the median. **EV**: strain harbors the pMMB206cam empty vector.

Figure 2.10

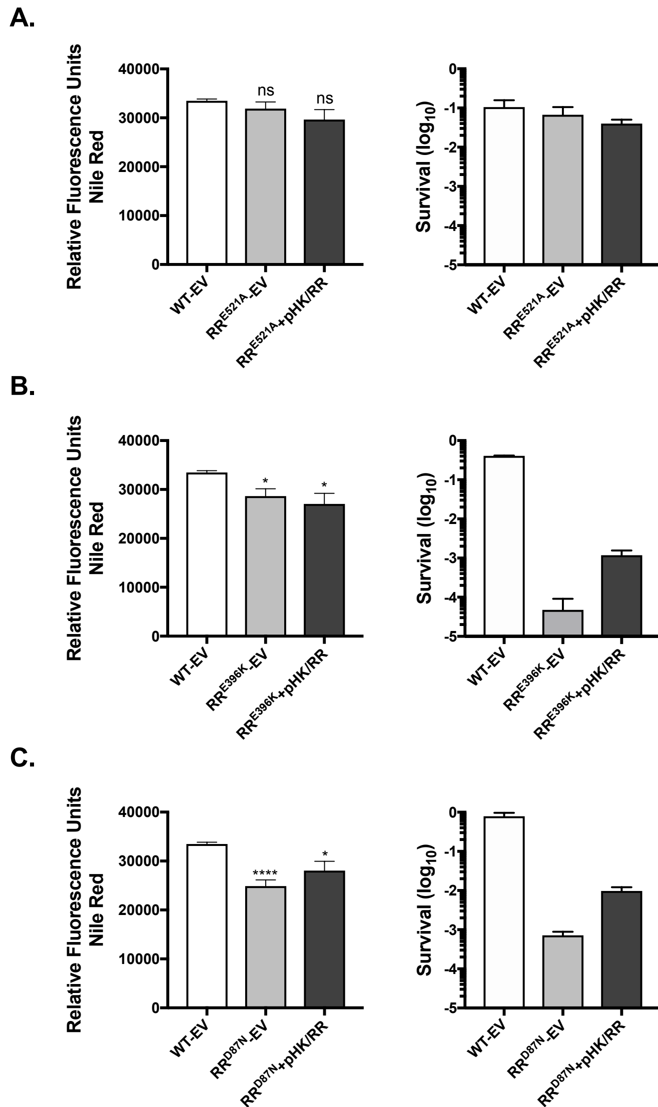


Fig. 2.10. The GGDEF domain and phosphoacceptor site of the RR contribute to PHB production and promote survival during prolonged nutrient deprivation.

PHB quantification and survival of E phase cultures after 24 h and 7 d L-cysteine deprivation, respectively. WT *L. pneumophila* were compared to **A.** RR^{E521A} PDE, **B.** RR^{E396K} DCG, and **C.** RR^{D87N} phosphoacceptor site point mutants, and the respective complemented strains bearing plasmid pHK/RR. PHB values represent the means ± SE of pooled Nile Red fluorescence data from triplicate samples in four independent experiments. A two-tailed Student's t-test was used to determine statistically significant differences in fluorescence compared to WT-EV (**ns**: no significance; *, $p < 0.05$; ****, $p < 0.0001$). Survival data is the ratio of CFU(day 7)/CFU(titer), and data is representative of results obtained in two or more independent experiments. **EV**: strain harbors the pMMB206cam empty vector.

Ectopic expression of *lpg0279* counteracts TCS function

The spatial proximity and co-regulation of *lpg0279* with the TCS-encoding genes *lpg0277* and *lpg0278* (Fig. 2.2) suggests a regulatory interaction. Although expression of the *lpg0279-77* operon is induced by RpoS (Fig. 2.5), we examined whether the product of *lpg0279* functions as an independent transcriptional regulator of the downstream TCS genes. In particular, we used RT-qPCR to analyze *lpg0278-77* mRNA levels in WT cells ectopically expressing *lpg0279* from IPTG-inducible plasmid p*lpg0279*. Despite a ~ 4-fold increase in *lpg0279* transcript compared to WT *L. pneumophila*, there was minimal change in *lpg0278-77* expression (Fig. 2.11A). Accordingly, we have no evidence that Lpg0279 alters *lpg0278-77* mRNA levels.

However, since constitutive expression of *lpg0279* in a WT background phenocopies the growth and pigmentation defects of the ΔRR and RR^{E396K} DCG mutants (Fig. 2.7 and 2.9), we postulated that Lpg0279 may function post-translationally as a negative regulator of TCS activity. To test this hypothesis, we transformed the RR^{E521A} PDE point mutant—which resembles WT in the transition from E to PE phase (Fig. 2.9B and C)— with plasmid p*lpg0279*. In parallel, we also transformed the RR^{E396K} mutant with p*lpg0279* to evaluate any additive effects of loss of DGC activity and gain of Lpg0279 function. After culturing both strains to an OD_{600} > 3.5 in the presence of 250 uM IPTG, we performed growth curve and pigmentation analyses. As expected, expression of *lpg0279* did not restore the capacity of the RR^{E396K} DCG mutant cells to differentiate to the PE phase, as judged by their minimal lag phase (Fig. 2.12A). However, *lpg0279* expression significantly shortened the lag phase of the RR^{E521A} PDE mutant to that of WT E phase cells (Fig. 2.12B). Assessment of pigment production yielded similar results, with *lpg0279* expression reducing pigment levels in the RR^{E521A}

PDE mutant but having no effect on the RR^{E396K} DCG mutant cells (Fig. 2.11B). Furthermore, when exposed to CDM lacking L-cysteine for 7 d, both the WT and RR^{E521A} PDE mutant strains harboring p*lpg0279* suffered a significant drop in cell viability compared to the respective parent strains (Fig. 2.11C). Accordingly, we favor a model in which the product of *lpg0279* negatively regulates the c-di-GMP pools generated by the TCS. The second messenger c-di-GMP then stimulates *L. pneumophila* to switch from a replicative state to a more resilient cell type better equipped to survive in low-nutrient environments.

Figure 2.11

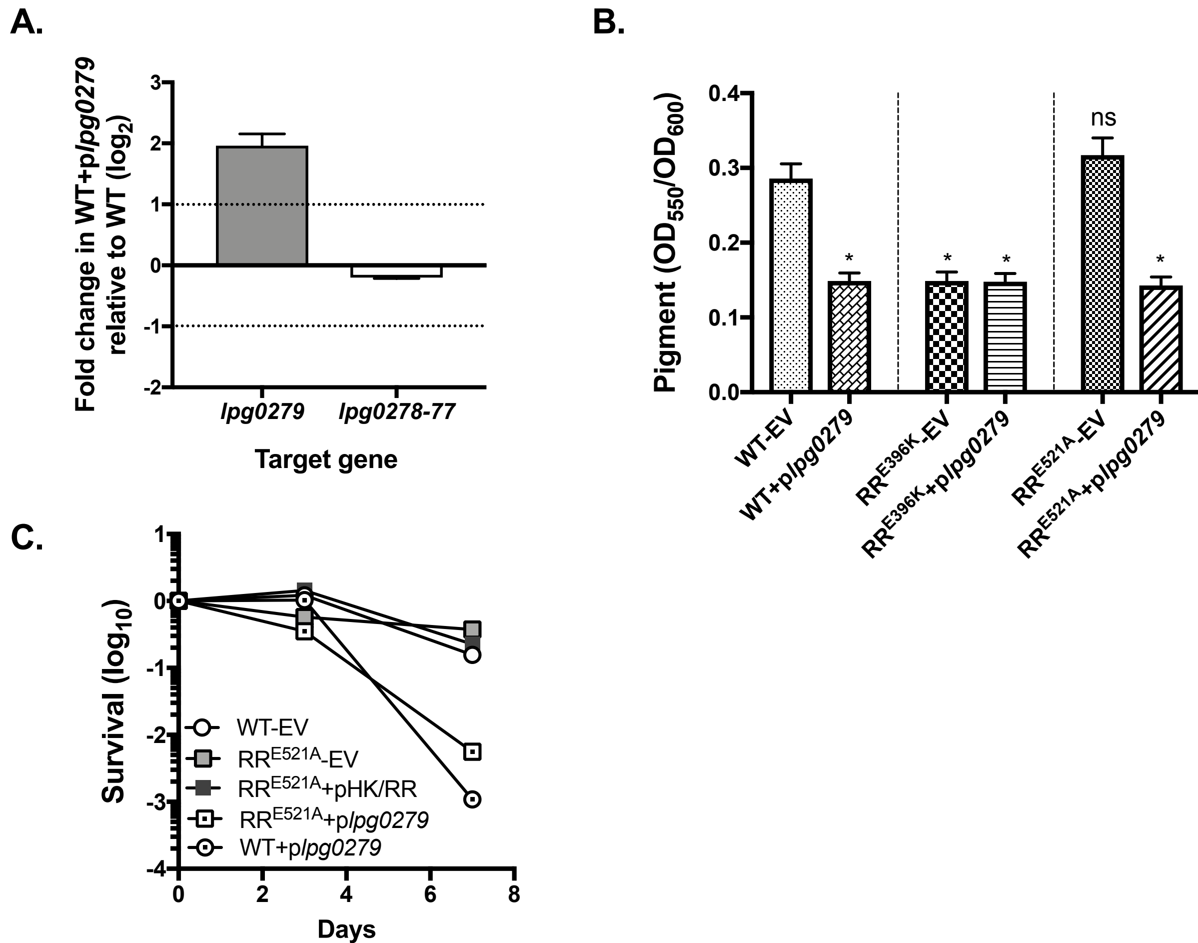


Fig. 2.11. Constitutively expressed lpg0279 is epistatic to RR^{E521A} PDE mutation.

A. RT-qPCR analysis of lpg0279 and lpg0278-77 gene expression in a strain constitutively expressing lpg0279 from an IPTG-inducible plasmid (plpg0279). Values calculated using the $2^{-\Delta\Delta Ct}$ method normalized to 16S ribosomal RNA expression and presented as Log2 fold-change relative to WT Lp02. Data presented are the means ± SE of triplicate samples performed in duplicate; similar results were obtained in at least one additional experiment. Dotted line represents the Log2 fold-change significance threshold of 1.0.

B. Pigment accumulation by PE phase WT L. pneumophila and the Lpg0277 RR^{E396K} CDG and RR^{E521A} PDE point mutants that do or do not constitutively express plpg0279. Shown are the means ± SE of four samples and are representative of results obtained in one other independent experiment. The Mann-Whitney test was used to determine statistically significant differences in pigmentation compared to WT (ns: not significant; *, $p < 0.05$).

C. Survival of WT L. pneumophila and the RR^{E521A} PDE mutant that do or do not constitutively express plpg0279 or the complementing pHK/RR plasmid. All strains were cultured in AYE to E phase, resuspended in CDM without L-cystine, and incubated at 37°C on an orbital shaker for up to 7 days. At the times shown, culture aliquots were serially diluted for CFU enumeration on CYET. Symbols shown are the ratio of CFU(day)/CFU(titer) of duplicate samples, and data is representative of results obtained in three independent experiments. EV: strain harbors the pMMB206cam empty vector.

Figure 2.12

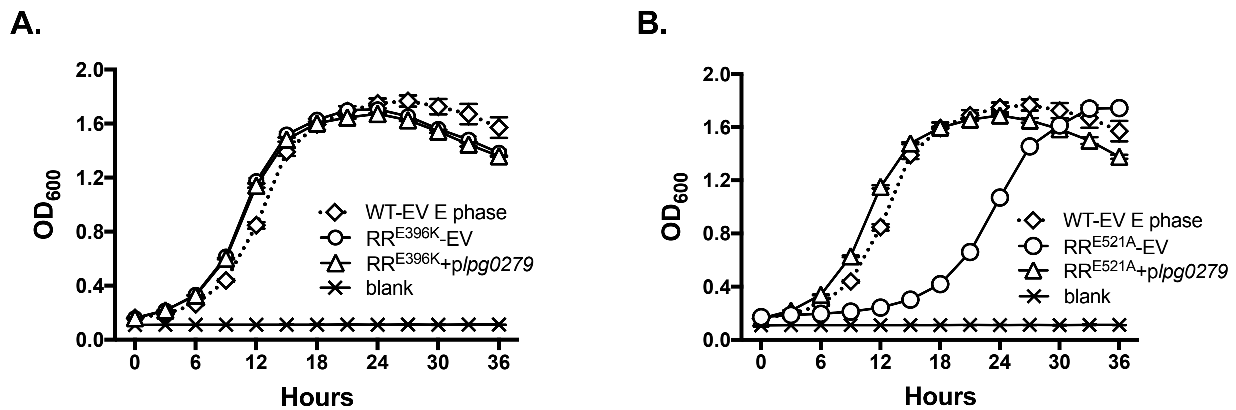


Fig. 2.12. Disruption of the Lpg0277 RR^{E521A} PDE domain is not sufficient to suppress the differentiation defect of PE phase *L. pneumophila* constitutively expressing *lpg0279*.

Growth kinetics of PE phase WT *L. pneumophila* and the **A.** Lpg0277 RR^{E396K} DCG and **B.** RR^{E521A} PDE point mutants that harbor either p*lpg0279* or pMMB206 empty vector (EV). Symbols denote the means ± SE of triplicate samples, and data shown are representative of one additional independent experiment.

DISCUSSION

As an intracellular pathogen, *L. pneumophila* has evolved multiple mechanisms to survive and replicate in a wide variety of environments, ranging from freshwater protozoans and human lung macrophages to nutrient-poor natural or engineered water systems. To thrive in such diverse conditions, *L. pneumophila* responds to environmental stimuli by alternating between distinct cell types. Amino acid or fatty acid starvation triggers replicating *L. pneumophila* to transition to a highly motile and infectious transmissive form, and prolonged starvation stimulates further development to the hardy MIF cell type (8, 24, 47, 147). Using an *in vitro* culture model to analyze the switch between replicative, transmissive, and resilient cell types, here we identify as a regulatory component an operon designed to regulate cyclic-di-GMP metabolism. This operon consists of *lpg0279*, which encodes a protein of unknown function that is abundant in MIF cells (46), and *lpg0278-lpg0277*, which encodes a two-component system (TCS) (56). Together, Lpg0279 and the TCS equip *L. pneumophila* to respond to nutrient deprivation by differentiating to a non-replicative resilient cell type that generates pigment, accumulates PHB storage granules, and maintains viability.

The *lpg0279-77* operon is induced by the stationary phase sigma factor RpoS in response to nutrient limitation (Fig. 2.2 to 2.6). Indeed, to survive prolonged amino acid limitation, *L. pneumophila* require not only a functional TCS (Fig. 2.8B) but also RpoS (148). Thus, RpoS equips *L. pneumophila* to express factors that enhance resilience in nutrient-poor environments, in part by promoting TCS-mediated production of c-di-GMP (122, 148-150).

A second messenger molecule, c-di-GMP is a wide-spread regulator of multiple bacterial physiological processes, including biofilm formation, cell cycle progression,

and virulence gene expression (55, 151-153). The RR encoded by *lpg0277* is a bifunctional enzyme whose DCG and PDE domains can generate and degrade c-di-GMP production, respectively (84). When *L. pneumophila* Philadelphia-1 cells experience nutrient deprivation, activation of the TCS is predicted to increase c-di-GMP levels, based on several genetic tests of RR function. In particular, point mutations in either the RR DGC domain (Fig. 2.9A and C, 2.10B) or its phosphoacceptor site (Fig. 2.9D and E, 2.10C) phenocopy the ΔRR mutant (Fig. 2.7B and D, 2.8), whereas the PDE domain point mutant resembles WT (Fig. 2.9B and C, 2.10A). Our observations are consistent with the studies by Pecastaings and colleagues of this locus in the *L. pneumophila* Lens strain: after 5 days culture on solid bacteriology medium, mutants lacking the homologous RR Lpl0329 contain less intracellular c-di-GMP than do WT cells (60). Thus, in non-replicating *L. pneumophila* cells, the DCG activity of RR Lpl0329 likely predominates. On the other hand, using proteins purified from the *L. pneumophila* Lens strain, Levet-Paulo and colleagues demonstrated that phosphorylation of the RR Lpl0329 reduced its DCG activity but left PDE activity unaltered (56). These biochemical experiments suggest that the TCS phosphorelay can decrease the local c-di-GMP level. Perhaps the differences between these *in vivo* studies and *in vitro* biochemical studies indicate that the enzymatic activity of RR Lpl0329 can be modulated not only through phosphorylation by its cognate HK, but also by other regulatory factor(s) that do not co-purify with the HK or RR proteins.

One factor that does functionally interact with the TCS is Lpg0279, a protein that is conserved among *L. pneumophila*, abundant in MIF cells (46), and encoded on the *lpg0279-0277* mRNA (Fig. 2.2). Consistent with a function in MIF cells, *L. pneumophila* do not require Lpg0279 to transition from E to PE phase in broth. However,

constitutive expression of *lpg0279* prevents replicating WT *L. pneumophila* from differentiating to the PE transmissive form, similar to loss of TCS function (Fig. 2.7). Moreover, our analysis of single and double mutant strains predicts that the MIF protein Lpg0279, by an unknown mechanism, reduces c-di-GMP output from the TCS (Figs. 2.11 and 2.12).

One clue to Lpg0279 function is its F-box and Intracellular Signal Transduction (FIST) domain, first recognized in 2007 as a component of signaling pathways in diverse prokaryotic and eukaryotic species (154). In *Pseudomonas aeruginosa*, the FIST domain of protein Pa1975 (NosP) senses nitric oxide (NO) and inhibits autophosphorylation of it co-cistronic histidine kinase (155). This decreases phosphotransfer and activation of downstream effectors, ultimately promoting biofilm dispersal. Similar indirect mechanisms involving NO-responsive regulation of downstream histidine kinases have been identified in *Vibrio harveyi* (156) and *Shewanella oneidensis* (157, 158), the latter involving a class of NO sensors deemed Haem-Nitric oxide/Oxygen (H-NOX) binding proteins. In *L. pneumophila*, an H-NOX protein (Hnox1) is genetically and functionally linked to another bi-functional GGDEF-EAL protein, Lpg1057; together this protein pair directly regulates biofilm formation (87). By analogy to these systems, a model that warrants testing is that Lpg0279 modulates TCS production of c-di-GMP in response to nitric oxide stress, potentially by regulating HK autokinase activity.

In response to nutrient limitation, the TCS induces accumulation of two factors that enhance persistence of environmental *L. pneumophila*: pyomelanin (Fig. 2.9C) and PHB (Fig. 2.8A). Derived via polymerization of homogentisic acid (HGA), pyomelanin is a soluble extracellular pigment that not only protects *L. pneumophila* from the

damaging effects of light (143), but also possesses ferric reductase activity that contributes to iron uptake (144). A recent study by Levin and colleagues (159) reveals that secreted HGA itself possesses antimicrobial activity. To generate a reserve energy source, *L. pneumophila* increases production of PHB lipid granules at the transition to PE phase (48, 145). PHB formation is a complex process that is not fully understood but involves numerous enzymatic steps including the generation of the precursor acetyl-CoA (145). In the environmental di-morphic bacterium *Bdellovibrio bacteriovorus*, an acyl-CoA dehydrogenase central to energy metabolism was recently demonstrated to directly and specifically bind to c-di-GMP (160). Thus, it is conceivable that in *L. pneumophila* c-di-GMP alters PHB stores indirectly by modulating generation of PHB precursors. Alternatively, a key enzyme in PHB generation, such as PHB polymerase (145), could be a c-di-GMP effector. However, since *L. pneumophila* that lack the RR still generate some PHB, this biosynthetic pathway is likely controlled by multiple regulators. More detailed biochemical and genetic studies can identify the downstream effectors of RR-generated c-di-GMP. Candidates for the TCS regulon include not only genes involved in pyomelanin and PHB biosynthesis, but other *L. pneumophila* genes induced in response to nutrient limitation (161).

Considering our genetic data in the context of the current literature, we favor the following working model for the signal transduction pathway encoded by *lpg0279-77* (Fig. 2.13). When conditions are favorable for *L. pneumophila* replication, the TCS is expressed at a low basal level and the Lpg0279 protein suppresses any production of cyclic-di-GMP, perhaps by inhibiting HK autophosphorylation. When nutrients become scarce, the stationary phase sigma factor RpoS increases transcription of the *lpg0279-77* operon. In addition, modulation of TCS function by Lpg0279 is relieved, possibly by NO

or another stress signal, and subsequent phosphorelay from the HK to the RR amplifies DCG activity. Accumulation of cellular c-di-GMP promotes differentiation by PE phase *L. pneumophila*, production of pigment and PHB, and survival in nutrient-poor conditions.

This study extends the understanding of the regulatory circuit that governs the *L. pneumophila* life cycle. When nutrients become limiting within host cells, the stringent response alarmone ppGpp coordinates differentiation of intracellular *L. pneumophila* to a motile, infectious form equipped for transmission between host cells. In nutrient poor environments, the second messenger cyclic-di-GMP promotes *L. pneumophila* differentiation into a cell type equipped for extracellular persistence. Defining c-di-GMP regulatory networks is a challenging endeavor, due to the spatial and temporal sequestering of c-di-GMP signaling, as well as the multiple enzymes that contribute to c-di-GMP metabolism in *L. pneumophila* and other bacteria (55). Accordingly, this genetic analysis of the signal transduction system comprised of the Lpg0279 MIF protein and the Lpg0278-Lpg0277 TCS can guide future molecular and biochemical studies to delineate how c-di-GMP promotes resilience of environmental *L. pneumophila*.

Figure 2.13

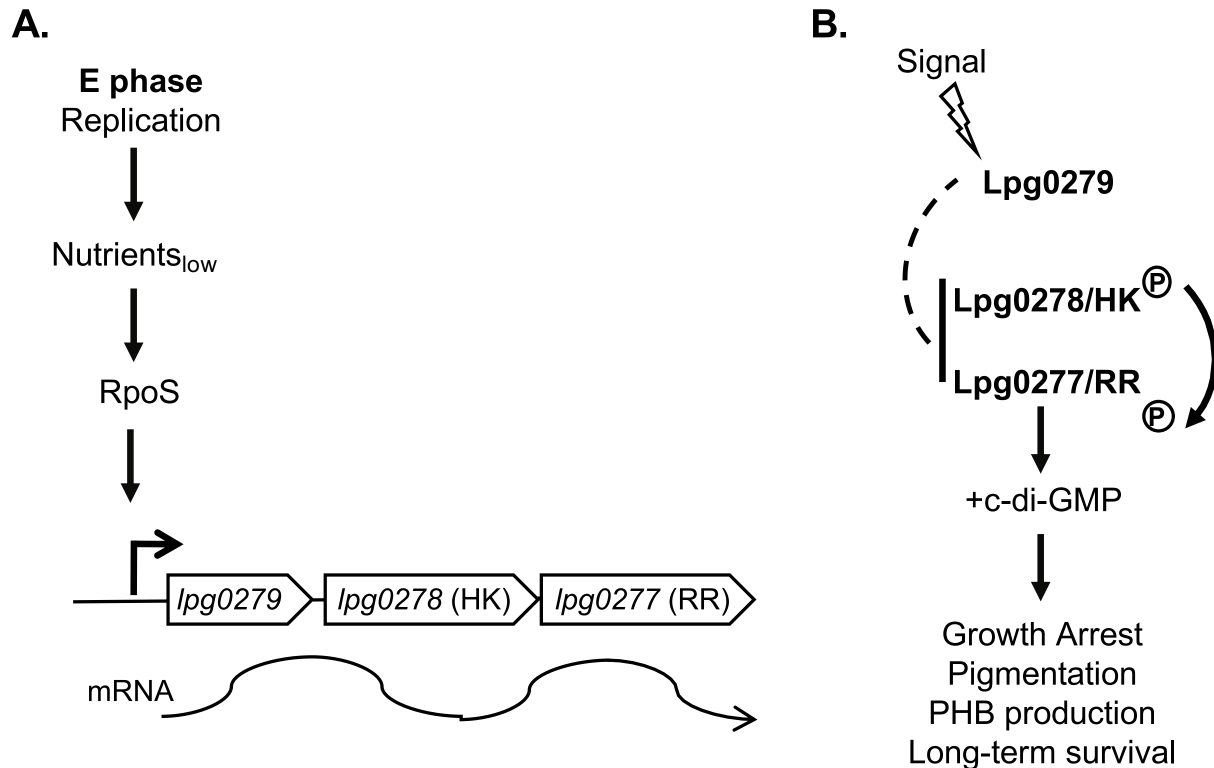


Fig. 2.13. Model for lpg0279-77 regulation of *L. pneumophila* differentiation.

A. When amino acids become limiting, RpoS equips replicating L. pneumophila to induce transcription of the lpg0279-0277 operon which encodes a putative repressor and a Two Component System.

B. Lpg0279 suppresses signaling by the Lpg0278/0277 TCS. In response to prolonged environmental stress, the TCS is derepressed and HK Lpg0278 phosphorylates RR Lpg0277. The DCG domain of RR Lpg0277 generates c-di-GMP, which arrests L. pneumophila replication, triggers accumulation of pigment and PHB storage granules, and promotes survival in nutrient-poor environments. Dashed line represents predicted function of Lpg0279 based on genetic tests.

MATERIALS AND METHODS

Bacterial strains and culture conditions

The bacterial strains utilized in this study are listed in Supplementary Material **Table S1**. Except where indicated, all *L. pneumophila* strains were cultured in ACES (Sigma)-buffered yeast extract (AYE) broth at pH = 6.9, supplemented with 0.1 mg/ml thymidine, 0.4 mg/ml L-cysteine, and 0.135 mg/ml ferric nitrate (AYET), or on solid medium containing AYET supplemented with 15 g/L agar and 2 g/L charcoal (CYET). Chemically defined medium (CDM) was prepared as previously described (139), except that ferric pyrophosphate was omitted and either L-cysteine/L-cystine, L-methionine, or L-serine where indicated. Where necessary for plasmid maintenance, media were supplemented with chloramphenicol (5 μg/ml) and/or kanamycin (10 μg/mL). All *E. coli* strains were cultured using Luria-Bertani (LB) broth or agar, supplemented where necessary with ampicillin (100 ug/ml), chloramphenicol (25 μg/ml) or kanamycin (25 μg/ml). To induce gene expression from the pMMB206cam plasmid, 250 μM isopropyl β-D-1-thiogalactopyranoside (IPTG; Gold Biotechnology) was added to growth media; all pMMB206cam-harboring strains used in experiments were exposed to IPTG from the point of initial inoculation.

Bacteria from frozen stocks were struck onto CYET plates (plus IPTG and/or antibiotics where necessary) every 1-2 weeks and incubated at 37°C for ≥ 3 d until colonies developed. For experiments, colonies were inoculated into AYET and cultured overnight at 37°C on an orbital shaker to exponential (E) phase ($OD_{600} < 2.5$) and then subcultured in AYET for a second overnight incubation until the desired growth stage: E phase or post-exponential (PE) phase ($OD_{600} > 3.5$).

Plasmids and primers

All plasmids and primers utilized in this study are listed in Supplementary Material **Table S2**. Plasmid p*lpg0279-gfp* was constructed by amplifying the 832 bp directly 5' of the *lpg0279* ORF using primers EH21 and EH43, which encode BamHI and XbaI restriction sites, respectively. After digestion, the fragment was then ligated into the GFP reporter plasmid pBH6119, 5' of a promoterless *gfpmut3* gene (24, 131). Plasmids pHK/RR and p*lpg0279* were constructed by amplifying either a 3.6 kb fragment containing *lpg0278* through *lpg0277* using primers EH69 and EH70 or a 1.2 kb fragment containing *lpg0279* using primers 79OE-F and 79OE-R, which each encode BamHI and HindIII restriction sites; primer 79OE-F also encodes an optimal ribosome binding site (84). Following restriction enzyme digestion, the fragments were ligated into the IPTG-inducible plasmid pMMB206cam. Correct placement and orientation of each insert was verified by PCR and/or DNA sequencing.

Mutant strain construction

The laboratory strain Lp02, a thymidine auxotroph derived from the clinical isolate Philadelphia-1 (4), was utilized as the parent strain for all constructs. Deletion mutants were generated by homologous recombination as previously described (162) using the primers listed in **Table S2**. Briefly, each gene of interest along with ~ 700 bp of 3' and 5' flanking DNA was amplified and cloned into the vector pGEM-T Easy (Promega) to create pGEM*lpg0277*, pGEM*lpg0280*, and pGEM*lpg0279*. The kanamycin cassette from pKD4 was amplified using primers comprised of the oligos PO and P2 along with ~ 36 bp of DNA sequence homologous to the regions 3' and 5' of each gene of interest. Following allelic exchange in the *E. coli* λ-red recombinase strain DY330,

candidate colonies were screened by PCR and transformed into *E. coli* host strain DH5α. Point mutants RR^{E396K}, RR^{E521A}, and RR^{D87N} were created using the QuikChange XLII Site-Directed Mutagenesis Kit (Agilent) with plasmid pGEM*lpg0277* serving as a template and using primers sets E396K-F/E396K-R, E521A-F/E521A-R, and D87N-F/D87N-R, respectively. The recombinant alleles (*lpg0277::kan*, *lpg0278::kan* and *lpg0279::kan*) and point mutant alleles were amplified by PCR using each relevant primer pair (77del-F/77del-R, 78del-F/78del-R or 79del-F/79del-R) and introduced into Lp02 by natural transformation. Where indicated in **Table S1**, the kanamycin cassette was subsequently excised by Flp recombinase as previously described (163). All mutations were confirmed by DNA sequencing.

Transformation with the plasmids identified in **Table S2** was conducted by electroporating isolated plasmid DNA (Qiagen) into 50 μl competent cells at 1.8 kV, 100 W and 25 μF using 1 mm cuvettes. Cells were then transferred to 950 μL AYET and incubated at 37°C for 1 h on an orbital shaker before plating on selective media. Also constructed were control strains that carry the corresponding pBH6119 or pMMB206cam empty vector.

RNA isolation

To isolate RNA for analysis, 0.5-1.0 ml of bacterial culture in AYET at $OD_{600} > 3.0$ was collected by centrifugation at 12,000 x *g*. The pellet was resuspended in an equal volume of TRIzol reagent and then purified using the Direct-zol RNA MiniPrep kit (Zymo Research). All RNA preparations were treated with DNase I Amplification Grade

or Turbo DNA-free (Invitrogen), and absence of genomic DNA was confirmed by PCR and gel electrophoresis.

End-point PCR experiments

To determine whether *lpg0279*, *lpg0278*, and *lpg0277* are co-transcribed, 800 ng of total RNA was used as a template to generate cDNA with the iScript cDNA Synthesis Kit (Bio-Rad). End-point PCR was then conducted using primer sets EH13/EH14 and EH1/EH2, which span the *lpg0279-lpg0278* and *lpg0278-lpg0277* intragenic regions, respectively. For the end-point PCR experiment examining co-transcription of *lpg0280* and *lpg0279*, cDNA synthesis was coupled with PCR amplification using 800 ng RNA, primer set EH55/56, and the SuperScript III One-Step RT-PCR System with Platinum *Taq* DNA Polymerase (Invitrogen). For all experiments, genomic DNA was used as a positive control, and reactions omitting the reverse transcriptase enzyme served as a negative control.

Reverse transcription quantitative PCR (RT-qPCR)

RT-qPCR was used to determine the expression levels of *lpg0279* (primer set EH101/102) and *lpg0278-77* (primer set EH122/EH123) in an Δ*rpoS* mutant and a strain constitutively expressing *lpg0279* (from IPTG-inducible plasmid p*lpg0279*), relative to WT Lp02. All strains were cultured to PE phase in AYET before RNA extraction and cDNA preparation as described above (except using 200 ng RNA for cDNA synthesis). qPCR was performed with an ABI 7500 Fast System, using iTaq Universal SYBR Green Supermix (Bio-Rad), following the manufacturer's reaction setup and thermocycling protocol. Samples were run in triplicate, using 1 ul of cDNA per

reaction. Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method (164), with 16S rRNA as the endogenous control.

Growth curves

Bacterial growth kinetics were analyzed by culturing *L. pneumophila* to E or PE phase in AYET as indicated, then collecting 1 ml aliquots by centrifugation at 5,000 x *g* for 5 minutes. The pellet was resuspended to an OD_{600} of ~ 0.1 in 1 ml fresh AYET supplemented with chloramphenicol and ITPG, and 250 μl aliquots were dispensed into triplicate wells of a sterile 100x Honeycomb Plate (Fisher Scientific). The plates were transferred to a Bioscreen C plate reader and incubated for 36 h at 37°C with continuous shaking, with OD_{600} measurements taken at 3 h intervals.

Pigmentation

To analyze pigment accumulation, strains were cultured as described above to PE phase and then incubated at 37°C for an additional 1-3 days. Next, 0.5 ml samples were centrifuged at 16,000 x *g* for 5 min, 200 μl aliquots of each supernatant were placed in a 96-well plate, and then their absorbance at OD_{550} was quantified on a plate reader. To normalize pigment values to cell density, each cell pellet was resuspended in PBS to its original volume, and then the OD_{600} of 100 μl aliquots was quantified on a plate reader. All measurements were performed in duplicate.

GFP transcriptional reporter experiments

To analyze activity of the *lpg0279-0277* promoter, strains EH224, EH97 and EH102 which each harbor plasmid p*lpg0279-gfp* were cultured overnight to E phase. These cultures were then diluted to an OD_{600} of ~ 0.8 in either AYE media, or CDM lacking either L-cysteine/L-cystine, L-serine, or L-methionine, as indicated. The bacterial suspensions were then supplemented aseptically with 0.135 mg/ml ferric nitrate (AYE only), and/or 2.27 mM, 1.14 mM or 0.7 mM L-cysteine, as indicated. All cultures were then further incubated at 37°C for 10-12 h on an orbital shaker. Measurements were taken at 2-3 h intervals by centrifuging 800 μl aliquots, resuspending the pellet in an equal volume of PBS, and quantifying fluorescence of triplicate 200 μl samples at $485_{EX}/528_{EM}$ on a Biotek plate reader. To normalize all fluorescence readings to cell density, the OD_{600} of a 1/10 dilution of each cell suspension was quantified with a spectrophotometer.

PHB measurement by Nile Red staining

To analyze intracellular lipid (PHB) content, 4-6 ml aliquots of E phase cultures were first collected by centrifugation (5 min at 5,000 x *g*) and the cell pellets resuspended in an equal volume of CDM lacking L-cysteine/L-cystine, and supplemented with thymidine (0.1 mg/ml), chloramphenicol (25 μg/ml), and IPTG (250 μM). Cultures were then incubated for 24 h at 37°C on an orbital shaker. PHB content was quantified for the initial E phase cultures and again following the 24 h incubation using the fluorescent dye Nile Red (Invitrogen) as described (48), with the following modifications. Briefly, aliquots of bacterial cultures were collected by centrifugation and resuspended in an equal volume of deionized water before fixing the

cells with 1% (v/v) formaldehyde at room temperature for 30 min. After washing to remove the formaldehyde, cell density was adjusted to OD_{600} = 0.5 in 1 ml of deionized water, and the cells were stained by adding 1 μl of a 25 mM Nile Red stock solution suspended in DMSO. The cells were incubated at room temperature in the dark for 1 h, and then 200 μl aliquots were measured in triplicate on a Biotek plate reader at $545_{EX}/600_{EM}$.

Survival assay

To assess long-term survival of *L. pneumophila* in the absence of L-cysteine, cultures prepared as for PHB measurement described above were incubated at 37°C on an orbital shaker for 7 days. At the times indicated, duplicate samples were removed, serially diluted, and plated to enumerate CFUs on CYET.

TABLES

Table 2.1. Strains used in this study

Strain	**Relevant properties**	**Source**
E. coli		
DH5α	*supE44 ΔlacU169 (80 lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Laboratory collection
DY330	W3110 *ΔlacU169 gal490 λcI857* Δ(*cro-bioA*)	(165)
EH207	DH5α p*lpg0279-gfp*	This work
EH272	DH5α p*lpg0279-77*	This work
EH140	DH5α p*lpg0279*	This work
L. pneumophila	**Relevant properties**	**Source**
MB110	Lp02 wild type; *thyA hsdR rpsL* (Str^{r})	(166)
EH284	Lp02 pMMB206cam	This work
EH286	Lp02 Δ*lpg0277*::*FRT* pMMB206cam	This work
EH276	Lp02 Δ*lpg0277*::*FRT* p*lpg0278-77*	This work
EH357	Lp02 Δ*lpg0278*::*FRT* pMMB206cam	This work
EH352	Lp02 Δ*lpg0278*::*FRT* p*lpg0278-77*	This work
EH350	Lp02 Δ*lpg0279*::*FRT-kan-FRT* pMMB206cam	This work
EH160	Lp02 Δ*lpg0279*::*FRT-kan-FRT* p*lpg0279*	This work
EH151	Lp02 p*lpg0279*	This work
EH344	Lp02 $Lpg0277^{E396K}$ pMMB206cam	This work
EH311	Lp02 $Lpg0277^{E396K}$ p*lpg0278-77*	This work
EH346	Lp02 $Lpg0277^{E521A}$ pMMB206cam	This work
EH314	Lp02 $Lpg0277^{E521A}$ p*lpg0278-77*	This work
EH102	Lp02 p*flaA-gfp*	This work
EH224	Lp02 p*lpg0279-gfp*	This work
EH97	Lp02 pBH6119	This work
MB410	Lp02 Δ*fliA::kan*	(29)
MB443	Lp02 Δ*rpoS::kan*	(27)
MB696	Lp02 Δ*relA::kan*	(26)
MB413	Lp02 Δ*letA::kan*	(29)
MB416	Lp02 Δ*letS::kan*	(29)
EH358	Lp02 Δ*fliA* p*lpg0279-gfp*	This work
EH360	Lp02 Δ*rpoS* p*lpg0279-gfp*	This work

EH366	Lp02 Δ*relA* p*lpg0279-gfp*	This work
EH362	Lp02 Δ*letA* p*lpg0279-gfp*	This work
EH364	Lp02 Δ*letS* p*lpg0279-gfp*	This work
MB1230	Lp02 Δ*letA::kan* p*flaA-gfp*	(29)
EH370	Lp02 Δ*letS::kan* p*flaA-gfp*	This work
MB1234	Lp02 Δ*rpoS::kan* p*flaA-gfp*	(27)
MB1014	Lp02 Δ*relA::kan* p*flaA-gfp*	(26)
EH368	Lp02 Δ*fliA::kan* p*flaA-gfp*	This work
EH373	Lp02 Lpg0277$^{\text{E521A}}$ p*lpg0279*	This work
EH375	Lp02 Lpg0277$^{\text{E396K}}$ p*lpg0279*	This work

Table 2.2. Primers and plasmids used in this study

Primers		
Construction of *lpg0279-gfp*		
EH21	AAA**GGATCC**AAATATGGTTCACACCCTGAAAAACTC GCAAC	Amplify 5' of *lpg0279* adding BamHI and XbaI
EH43	AAA**TCTAGA**ATTGCTGTGACTTGGATTTATCTTATG ATTAATTATAG	
Construction of complementing vector p*lpg0278-77*		
EH69	TATA**GGATCC**ATGACAGAAATGCATCGGTTGTTGC	Amplify *lpg0278-lpg0277* adding BamHI and HindIII
EH70	AAAT**AAGCTT**TTATTTTGTCCTATTTCCTTGCAGAAG TTTTTC	
Construction of vector p*lpg0279* (optimal RBS in *italics*)		
79OE-F	AAA**GGATCC***GAAGGAGATATACAT*TCACAGCAATAT GATGAAAATTGAATCATTTC	Amplify *lpg0279* adding BamHI and HindIII
79OE-R	AAA**AAGCTT**CTTTTTGGTTTATGGACTCTCTAACAG GGTCG	
Construction of deletion mutants (PO and P2 oligos from pKD4 underlined)		
77del-F	ACTGTCCGCTATAAAAACCATGCTTGACGAAAAC	Amplify *lpg0277* + ~700bp flanking DNA
77del-R	AAGAGAGTATGAAGAACTTGTTACAACGCATGGTGG	
77PO-F	TTTGCCCATCAATCCCAAAACCTTGAGTACGAGGTAG AATGTGTAGGCTGGAGCTGCTTC	Amplify Kan cassette with homology to *lpg0277*
77P2-R	CGGATAGCTCTTATAGATGCCAATGCGATTTTTATCC ATATGAATATCCTCCTTAGTTCC	
78del-F	GAAAAATTGTAGACCATTGCATTGGGGCAGTAGG	Amplify *lpg0278* + ~700bp flanking DNA
78del-R	TTGAGTAATTGCCTTGACATATACTGAATGAGTTGGG	
78PO-F	TAGAGAGTCCATAAACCAAAAAGGAATTCAGTAATA ATGTGTGTAGGCTGGAGCTGCTTC	Amplify Kan cassette with

78PO-R	AAAGATTCAGTACTCATTTCTACCTCGTACTCAAGGCATATGAATATCCTCCTTAGTTCC	homology to *lpg0278*
79del-F	AATACTTAAAGGAAACCCATCCGACCATTCAAGC	Amplify *lpg0279* + ~700bp flanking DNA
79del-R	GTTTTCGTCAAGCATGGTTTTTATAGCGGACAG	
79PO-F	ACTATAATTAATCATAAGATAAATCCAAGTCACAGCAATTGTGTAGGCTGGAGCTGCTTC	Amplify Kan cassette with homology to *lpg0279*
79PO-R	TGCATTTCTGTCATTATTACTGAATTCCTTTTTGGTCATATGAATATCCTCCTTAGTTCC	
Construction of point mutants		
E396K-F	AAAGCATTACAAACTTATCCCCACCAAGTCTGGCG	
E396K-R	CGCCAGACTTGGTGGGGATAAGTTTGTAATGCTTT	
E521A-F	TCCACCGGATTAATGCTGCCATGGAACGAATCTCA	
E521A-R	TGAGATTCGTTCCATGGCAGCATTAATCCGGTGGA	
D87N-F	GGGGCATTCTTATGTTAACAAAAGCGAGTGGGTATG	
D87N-R	CATACCCACTCGCTTTTGTTAACATAAGAATGCCCC	
qPCR		
EH102	CAATCACTGCCTCCCAAATC	123 bp internal to *lpg0279*
EH103	ACTCTCTAACAGGGTCGTAATG	
EH122	GCAAAGGAGCTCAATTTGTATTAG	115 bp spanning *lpg0278-lpg0277*
EH123	GATTGCCGGGTTGTCATC	
Other oligos		
EH13	TCGGCAGAAGATTGGTATTAGG	Fig 2.2A, set A/B
EH14	GCAATAAATTGAGTCCAACTCTCC	
EH1	GGATCTGGGTGGTACACTTTATG	Fig 2.2A, set C/D
EH2	CAATGCCTTGAGTCGCTACA	
EH55	TGAGTACTATCGATGCCATAAAATCTCT	Fig 2.2A, set E/F
EH56	AGGCTTGAGCCAATTCTTCAATT	

Plasmids		
pGEMT-easy	Cloning vector; Amp^R	Promega
pMMB206cam	Broad host range vector; pMMB66EH derivative; Cam^R	(19)
pKD4	Source of *FRT-kan-FRT* allele	(167)
pBH6119	~800 bp XbaI/PstI fragment (containing GFP gene from p*GFPmut3*) inserted into pJB98 at XbaI and PstI sites; Amp^R; tdΔi	(24)
p*flaA-gfp*	pBH6119 with ~300 bp of *flaA* promoter region cloned into *Bam*HI and *Xba*I sites; GFP transcriptional reporter plasmid	This work
p*lpg0279-gfp*	pBH6119 with ~830 bp of the *lpg0279-77* promoter region cloned into *Bam*HI and *Xba*I sites; GFP transcriptional reporter plasmid	This work
pHK/RR	pMMB206cam with ~3.6 kb PCR product of the *lpg0278* through *lpg0277* ORFs cloned into *Bam*HI and *Hind*II sites; IPTG-inducible plasmid; Cam^R	This work
p*lpg0279*	pMMB206cam with ~1.2 kb PCR product containing the *lpg0279* ORF and optimal RBS cloned into *Bam*HI and *Hind*II sites; IPTG-inducible plasmid; Cam^R	This work

CHAPTER 3

Discussion and Future Directions

Introduction

Years of dedicated research have illuminated the complex processes involved in coordinating the intracellular switch between a replicative and transmissive state in the waterborne pathogen *Legionella pneumophila*. Studies examining this biphasic switch have identified multiple cellular factors including enzymes such as RelA and SpoT, the stringent response alarmone ppGpp, alternate sigma factors such as FliA and RpoS, and two-component systems including LetA/S (8). However, there has been a shortage of research in two key areas: the role of the nucleotide messenger c-di-GMP in the *L. pneumophila* life cycle and the genetic pathways and regulatory factors that support extracellular survival. The first deficiency is remarkable given the number of genes in the *L. pneumophila* genome that code for enzymes that make and break c-di-GMP (84). In fact, the *L. pneumophila* Philadelphia-1 genome codes for 22 different c-di-GMP metabolizing enzymes (84), and despite the plasticity of the *Legionella* genome, most of these genes are conserved in at least two other strains of *L. pneumophila*, Paris and Lens (Fig. 1.2). This conservation indicates that c-di-GMP likely plays a significant role in multiple cellular processes. The second deficiency in understanding *L. pneumophila* extracellular survival is unfortunate given the ubiquity of this bacterium in both natural and engineered water sources, especially in light of the difficulty in remediating contaminated systems.

In this dissertation, I show that a two-component system (TCS) encoded by *lpg0278-lpg0277*, which generates c-di-GMP, promotes *L. pneumophila* cellular differentiation from the replicative to stationary phase in a broth model. I also show that this TCS contributes to survival in low-nutrient conditions. As the gene expression profile of *in vitro* cultures reproduces the intracellular replicative to transmissive switch in *L. pneumophila* (12), this TCS therefore represents an additional regulatory element governing this pathogen's intracellular life cycle. Additionally, I demonstrate that a MIF-related protein (Lpg0279) likely acts as a regulator of the TCS, representing the first link between MIF cells and cyclic-di-GMP metabolism in *L. pneumophila* (Fig. 2.13). These results and a few promising avenues for further research will be detailed below.

MIF cell development

MIF cells are highly infectious yet metabolically dormant "cyst-like" cells that develop only in amoeba and other ciliated protozoans, which are *Legionella*'s natural host. As *L. pneumophila* cells that survive grazing by amoeba in the environment are likely to have also been generated within amoeba (*i.e.*, are not progeny from macrophages), this environmentally resistant form of *L. pneumophila* is likely the predominant cell type colonizing water systems and causing disease in humans. Thus, an understanding of the genetic mechanisms that drive MIF cell development would be valuable from both a medical and engineering standpoint. As the TCS consisting of Lpg0278-77 is associated with a protein that is prevalent in MIF cells (Lpg0279), to what extent does this locus contribute to MIF formation in *L. pneumophila*? Unfortunately, our current inability to generate MIF cells in broth cultures (or in

macrophages), and the lack of reliable MIF-specific differentiation markers, complicates their study in the lab (8). Most of the research done to date has focused on the structural characteristics and enhanced stress resistance of MIF cells, and only a few MIF-associated genes have been identified and even fewer have been fully characterized. One such gene is *magA* (MIF-associated gene). Although the MagA protein is almost 7x more abundant in MIF cells than *in vitro* stationary phase cells, mutational studies indicate it is not required for MIF generation (168). This could be due to genetic redundancy, contribution to non-structural processes that aren't identified by the assays employed, or MagA could simply be dispensable for either MIF generation or function despite the abundancy. The latter seems plausible as *magA* is encoded on a mobile pathogenicity island (LpPI-1) that is unique to the Philadelphila-1 strain (168), and already shown to increase fitness in macrophages and enhance oxidative stress resistance *in vitro* (169). Lpg0279 has also been identified as abundant in MIF cells (8, 46), but unlike *magA*, the *lpg0279* gene is also present in the Lens, Paris, Alcoy, and Corby strains of *L. pneumophila* (Fig. 1.2). Also, as there was no defect associated with loss of *lpg0279* in our *in vitro* assays, this protein may be more relevant to MIF morphogenesis in a protozoan host than MagA.

Interestingly though, expression of *magA* is dependent on a protein that itself has been identified as essential for full MIF cell differentiation—Integration Host Factor (IHF) (170). IHF belongs to a class of DNA-binding proteins that can modify DNA structure and thereby alter efficiency of cellular processes such as replication, transcription, and translation (171, 172). Not only are MagA protein levels diminished in an IHF mutant, those cells fail to replicate in amoeba and lack several distinctive morphologies associated with the MIF form such as an altered cell membrane and

prominent PHB inclusions (170). The latter phenotype is particularly relevant to our findings as *L. pneumophila* lacking the Lpg0278-77 TCS, which we hypothesize is regulated by the MIF-related protein Lpg0279, accumulate less PHB (Fig. 2.8A). Even though our GFP-reporter work (Fig. 2.5A), qPCR data (Fig. 2.5C) and *in silico* analysis (Fig. 2.3) confirm the role of RpoS in promoting *lpg0279-77* expression, further study could investigate whether IHF acts as an additional regulator of this locus. Such co-regulation is feasible as expression of *magA* (and *ihf* itself in an autoregulatory manner) is jointly controlled by IHF and RpoS (170). Furthermore, possible IHF binding sites (based on the *E. coli* consensus sequence) are located in the *lpg0279* promoter region, which are similar to those identified in the promoter regions for *ihfA* and *ihfB* in *L. pneumophila* (138)(Fig. 3.1). One method of examining this potential relationship is by comparing *lpg0279* and/or *lpg0278-77* mRNA transcripts by qPCR in an Δ*ihf* mutant and WT strain. If the results of the qPCR suggest IHF involvement in *lpg0279-77* expression, identifying the specific binding site would be a logical follow-up. There are several approaches to exploring protein-DNA interactions, including traditional Electrophoretic Mobility Shift Assays (EMSA) to more sophisticated high-throughput measures (173).

Figure 3.1

A.

5'-TTAAAAAATGGCAAAATCGCGATAATTAACAGGAATGAATCTTTTATGAAAATAATTGGCTTTATCA
IHF #1
CAAAAAACATTCGGGTTCATATAACAGATTTTTATCACTAATATTGCAATAGACAGAACCTCCACTTCAT
IHF #2
TGATCCTTTTGAACATATACTATAATTAATCATAAGATAAATCCAAGTCACAGCAAT*ATGATGAAAATT*
RpoS IHF #3 *lpg0279*

B.

E. coli IHF-binding consensus sequence	(A/T)ATCAANNNNTT(A/G)
L. pneumophila IHF-binding in *ihfA* #1	**TATCCACAAATAA**
L. pneumophila IHF-binding in *ihfA* #2	**AATCAGAAATACA**
L. pneumophila IHF-binding in *ihfB* #1	**AATCAAATGG**GAA
L. pneumophila IHF-binding in *lpg0279* #1	**TATCACAAAA**AAC
L. pneumophila IHF-binding in *lpg0279* #2	**TATCACTAATATT**
L. pneumophila IHF-binding in *lpg0279* #3	**AATCA**T**AAGATAA**

Fig. 3.1. Candidate IHF-binding sites in the *lpg0279* promoter region

A. Sequence consisting of ~200 nt directly 5' of the *lpg0279* start codon, indicating three potential IHF-binding sites (underlined).

B. Comparison the *E. coli* IHF-binding consensus sequence with the three sites identified in the *ihfA* and *ihfB* promoter regions in *L. pneumophila* that bind recombinant IHF (138), and the potential sequences in the *lpg0279* promoter region labeled in **A.** Nucleotides that match the consensus sequence are in bold.

RpoS regulation

As noted above, a common thread between *lpg0279, ihf*, and *magA* is that they all code for proteins that are abundant in MIF cells, and all are regulated by RpoS. In fact, Δ*rpoS* mutants themselves do not differentiate into MIF cells, which could explain why they either fail to replicate or are digested within protozoan hosts (34, 45). One interesting facet of RpoS in *L. pneumophila* is that, unlike other bacteria such as *E. coli* and *V. cholerae*, it is dispensable for stationary-phase associated stress resistance, despite the correlation between RpoS activity and increased production of the stress alarmone ppGpp (33, 34). Neither is RpoS required for *L. pneumophila* replication within human macrophages (34, 132). Thus, it is logical to infer that RpoS primarily regulates traits that promote survival in the extracellular niche such as differentiation to the MIF form, the cell type that survives grazing by amoeba in the natural environment.

As *lpg0279-77* expression is clearly controlled by RpoS, one unanswered question is whether this locus, and *lpg0279* in particular, is required for MIF differentiation in protozoan cells. Loss of *lpg0279* does not affect *in vitro* growth or the transition to stationary phase as evidenced by pigment production (Fig. 2.7C and D). Because overexpression of *lpg0279* does produce growth and survival defects similar to what occurs with loss of the co-regulated *lpg0278-77* (Fig. 2.7, 2.8 and 2.11C), we surmised that Lpg0279 initially acts as a negative inhibitor of the TCS. However, given that the *lpg0279* is a MIF-associated protein, it is conceivable that this protein might also contribute to additional developmental pathways inside a protozoan host. Thus, follow-up experiments could examine the ability of the Δ*lpg0279* mutant to successfully replicate within amoeba or to differentiate into cells that possess the morphological traits characteristic of MIF cells as assessed by electron microscopy (47). Such

experiments examining the contribution of *lpg0279* to MIF generation would provide further insight into the role of this gene in the *L. pneumophila* developmental cycle, apart from its hypothesized regulation of the c-di-GMP-generating TCS encoded by *lpg0277-78*.

Planktonic survival and/or VBNC state

In addition to being required for MIF-cell development, RpoS is also essential for survival of planktonic *L. pneumophila* in water (148). In this respect, it makes sense that the RpoS regulon includes the *lpg0279-77* locus, which supports survival in low-nutrient aqueous conditions in the absence of a host cell. Thus, this work not only identifies a genetic pathway that contributes to survival in the water environment, it is the first to examine how c-di-GMP signaling in *L. pneumophila* impacts cellular processes other than biofilm regulation or intracellular infection.

Based on our data, we believe that the bifunctional GGDEF/EAL response regulator (RR) encoded by *lpg0277* acts primarily as a DCG enzyme under the conditions examined, and this activity increases following phosphorylation by its cognate histidine kinase (HK) encoded by *lpg0278* (Fig. 2.13). This finding is in agreement with other studies that show a decrease in c-di-GMP upon deletion of the *lpg0277* gene (60, 84). Interestingly, Pecastaings *et al.* (60) discovered that *lpg0277* was involved in biofilm regulation, specifically biofilm dispersal, despite contributing to an increase in cellular c-di-GMP pools. This is contrary to most biofilm-forming bacteria that utilize c-di-GMP, where low levels are associated with motility and high levels contribute to sessility and biofilm formation (59).

L. pneumophila in the environment exist either as intracellular parasites of amoeba or other protozoans, as residents of multi-species biofilms, or as free-living bacteria. Given that *lpg0277* (and by extension the TCS) appears dispensable for replication in amoeba (85) and promotes biofilm dispersal (60), it is possible that c-di-GMP generated by the TCS is important for *L. pneumophila* extracellular survival in the planktonic state. This is supported by my data showing loss of viability in a Δ*lpg0277* mutant exposed to low-nutrient conditions (Fig. 2.8B).

One additional possibility is that the full *lpg0279-77* locus promotes entry into the VBNC state, a transition that is more successful for MIF cells than their *in vitro* stationary phase counterparts (174). In a study examining the effects of starvation on VBNC development, a much larger proportion of MIF cells (70-90%) survived the transition compared to stationary phase cells (5-20%) (174). Furthermore, VBNC cells derived from MIFs retained their unique morphology and high degree of stress resistance (174). Although VBNCs are a challenge to study due to the characteristic inability to be cultured on standard media and lack of protocols to trigger efficient resuscitation, any remediation protocol designed to counter *L. pneumophila* contamination should account for the likely large percentage of MIF-derived VBNC cells in that system. Confirming the contribution of the *lpg0279-77* locus to either MIF formation and/or the VBNC state would establish an important link between cyclic-di-GMP and the generation of a resilient form of the pathogen in a free-living, planktonic state.

Final considerations

One drawback to being among the first to characterize a genetic locus is that there is little experimental data from other labs to draw upon. Although the bifunctional enzyme encoded by *lpg0277* has been investigated for its contribution to biofilm regulation, and the enzymatic relationship between the Lpg0277 RR and its cognate Lpg0278 HK has been investigated biochemically, the work provided here is the first to examine the relationship between this TCS and a MIF-associated protein (Lpg0279) and identify the contribution of this locus to both *L. pneumophila* cellular differentiation and extracellular persistence.

As noted above, further research could examine the connection between Lpg0279 and/or the TCS to other cellular factors involved in regulating the *L. pneumophila* life cycle (such as IHF), and contribution of the *lpg0279-77* locus to MIF or VBNC development. In addition, there are a few peculiarities with respect to this locus that could be the subject of additional studies, particularly from a biochemical standpoint. My initial interest in Lpg0279 arose from its identification as a protein that was prevalent in MIF cells, but not readily detectable in progeny from macrophages (8, 46), which suggests that *lpg0279* is only expressed in MIF cells (175). However, my RNA and GFP experimental data belies that conclusion, as it clearly shows that *lpg0279* is co-transcribed with *lpg0278-77* upon transition to PE phase *in vitro*. So how to explain this discrepancy? Bacteria are masters at controlling gene expression, and it is not uncommon for co-cistronic genes to be translated at different rates (176). Perhaps *lpg0279* is initially translated at low levels, at a rate sufficient for Lpg0279 to block c-di-GMP synthesis by the TCS until an unknown signal alleviates this repression, as suggested by our model (Fig. 2.13). Then perhaps this directional control is reversed in

later stages of differentiation and the TCS affects *lpg0279* expression. An intriguing model involves cyclic-di-GMP produced by the TCS enhancing translation of *lpg0279* mRNA in protozoan cell. For example, the 5' UTR of *lpg0279* could possess a translation riboswitch that is "turned on" by binding c-di-GMP. This altered activity, which would not be evident from an *in vitro* or macrophage infection assay, could explain the increase in Lpg0279 protein in MIF cells.

There would be two potential outcomes of a system in which *lpg0279* translation is temporally regulated. One, the increase in *lpg0279* translation following TCS de-repression could entail a repurposing of this protein to contribute to MIF cell generation by an unknown mechanism. For example, Lpg0279 in this context could act as a transcription factor, or function in a signal transduction cascade that promotes MIF-related traits. The presence of a FIST domain in Lpg0279 suggests such activity could be responsive to cellular NO levels. Thus Lpg0279 would function as a "moonlighting" protein—an increasingly recognized phenomenon in which proteins have multiple, often unrelated, functions in a cell (177). The second outcome is that Lpg0279 continues to act to modulate c-di-GMP produced by the TCS. Due to the spatial and temporal sequestering of c-di-GMP signaling pathways, mutations in DCG or PDE enzymes often do not result in measurable changes in c-di-GMP levels, even when there is a corresponding phenotype (60). However, as noted by Pecastaings *et al.* (60), the enzymatic activity of Lpg0277 has the potential to alter cellular pools of c-di-GMP. Thus, tight control of Lpg0277 enzymatic function would be essential. Although Lpg0277 does possess the allosteric I-site that provides one means of reducing its DCG activity, ongoing regulation by Lpg0279 in the model just described would provide an additional layer of feedback control, ensuring a cap to c-di-GMP production.

Another area that remains to be addressed includes the intersection of c-di-GMP generated by Lpg0277 with the alarmone ppGpp that drives the stringent response, as both messenger molecules utilize GTP as a precursor. Despite the widespread utilization of both ppGpp and c-di-GMP in prokaryotes, only a few studies have examined the crosstalk between them. In *Mycobacterium smegmatis*, these molecules collaborate to promote lipid metabolism that is critical for antibiotic resistance (178). Perhaps a similar relationship exists in *L. pneumophila*, where both molecules are required for successful transition from a replicative to transmissive state. Alternately, competition over (and depletion of) the pool of GTP could act as a signal for other metabolic processes (179).

The research detailed in this dissertation represents the first characterization of a c-di-GMP utilizing signal transduction pathway that promotes both *L. pneumophila* cellular differentiation and environmental persistence. It provides a foundation for future studies aimed at elucidating the contribution of this messenger molecule to the *L. pneumophila* life cycle. Furthermore, the link between the c-di-GMP metabolizing TCS encoded by *lpg0278-77* and the MIF-related protein encoded by *lpg0279* indicates that c-di-GMP may play a major role in promoting the shift to this environmentally resilient cell type. Determining the key regulatory factors that promote MIF generation would be invaluable from both a control and remediation standpoint. It is possible that the elusive MIF-specific genetic marker may be a downstream effector of the *lpg0279-77* pathway, which would enable specific environmental surveillance of this cell type. Collectively, this work provides the groundwork for developing improved monitoring and control methods that will aid in the ongoing battle to eliminate pathogenic *L. pneumophila* from the built environment.

APPENDIX

How *Legionella* Defend Their Turf

Elisa D. Hughes and Michele S. Swanson

eLife 2019, doi: 10.7554/eLife.48695

Legionella pneumophila is a water-borne bacterium that takes up residence in engineered water systems, and if inhaled by susceptible people, it can cause a severe form of pneumonia known as Legionnaires' disease. According to the Centers for Disease Control and Prevention, *L. pneumophila* infections are on the rise and are now the leading cause of water-associated illness in the United States. However, a major problem is that decontamination methods often fail to eradicate every single bacterium, which allows populations of bacteria to build up again (180, 181).

One way that *Legionella* manages to persist in the environment is by associating with biofilms – microbial communities that attach to surfaces and encase themselves in a protective matrix (90). The microbes in these communities engage in behaviors that mutually benefit each other, such as forming food chains or making up the sugar chains of the biofilm's protective shield (182). Inhabitants of the biofilm must also repel other microbes that are unlikely to contribute to the community (183, 184). However, despite these biofilms being a threatening pool of infectious bacteria, little is known about how *L. pneumophila* socially behave. Now, in eLife, Tera Levin, Brian Goldspiel and Harmit Malik from the Fred Hutchinson Cancer Research Center report how highly

dense populations of *L. pneumophila* can inhibit the growth of other bacteria belonging to the same or related species of *Legionella* (159).

Levin *et al*. found that *L. pneumophila* secrete a molecule called homogenetisic acid, or HGA for short, which is produced by the amino acids tyrosine and phenylalanine as they convert into more complex molecules. *Legionella* primarily secretes HGA after the bacteria have stopped replicating, during a period known as the stationary phase. Once secreted, HGA combines with oxygen and self-assembles into long chains to form a dark brown pigment. This pigment, called pyomelanin, is known to protect *Legionella* against light damage and to help them acquire iron, which is an essential micronutrient (143, 185). Levin *et al*. show that HGA also has toxic properties that can defend *Legionella* communities from invading microbes.

These properties became evident when the researchers discovered that *L. pneumophila* mutants that fail to inhibit the growth of other *Legionella* bacteria did not produce HGA. Interestingly, secreted HGA is only toxic if oxygen is present in the environment, and Levin *et al*. argue that a reactive intermediate formed during the conversion of HGA to pyomelanin likely accounts for its inhibitory properties. HGA is also detoxified by reducing agents, including the amino acid cysteine, which must be present for *L. pneumophila* to grow in laboratory cultures.

One conundrum is how *L. pneumophila* avoids the toxic effects of HGA. As secretion of the inhibitor increases when the bacteria are in the stationary phase – a state typically associated with high bacterial density – Levin *et al*. speculated that genes involved in either quorum sensing (which leads to changes in gene regulation based on population density) or the stringent stress response may promote resistance to HGA. However, deleting known genes in each of these widespread cell-signaling pathways

does not alter resistance or susceptibility to HGA. Instead, *L. pneumophila* cells are only susceptible to inhibition when at low density, regardless of whether they are in the stationary or replicating phase. It therefore remains to be discovered how individual *L. pneumophila* cells at high density protect themselves from HGA.

Thinking about the natural habitats of *L. pneumophila*, Levin *et al.* envision individual bacterial cells sticking to a surface, such as the interior of a water pipe (Fig. A.1). As *L. pneumophila* begin to replicate, they form a small colony, then grow to form a large cooperative community, likely sharing resources liberated by digestive enzymes that they have secreted (186). After reaching a certain density, the residents protect their turf from other bacteria by releasing a pulse of HGA. Since the high concentration of bacteria in the colony also triggers resistance to HGA, the community is automatically protected from the inhibitor.

The work of Levin, Goldspiel and Malik provides an important insight into how *Legionella* persist within microbial communities, and also identifies several questions that warrant further investigation. First, as biofilms comprised of a single species are not common in nature (182), it would be useful to know more about the impact of HGA on non-*Legionella* bacteria. In addition, understanding how the reactive intermediate of HGA is able to inhibit *Legionella*, and how individuals within dense colonies of the pathogen acquire resistance, could significantly advance the broad field of microbiology ecology. Finally, it is tempting to speculate that the HGA inhibition and resistance pathways discovered by Levin *et al.* could help identify new ways of eliminating pathogenic *Legionella* from engineered water systems.

Figure A.1

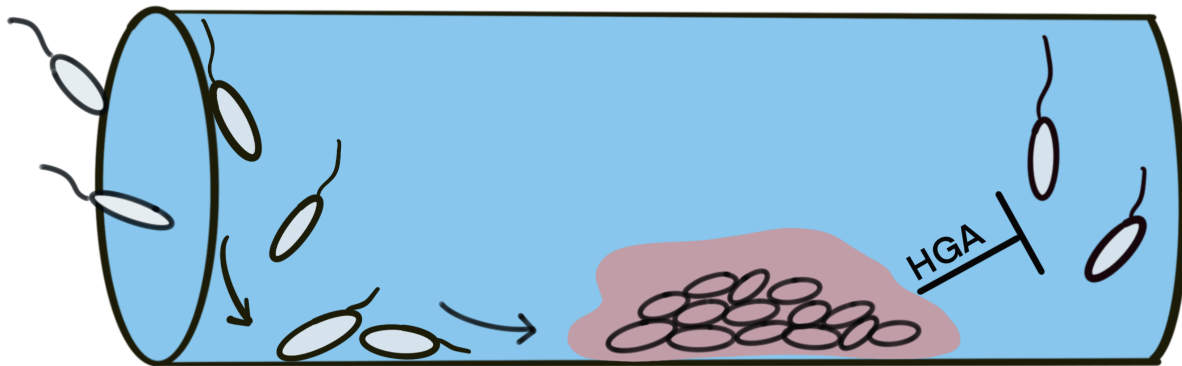


Fig. A.1. High-density colonies of *Legionella* protect themselves by secreting pulses of HGA.

The development of *Legionella* colonies begins with individual cells adhering to a surface, such as the inside of a water pipe. After reaching a certain density, the bacteria intensify the secretion of homogenetisic acid (HGA), which prevents other *Legionella* cells from joining the colony.

REFERENCES

1. Gomez-Valero L, Rusniok C, Rolando M, Neou M, Dervins-Ravault D, Demirtas J, Rouy Z, Moore RJ, Chen H, Petty NK, Jarraud S, Etienne J, Steinert M, Heuner K, Gribaldo S, Medigue C, Glockner G, Hartland EL, Buchrieser C. 2014. Comparative analyses of *Legionella* species identifies genetic features of strains causing Legionnaires' disease. Genome Biol 15:505.

2. Levin AS. 2009. Nosocomial legionellosis: prevention and management. Expert Rev Anti Infect Ther 7:57-68.

3. Joseph SJ, Cox D, Wolff B, Morrison SS, Kozak-Muiznieks NA, Frace M, Didelot X, Castillo-Ramirez S, Winchell J, Read TD, Dean D. 2016. Dynamics of genome change among *Legionella* species. Sci Rep 6:33442.

4. Rao C, Benhabib H, Ensminger AW. 2013. Phylogenetic reconstruction of the *Legionella pneumophila* Philadelphia-1 laboratory strains through comparative genomics. PLoS One 8:e64129.

5. D'Auria G, Jimenez-Hernandez N, Peris-Bondia F, Moya A, Latorre A. 2010. L*egionella pneumophila* pangenome reveals strain-specific virulence factors. BMC Genomics 11:181.

6. Swanson MS, Hammer BK. 2000. *Legionella pneumophila* pathogesesis: a fateful journey from amoebae to macrophages. Annu Rev Microbiol 54:567-613.

7. Segal G, Shuman HA. 1999. *Legionella pneumophila* utilizes the same genes to multiply within Acanthamoeba castellanii and human macrophages. Infect Immun 67:2117-24.

8. Robertson P, Abdelhady H, Garduno RA. 2014. The many forms of a pleomorphic bacterial pathogen-the developmental network of *Legionella pneumophila*. Front Microbiol 5:670.

9. Byrne B, Swanson MS. 1998. Expression of *Legionella pneumophila* virulence traits in response to growth conditions. Infect Immun 66:3029-34.

10. Horwitz MA. 1983. The Legionnaires' disease bacterium (*Legionella pneumophila*) inhibits phagosome-lysosome fusion in human monocytes. J Exp Med 158:2108-26.

11. Swanson MS, Isberg RR. 1995. Association of *Legionella pneumophila* with the macrophage endoplasmic reticulum. Infect Immun 63:3609-20.

12. Oliva G, Sahr T, Buchrieser C. 2018. The Life Cycle of *L. pneumophila*: Cellular differentiation is linked to virulence and metabolism. Front Cell Infect Microbiol 8:3.

13. Barlocher K, Welin A, Hilbi H. 2017. Formation of the *Legionella* replicative compartment at the crossroads of retrograde trafficking. Front Cell Infect Microbiol 7:482.

14. Zink SD, Pedersen L, Cianciotto NP, Abu-Kwaik Y. 2002. The Dot/Icm type IV secretion system of *Legionella pneumophila* is essential for the induction of apoptosis in human macrophages. Infect Immun 70:1657-63.

15. George JR, Pine L, Reeves MW, Harrell WK. 1980. Amino acid requirements of *Legionella pneumophila*. J Clin Microbiol 11:286-91.

16. Fonseca MV, Swanson MS. 2014. Nutrient salvaging and metabolism by the intracellular pathogen *Legionella pneumophila*. Front Cell Infect Microbiol 4:12.

17. Schunder E, Gillmaier N, Kutzner E, Eisenreich W, Herrmann V, Lautner M, Heuner K. 2014. Amino acid uptake and metabolism of *Legionella pneumophila* hosted by *Acanthamoeba castellanii*. J Biol Chem 289:21040-54.

18. Romeo T. 1998. Global regulation by the small RNA-binding protein CsrA and the non-coding RNA molecule CsrB. Mol Microbiol 29:1321-30.

19. Molofsky AB, Swanson MS. 2003. *Legionella pneumophila* CsrA is a pivotal repressor of transmission traits and activator of replication. Mol Microbiol 50:445-61.

20. Rasis M, 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 72:995-1010.

21. Sahr T, Rusniok C, Impens F, Oliva G, Sismeiro O, Coppee JY, Buchrieser C. 2017. The *Legionella pneumophila* genome evolved to accommodate multiple regulatory mechanisms controlled by the CsrA-system. PLoS Genet 13:e1006629.

22. Molofsky AB, Swanson MS. 2004. Differentiate to thrive: lessons from the *Legionella pneumophila* life cycle. Mol Microbiol 53:29-40.

23. Bruggemann H, Hagman A, Jules M, Sismeiro O, Dillies MA, Gouyette C, Kunst F, Steinert M, Heuner K, Coppee JY, Buchrieser C. 2006. Virulence strategies for infecting phagocytes deduced from the in vivo transcriptional program of *Legionella pneumophila*. Cell Microbiol 8:1228-40.

24. Hammer BK, Swanson MS. 1999. Co-ordination of *Legionella pneumophila* virulence with entry into stationary phase by ppGpp. Mol Microbiol 33:721-31.

25. Winther KS, Roghanian M, Gerdes K. 2018. Activation of the Stringent Response by Loading of RelA-tRNA Complexes at the Ribosomal A-Site. Mol Cell 70:95-105 e4.

26. Dalebroux ZD, Edwards RL, Swanson MS. 2009. SpoT governs *Legionella pneumophila* differentiation in host macrophages. Mol Microbiol 71:640-58.

27. Bachman MA, Swanson MS. 2001. RpoS co-operates with other factors to induce *Legionella pneumophila* virulence in the stationary phase. Mol Microbiol 40:1201-14.

28. Dalebroux ZD, Swanson MS. 2012. ppGpp: magic beyond RNA polymerase. Nat Rev Microbiol 10:203-12.

29. Hammer BK, Tateda ES, Swanson MS. 2002. A two-component regulator induces the transmission phenotype of stationary-phase *Legionella pneumophila*. Mol Microbiol 44:107-18.

30. Lange R, Hengge-Aronis R. 1991. Identification of a central regulator of stationary-phase gene expression in *Escherichia coli*. Mol Microbiol 5:49-59.

31. Chiang SM, Schellhorn HE. 2010. Evolution of the RpoS regulon: origin of RpoS and the conservation of RpoS-dependent regulation in bacteria. J Mol Evol 70:557-71.

32. Bachman MA, Swanson MS. 2004. Genetic evidence that *Legionella pneumophila* RpoS modulates expression of the transmission phenotype in both the exponential phase and the stationary phase. Infect Immun 72:2468-76.

33. Hovel-Miner G, Pampou S, Faucher SP, Clarke M, Morozova I, Morozov P, Russo JJ, Shuman HA, Kalachikov S. 2009. SigmaS controls multiple pathways associated with intracellular multiplication of *Legionella pneumophila*. J Bacteriol 191:2461-73.

34. Hales LM, Shuman HA. 1999. The *Legionella pneumophila rpoS* gene is required for growth within *Acanthamoeba castellanii*. J Bacteriol 181:4879-89.

35. Heuner K, Bender-Beck L, Brand BC, Luck PC, Mann KH, Marre R, Ott M, Hacker J. 1995. Cloning and genetic characterization of the flagellum subunit gene (*flaA*) of *Legionella pneumophila* serogroup 1. Infect Immun 63:2499-507.

36. Albert-Weissenberger C, Sahr T, Sismeiro O, Hacker J, Heuner K, Buchrieser C. 2010. Control of flagellar gene regulation in *Legionella pneumophila* and its relation to growth phase. J Bacteriol 192:446-55.

37. Heuner K, Dietrich C, Skriwan C, Steinert M, Hacker J. 2002. Influence of the alternative sigma(28) factor on virulence and flagellum expression of *Legionella pneumophila*. Infect Immun 70:1604-8.

38. Molofsky AB, Shetron-Rama LM, Swanson MS. 2005. Components of the *Legionella pneumophila* flagellar regulon contribute to multiple virulence traits, including lysosome avoidance and macrophage death. Infect Immun 73:5720-34.

39. Zschiedrich CP, Keidel V, Szurmant H. 2016. Molecular mechanisms of two-component signal transduction. J Mol Biol 428:3752-75.

40. Mascher T. 2006. Intramembrane-sensing histidine kinases: a new family of cell envelope stress sensors in Firmicutes bacteria. FEMS Microbiol Lett 264:133-44.

41. Edwards RL, Jules M, Sahr T, Buchrieser C, Swanson MS. 2010. The *Legionella pneumophila* LetA/LetS two-component system exhibits rheostat-like behavior. Infect Immun 78:2571-83.

42. Altegoer F, Rensing SA, Bange G. 2016. Structural basis for the CsrA-dependent modulation of translation initiation by an ancient regulatory protein. Proc Natl Acad Sci U S A 113:10168-73.

43. Valverde C, Lindell M, Wagner EG, Haas D. 2004. A repeated GGA motif is critical for the activity and stability of the riboregulator RsmY of *Pseudomonas fluorescens*. J Biol Chem 279:25066-74.

44. Sahr T, Bruggemann H, Jules M, Lomma M, Albert-Weissenberger C, Cazalet C, Buchrieser C. 2009. Two small ncRNAs jointly govern virulence and transmission in *Legionella pneumophila*. Mol Microbiol 72:741-62.

45. Faulkner G, Berk SG, Garduno E, Ortiz-Jimenez MA, Garduno RA. 2008. Passage through *Tetrahymena tropicalis* triggers a rapid morphological differentiation in *Legionella pneumophila*. J Bacteriol 190:7728-38.

46. Abdelhady H, Garduno RA. 2013. The progeny of *Legionella pneumophila* in human macrophages shows unique developmental traits. FEMS Microbiol Lett 349:99-107.

47. Garduno RA, Garduno E, Hiltz M, Hoffman PS. 2002. Intracellular growth of *Legionella pneumophila* gives rise to a differentiated form dissimilar to stationary-phase forms. Infect Immun 70:6273-83.

48. James BW, Mauchline WS, Dennis PJ, Keevil CW, Wait R. 1999. Poly-3-hydroxybutyrate in *Legionella pneumophila*, an energy source for survival in low-nutrient environments. Appl Environ Microbiol 65:822-7.

49. Schirmer T. 2016. C-di-GMP Synthesis: Structural Aspects of Evolution, Catalysis and Regulation. J Mol Biol 428:3683-701.

50. Christen B, Christen M, Paul R, Schmid F, Folcher M, Jenoe P, Meuwly M, Jenal U. 2006. Allosteric control of cyclic di-GMP signaling. J Biol Chem 281:32015-24.

51. Schmidt AJ, Ryjenkov DA, Gomelsky M. 2005. The ubiquitous protein domain EAL is a cyclic diguanylate-specific phosphodiesterase: enzymatically active and inactive EAL domains. J Bacteriol 187:4774-81.

52. Tchigvintsev A, Xu X, Singer A, Chang C, Brown G, Proudfoot M, Cui H, Flick R, Anderson WF, Joachimiak A, Galperin MY, Savchenko A, Yakunin AF. 2010. Structural insight into the mechanism of c-di-GMP hydrolysis by EAL domain phosphodiesterases. J Mol Biol 402:524-38.

53. Paul R, Abel S, Wassmann P, Beck A, Heerklotz H, Jenal U. 2007. Activation of the diguanylate cyclase PleD by phosphorylation-mediated dimerization. J Biol Chem 282:29170-7.

54. Jenal U, Reinders A, Lori C. 2017. Cyclic di-GMP: second messenger extraordinaire. Nat Rev Microbiol 15:271-284.

55. Romling U, Galperin MY, Gomelsky M. 2013. Cyclic di-GMP: the first 25 years of a universal bacterial second messenger. Microbiol Mol Biol Rev 77:1-52.

56. Levet-Paulo M, Lazzaroni JC, Gilbert C, Atlan D, Doublet P, Vianney A. 2011. The atypical two-component sensor kinase Lpl0330 from *Legionella pneumophila* controls the bifunctional diguanylate cyclase-phosphodiesterase Lpl0329 to modulate bis-(3'-5')-cyclic dimeric GMP synthesis. J Biol Chem 286:31136-44.

57. Bharati BK, Sharma IM, Kasetty S, Kumar M, Mukherjee R, Chatterji D. 2012. A full-length bifunctional protein involved in c-di-GMP turnover is required for long-term survival under nutrient starvation in *Mycobacterium smegmatis*. Microbiology 158:1415-27.

58. Römling U, Gomelsky M, Galperin MY. 2005. C-di-GMP: the dawning of a novel bacterial signalling system. Mol Microbiol 57:629-639.

59. Hengge R. 2009. Principles of c-di-GMP signalling in bacteria. Nat Rev Microbiol 7:263-73.

60. Pecastaings S, Allombert J, Lajoie B, Doublet P, Roques C, Vianney A. 2016. New insights into *Legionella pneumophila* biofilm regulation by c-di-GMP signaling. Biofouling 32:935-48.

61. Chan C, Paul R, Samoray D, Amiot NC, Giese B, Jenal U, Schirmer T. 2004. Structural basis of activity and allosteric control of diguanylate cyclase. Proc Natl Acad Sci U S A 101:17084-9.

62. Ross P, Weinhouse H, Aloni Y, Michaeli D, Weinberger-Ohana P, Mayer R, Braun S, de Vroom E, van der Marel GA, van Boom JH, Benziman M. 1987. Regulation of cellulose synthesis in *Acetobacter xylinum* by cyclic diguanylic acid. Nature 325:279-81.

63. Simm R, Morr M, Kader A, Nimtz M, Romling U. 2004. GGDEF and EAL domains inversely regulate cyclic di-GMP levels and transition from sessility to motility. Mol Microbiol 53:1123-34.

64. Cotter PA, Stibitz S. 2007. c-di-GMP-mediated regulation of virulence and biofilm formation. Curr Opin Microbiol 10:17-23.

65. Karaolis DK, Rashid MH, Chythanya R, Luo W, Hyodo M, Hayakawa Y. 2005. c-di-GMP (3'-5'-cyclic diguanylic acid) inhibits *Staphylococcus aureus* cell-cell interactions and biofilm formation. Antimicrob Agents Chemother 49:1029-38.

66. Schirmer T, Jenal U. 2009. Structural and mechanistic determinants of c-di-GMP signalling. Nat Rev Microbiol 7:724-35.

67. Chou SH, Galperin MY. 2016. Diversity of Cyclic Di-GMP-Binding Proteins and Mechanisms. J Bacteriol 198:32-46.

68. Alm RA, Bodero AJ, Free PD, Mattick JS. 1996. Identification of a novel gene, *pilZ*, essential for type 4 fimbrial biogenesis in *Pseudomonas aeruginosa*. J Bacteriol 178:46-53.

69. Hufnagel DA, DePas WH, Chapman MR. 2014. The disulfide bonding system suppresses CsgD-independent cellulose production in *Escherichia coli*. J Bacteriol 196:3690-9.

70. Hickman JW, Harwood CS. 2008. Identification of FleQ from *Pseudomonas aeruginosa* as a c-di-GMP-responsive transcription factor. Mol Microbiol 69:376-89.

71. Lee VT, Matewish JM, Kessler JL, Hyodo M, Hayakawa Y, Lory S. 2007. A cyclic-di-GMP receptor required for bacterial exopolysaccharide production. Mol Microbiol 65:1474-84.

72. Whitney JC, Colvin KM, Marmont LS, Robinson H, Parsek MR, Howell PL. 2012. Structure of the cytoplasmic region of PelD, a degenerate diguanylate cyclase receptor that regulates exopolysaccharide production in *Pseudomonas aeruginosa*. J Biol Chem 287:23582-93.

73. Sudarsan N, Lee ER, Weinberg Z, Moy RH, Kim JN, Link KH, Breaker RR. 2008. Riboswitches in eubacteria sense the second messenger cyclic di-GMP. Science 321:411-3.

74. Ramesh A. 2015. Second messenger - Sensing riboswitches in bacteria. Semin Cell Dev Biol 47-48:3-8.

75. Smith KD, Lipchock SV, Ames TD, Wang J, Breaker RR, Strobel SA. 2009. Structural basis of ligand binding by a c-di-GMP riboswitch. Nat Struct Mol Biol 16:1218-23.

76. Bordeleau E, Purcell EB, Lafontaine DA, Fortier LC, Tamayo R, Burrus V. 2015. Cyclic di-GMP riboswitch-regulated type IV pili contribute to aggregation of *Clostridium difficile*. J Bacteriol 197:819-32.

77. Roop RM, Kariisa AT, Weeks K, Tamayo R. 2016. The RNA Domain Vc1 Regulates Downstream Gene Expression in Response to Cyclic Diguanylate in *Vibrio cholerae*. Plos One 11.

78. Tamayo R. 2019. Cyclic diguanylate riboswitches control bacterial pathogenesis mechanisms. PLoS Pathog 15:e1007529.

79. Seshasayee AS, Fraser GM, Luscombe NM. 2010. Comparative genomics of cyclic-di-GMP signalling in bacteria: post-translational regulation and catalytic activity. Nucleic Acids Res 38:5970-81.

80. Sarenko O, Klauck G, Wilke FM, Pfiffer V, Richter AM, Herbst S, Kaever V, Hengge R. 2017. More than enzymes that make or break cyclic di-GMP-Local signaling in the interactome of GGDEF/EAL domain proteins of *Escherichia coli*. MBio 8.

81. Hickman JW, Tifrea DF, Harwood CS. 2005. A chemosensory system that regulates biofilm formation through modulation of cyclic diguanylate levels. Proc Natl Acad Sci U S A 102:14422-7.

82. Sommerfeldt N, Possling A, Becker G, Pesavento C, Tschowri N, Hengge R. 2009. Gene expression patterns and differential input into curli fimbriae regulation of all GGDEF/EAL domain proteins in *Escherichia coli*. Microbiology 155:1318-31.

83. Abel S, Bucher T, Nicollier M, Hug I, Kaever V, Abel Zur Wiesch P, Jenal U. 2013. Bi-modal distribution of the second messenger c-di-GMP controls cell fate and asymmetry during the *Caulobacter* cell cycle. PLoS Genet 9:e1003744.

84. Levi A, Folcher M, Jenal U, Shuman HA. 2011. Cyclic diguanylate signaling proteins control intracellular growth of *Legionella pneumophila*. MBio 2:e00316-10.

85. Allombert J, Lazzaroni JC, Bailo N, Gilbert C, Charpentier X, Doublet P, 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 82:1222-33.

86. Simm R, Morr M, Remminghorst U, Andersson M, Romling U. 2009. Quantitative determination of cyclic diguanosine monophosphate concentrations in nucleotide extracts of bacteria by matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry. Anal Biochem 386:53-8.

87. Carlson HK, Vance RE, Marletta MA. 2010. H-NOX regulation of c-di-GMP metabolism and biofilm formation in *Legionella pneumophila*. Mol Microbiol 77:930-42.

88. Guo Y, Marletta MA. 2019. Structural insight into H-NOX gas sensing and cognate signaling protein regulation. ChemBioChem 20:7-19.

89. Declerck P. 2010. Biofilms: the environmental playground of *Legionella pneumophila*. Environ Microbiol 12:557-66.

90. Abu Khweek A, Amer AO. 2018. Factors mediating environmental biofilm formation by *Legionella pneumophila*. Front Cell Infect Microbiol 8:38.

91. Waak MB, LaPara TM, Halle C, Hozalski RM. 2018. Occurrence of *Legionella spp*. in water-main biofilms from two drinking water distribution systems. Environ Sci Technol 52:7630-7639.

92. Stewart CR, Muthye V, Cianciotto NP. 2012. *Legionella pneumophila* persists within biofilms formed by *Klebsiella pneumoniae*, *Flavobacterium sp*., and *Pseudomonas fluorescens* under dynamic flow conditions. PLoS One 7:e50560.

93. Declerck P, Behets J, van Hoef V, Ollevier F. 2007. Replication of *Legionella pneumophila* in floating biofilms. Curr Microbiol 55:435-40.

94. Murga R, Forster TS, Brown E, Pruckler JM, Fields BS, Donlan RM. 2001. Role of biofilms in the survival of *Legionella pneumophila* in a model potable-water system. Microbiology 147:3121-6.

95. Mampel J, Spirig T, Weber SS, Haagensen JA, Molin S, 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 72:2885-95.

96. Shaheen M, Scott C, Ashbolt NJ. 2019. Long-term persistence of infectious *Legionella* with free-living amoebae in drinking water biofilms. Int J Hyg Environ Health 222:678-686.

97. van der Kooij D. 1998. Potential for biofilm development in drinking water distribution systems. J Appl Microbiol 85 Suppl 1:39S-44S.

98. Xu HS, Roberts N, Singleton FL, Attwell RW, Grimes DJ, Colwell RR. 1982. Survival and viability of nonculturable *Escherichia coli* and *Vibrio cholerae* in the estuarine and marine environment. Microb Ecol 8:313-23.

99. Li L, Mendis N, Trigui H, Oliver JD, Faucher SP. 2014. The importance of the viable but non-culturable state in human bacterial pathogens. Front Microbiol 5:258.

100. Environmental Protection Agency. Legionella: Drinking Water Health Advisory.

101. Epalle T, Girardot F, Allegra S, Maurice-Blanc C, Garraud O, Riffard S. 2015. Viable but not culturable forms of *Legionella pneumophila* generated after heat shock treatment are infectious for macrophage-like and alveolar epithelial cells after resuscitation on *Acanthamoeba polyphaga*. Microb Ecol 69:215-24.

102. Whiley H, Bentham R, Brown MH. 2017. *Legionella* persistence in manufactured water systems: pasteurization potentially selecting for thermal tolerance. Front Microbiol 8:1330.

103. Flannery B, Gelling LB, Vugia DJ, Weintraub JM, Salerno JJ, Conroy MJ, Stevens VA, Rose CE, Moore MR, Fields BS, Besser RE. 2006. Reducing *Legionella* colonization in water systems with monochloramine. Emerg Infect Dis 12:588-96.

104. Alleron L, Merlet N, Lacombe C, Frere J. 2008. Long-term survival of *Legionella pneumophila* in the viable but nonculturable state after monochloramine treatment. Curr Microbiol 57:497-502.

105. Schrammel B, Cervero-Arago S, Dietersdorfer E, Walochnik J, Luck C, Sommer R, Kirschner A. 2018. Differential development of *Legionella* sub-populations during short- and long-term starvation. Water Res 141:417-427.

106. Dietersdorfer E, Kirschner A, Schrammel B, Ohradanova-Repic A, Stockinger H, Sommer R, Walochnik J, Cervero-Arago S. 2018. Starved viable but non-culturable (VBNC) *Legionella* strains can infect and replicate in amoebae and human macrophages. Water Res 141:428-438.

107. Fraser DW, Tsai TR, Orenstein W, Parkin WE, Beecham HJ, Sharrar RG, Harris J, Mallison GF, Martin SM, McDade JE, Shepard CC, Brachman PS. 1977. Legionnaires' disease: description of an epidemic of pneumonia. N Engl J Med 297:1189-97.

108. Mautino FJ. 2019. Performance Audit of Legionnaires’ Disease at the Quincy Veterans’ Home.

109. Garrison LE, Kunz JM, Cooley LA, Moore MR, Lucas C, Schrag S, Sarisky J, Whitney CG. 2016. Vital signs: deficiencies in environmental control identified in outbreaks of Legionnaires' Disease - North America, 2000-2014. MMWR Morb Mortal Wkly Rep 65:576-84.

110. Zahran S, McElmurry SP, Kilgore PE, Mushinski D, Press J, Love NG, Sadler RC, Swanson MS. 2018. Assessment of the Legionnaires' disease outbreak in Flint, Michigan. Proc Natl Acad Sci U S A 115:E1730-E1739.

111. Lapierre P, Nazarian E, Zhu Y, Wroblewski D, Saylors A, Passaretti T, Hughes S, Tran A, Lin Y, Kornblum J, Morrison SS, Mercante JW, Fitzhenry R, Weiss D, Raphael BH, Varma JK, Zucker HA, Rakeman JL, Musser KA. 2017. Legionnaires' Disease outbreak caused by endemic strain of *Legionella pneumophila*, New York, New York, USA, 2015. Emerg Infect Dis 23:1784-1791.

112. Whitney EA, Blake S, Berkelman RL. 2017. Implementation of a *Legionella* ordinance for multifamily housing, Garland, Texas. J Public Health Manag Pract 23:601-607.

113. Dooling KL, Toews K-A, Hicks LA, Garrison LE, Brian Bachaus, Zansky S, Carpenter LR, Bill Schaffner, Parker E, Petit S, Thomas A, Thomas S, Mansmann R, Morin C, White B, Langley GE. 2015. Active bacterial core surveillance for Legionellosis — United States, 2011–2013. MMWR Morbidity Mortality Weekly Report 64:1190-1193.

114. Correia AM, Ferreira JS, Borges V, Nunes A, Gomes B, Capucho R, Goncalves J, Antunes DM, Almeida S, Mendes A, Guerreiro M, Sampaio DA, Vieira L, Machado J, Simoes MJ, Goncalves P, Gomes JP. 2016. Probable person-to-person transmission of Legionnaires' Disease. N Engl J Med 374:497-8.

115. Beaute J, Sandin S, de Jong B, Hallstrom LP, Robesyn E, Giesecke J, Sparen P, On Behalf Of The European Legionnaires' Disease Surveillance N. 2019. Factors associated with Legionnaires' disease recurrence in hotel and holiday rental accommodation sites. Euro Surveill 24.

116. Cowgill KD, Lucas CE, Benson RF, Chamany S, Brown EW, Fields BS, Feikin DR. 2005. Recurrence of Legionnaires disease at a hotel in the United States Virgin Islands over a 20-year period. Clin Infect Dis 40:1205-7.

117. Lasheras A, Boulestreau H, Rogues AM, Ohayon-Courtes C, Labadie JC, Gachie JP. 2006. Influence of amoebae and physical and chemical characteristics of water on presence and proliferation of Legionella species in hospital water systems. Am J Infect Control 34:520-5.

118. Scaturro M, Dell'eva I, Helfer F, Ricci ML. 2007. Persistence of the same strain of *Legionella pneumophila* in the water system of an Italian hospital for 15 years. Infect Control Hosp Epidemiol 28:1089-92.

119. Neil K, Berkelman R. 2008. Increasing incidence of Legionellosis in the United States, 1990–2005: changing epidemiologic trends. Clin Infect Dis 47:591-599.

120. Soda EA, Barskey AE, Shah PP, Schrag S, Whitney CG, Arduino MJ, Reddy SC, Kunz JM, Hunter CM, Raphael BH, Cooley LA. 2017. Vital signs: health care-associated Legionnaires' Disease surveillance data from 20 States and a large metropolitan area-United States, 2015. Am J Transplant 17:2215-2220.

121. Collier SA, Stockman LJ, Hicks LA, Garrison LE, Zhou FJ, Beach MJ. 2012. Direct healthcare costs of selected diseases primarily or partially transmitted by water. Epidemiol Infect 140:2003-13.

122. Mendis N, McBride P, Faucher SP. 2015. Short-Term and Long-Term Survival and Virulence of *Legionella pneumophila* in the defined freshwater medium Fraquil. PLoS One 10:e0139277.

123. Pine L, George JR, Reeves MW, Harrell WK. 1979. Development of a chemically defined liquid medium for growth of *Legionella pneumophila*. J Clin Microbiol 9:615-26.

124. Abbott ZD, Yakhnin H, Babitzke P, Swanson MS. 2015. *csrR*, a Paralog and Direct Target of CsrA, promotes *Legionella pneumophila* resilience in water. MBio 6:e00595.

125. Tamayo R, Pratt JT, Camilli A. 2007. Roles of cyclic diguanylate in the regulation of bacterial pathogenesis. Annu Rev Microbiol 61:131-148.

126. Stock AM, Robinson VL, Goudreau PN. 2000. Two-component signal transduction. Annu Rev Biochem 69:183-215.

127. Tao J, Li C, Luo C, He C. 2014. RavA/RavR two-component system regulates *Xanthomonas campestris* pathogenesis and c-di-GMP turnover. FEMS Microbiol Lett 358:81-90.

128. Samrakandi MM, Cirillo SL, Ridenour DA, Bermudez LE, Cirillo JD. 2002. Genetic and phenotypic differences between *Legionella pneumophila* strains. J Clin Microbiol 40:1352-62.

129. Cazalet C, Rusniok C, Bruggemann H, Zidane N, Magnier A, Ma L, Tichit M, Jarraud S, Bouchier C, Vandenesch F, Kunst F, Etienne J, Glaser P, Buchrieser C. 2004. Evidence in the *Legionella pneumophila* genome for exploitation of host cell functions and high genome plasticity. Nat Genet 36:1165-73.

130. Osbourn AE, Field B. 2009. Operons. Cell Mol Life Sci 66:3755-75.

131. Cormack BP, Valdivia RH, Falkow S. 1996. FACS-optimized mutants of the green fluorescent protein (GFP). Gene 173:33-8.

132. Abu-Zant A, Asare R, Graham JE, 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 74:3021-6.

133. Appelt S, Heuner K. 2017. The flagellar regulon of *Legionella*-a review. Front Cell Infect Microbiol 7:454.

134. Conway T, Creecy JP, Maddox SM, Grissom JE, Conkle TL, Shadid TM, Teramoto J, San Miguel P, Shimada T, Ishihama A, Mori H, Wanner BL. 2014. Unprecedented high-resolution view of bacterial operon architecture revealed by RNA sequencing. MBio 5:e01442-14.

135. Tanaka K, Kusano S, Fujita N, Ishihama A, Takahashi H. 1995. Promoter determinants for *Escherichia coli* RNA polymerase holoenzyme containing sigma 38 (the *rpoS* gene product). Nucleic Acids Res 23:827-34.

136. Espinosa-Urgel M, Chamizo C, Tormo A. 1996. A consensus structure for sigma S-dependent promoters. Mol Microbiol 21:657-9.

137. Lee SJ, Gralla JD. 2001. Sigma38 (*rpoS*) RNA polymerase promoter engagement via -10 region nucleotides. J Biol Chem 276:30064-71.

138. Pitre CA, Tanner JR, Patel P, Brassinga AK. 2013. Regulatory control of temporally expressed integration host factor (IHF) in *Legionella pneumophila*. Microbiology 159:475-92.

139. Warren WJ, Miller RD. 1979. Growth of Legionnaires disease bacterium (*Legionella pneumophila*) in chemically defined medium. J Clin Microbiol 10:50-5.

140. Ewann F, Hoffman PS. 2006. Cysteine metabolism in *Legionella pneumophila*: characterization of an L-cystine-utilizing mutant. Appl Environ Microbiol 72:3993-4000.

141. Zusman T, Gal-Mor O, Segal G. 2002. Characterization of a *Legionella pneumophila relA* insertion mutant and roles of RelA and RpoS in virulence gene expression. J Bacteriol 184:67-75.

142. Bachman MA, Swanson MS. 2004. The LetE protein enhances expression of multiple LetA/LetS-dependent transmission traits by *Legionella pneumophila*. Infect Immun 72:3284-93.

143. Steinert M, Engelhard H, Flugel M, Wintermeyer E, Hacker J. 1995. The Lly protein protects *Legionella pneumophila* from light but does not directly influence its intracellular survival in *Hartmannella vermiformis*. Appl Environ Microbiol 61:2428-2430.

144. Chatfield CH, Cianciotto NP. 2007. The secreted pyomelanin pigment of *Legionella pneumophila* confers ferric reductase activity. Infect Immun 75:4062-70.

145. Gillmaier N, Schunder E, Kutzner E, Tlapak H, Rydzewski K, Herrmann V, Stammler M, Lasch P, Eisenreich W, Heuner K. 2016. Growth-related Metabolism of the Carbon Storage Poly-3-hydroxybutyrate in *Legionella pneumophila*. J Biol Chem 291:6471-82.

146. Greenspan P. 1985. Nile red: a selective fluorescent stain for intracellular lipid droplets. The Journal of Cell Biology 100:965-973.

147. Edwards RL, Dalebroux ZD, Swanson MS. 2009. *Legionella pneumophila* couples fatty acid flux to microbial differentiation and virulence. Mol Microbiol 71:1190-1204.

148. Trigui H, Dudyk P, Oh J, Hong JI, Faucher SP. 2015. A regulatory feedback loop between RpoS and SpoT supports the survival of *Legionella pneumophila* in water. Appl Environ Microbiol 81:918-28.

149. Skaliy P, McEachern HV. 1979. Survival of the Legionnaires' disease bacterium in water. Ann Intern Med 90:662-3.

150. Paszko-Kolva C, Shahamat M, Colwell RR. 1992. Long-term survival of *Legionella pneumophila* serogroup 1 under low-nutrient conditions and associated morphological changes. FEMS Microbiol Ecol 11:45-55.

151. Duerig A, Abel S, Folcher M, Nicollier M, Schwede T, Amiot N, Giese B, Jenal U. 2009. Second messenger-mediated spatiotemporal control of protein degradation regulates bacterial cell cycle progression. Genes Dev 23:93-104.

152. He M, Zhang JJ, Ye M, Lou Y, Yang XF. 2014. Cyclic Di-GMP receptor PlzA controls virulence gene expression through RpoS in *Borrelia burgdorferi*. Infect Immun 82:445-52.

153. Conner JG, Zamorano-Sanchez D, Park JH, Sondermann H, Yildiz FH. 2017. The ins and outs of cyclic di-GMP signaling in *Vibrio cholerae*. Curr Opin Microbiol 36:20-29.

154. Borziak K, Zhulin IB. 2007. FIST: a sensory domain for diverse signal transduction pathways in prokaryotes and ubiquitin signaling in eukaryotes. Bioinformatics 23:2518-21.

155. Hossain S, Boon EM. 2017. Discovery of a novel nitric oxide binding protein and nitric-oxide-responsive signaling pathway in *Pseudomonas aeruginosa*. ACS Infect Dis 3:454-461.

156. Henares BM, Higgins KE, Boon EM. 2012. Discovery of a nitric oxide responsive quorum sensing circuit in *Vibrio harveyi*. ACS Chem Biol 7:1331-6.

157. Price MS, Chao LY, Marletta MA. 2007. *Shewanella oneidensis* MR-1 H-NOX regulation of a histidine kinase by nitric oxide. Biochemistry 46:13677-83.

158. Hossain S, Nisbett LM, Boon EM. 2017. Discovery of two bacterial nitric oxide-responsive proteins and their roles in bacterial biofilm begulation. Acc Chem Res 50:1633-1639.

159. Levin TC, Goldspiel BP, Malik HS. 2019. Density-dependent resistance protects *Legionella pneumophila* from its own antimicrobial metabolite, HGA. Elife 8.

160. Rotem O, Nesper J, Borovok I, Gorovits R, Kolot M, Pasternak Z, Shin I, Glatter T, Pietrokovski S, Jenal U, Jurkevitch E. 2016. An extended cyclic di-GMP network in the predatory bacterium *Bdellovibrio bacteriovorus*. J Bacteriol 198:127-37.

161. Li L, Mendis N, Trigui H, Faucher SP. 2015. Transcriptomic changes of *Legionella pneumophila* in water. BMC Genomics 16:637.

162. Bryan A, Abbott ZD, Swanson MS. 2013. Constructing unmarked gene deletions in *Legionella pneumophila*. Methods Mol Biol 954:197-212.

163. Bryan A, Harada K, Swanson MS. 2011. Efficient generation of unmarked deletions in *Legionella pneumophila*. Appl Environ Microbiol 77:2545-8.

164. Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25:402-8.

165. Yu D, Ellis HM, Lee EC, Jenkins NA, Copeland NG, Court DL. 2000. An efficient recombination system for chromosome engineering in *Escherichia coli*. Proc Natl Acad Sci U S A 97:5978-83.

166. Berger KH, Isberg RR. 1993. Two distinct defects in intracellular growth complemented by a single genetic locus in *Legionella pneumophila*. Mol Microbiol 7:7-19.

167. Datsenko KA, Wanner BL. 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc Natl Acad Sci U S A 97:6640-5.

168. Brassinga AK, Hiltz MF, Sisson GR, Morash MG, Hill N, Garduno E, Edelstein PH, Garduno RA, Hoffman PS. 2003. A 65-kilobase pathogenicity island is unique to Philadelphia-1 strains of *Legionella pneumophila*. J Bacteriol 185:4630-7.

169. Flynn KJ, Swanson MS. 2014. Integrative conjugative element ICE-betaox confers oxidative stress resistance to *Legionella pneumophila in vitro* and in macrophages. MBio 5:e01091-14.

170. Morash MG, Brassinga AK, Warthan M, Gourabathini P, Garduno RA, Goodman SD, Hoffman PS. 2009. Reciprocal expression of integration host factor and HU in the developmental cycle and infectivity of *Legionella pneumophila*. Appl Environ Microbiol 75:1826-37.

171. Friedman DI. 1988. Integration host factor: a protein for all reasons. Cell 55:545-54.

172. Lynch TW, Read EK, Mattis AN, Gardner JF, Rice PA. 2003. Integration Host Factor: Putting a Twist on Protein–DNA Recognition. J Mol Biol 330:493-502.

173. Geertz M, Maerkl SJ. 2010. Experimental strategies for studying transcription factor-DNA binding specificities. Brief Funct Genomics 9:362-73.

174. Al-Bana BH, Haddad MT, Garduno RA. 2014. Stationary phase and mature infectious forms of *Legionella pneumophila* produce distinct viable but non-culturable cells. Environ Microbiol 16:382-95.

175. Bonfiglio G, Lanzafame A, Santini L, Mattina R. 2004. *In vitro* activity of thiamphenicol, erythromycin and fluoroquinolones against *Legionella pneumophila*. J Chemother 16:502-3.

176. Li GW. 2015. How do bacteria tune translation efficiency? Curr Opin Microbiol 24:66-71.

177. Huberts DH, van der Klei IJ. 2010. Moonlighting proteins: an intriguing mode of multitasking. Biochim Biophys Acta 1803:520-5.

178. Gupta KR, Kasetty S, Chatterji D. 2015. Novel functions of (p)ppGpp and cyclic di-GMP in mycobacterial physiology revealed by phenotype microarray analysis of wild-type and isogenic strains of *Mycobacterium smegmatis*. Appl Environ Microbiol 81:2571-8.

179. Buckstein MH, He J, Rubin H. 2008. Characterization of nucleotide pools as a function of physiological state in *Escherichia coli*. J Bacteriol 190:718-26.

180. Cervero-Arago S, Rodriguez-Martinez S, Puertas-Bennasar A, Araujo RM. 2015. Effect of common drinking water disinfectants, chlorine and heat, on free *Legionella* and Amoebae-Associated *Legionella*. PLoS One 10:e0134726.

181. Berjeaud JM, Chevalier S, Schlusselhuber M, Portier E, Loiseau C, Aucher W, Lesouhaitier O, Verdon J. 2016. *Legionella pneumophila*: the paradox of a highly sensitive opportunistic waterborne pathogen able to persist in the environment. Front Microbiol 7:486.

182. Elias S, Banin E. 2012. Multi-species biofilms: living with friendly neighbors. FEMS Microbiol Rev 36:990-1004.

183. Drescher K, Nadell CD, Stone HA, Wingreen NS, Bassler BL. 2014. Solutions to the public goods dilemma in bacterial biofilms. Curr Biol 24:50-55.

184. Ozkaya O, Xavier KB, Dionisio F, Balbontin R. 2017. Maintenance of microbial cooperation mediated by public goods in single- and multiple-trait scenarios. J Bacteriol 199.

185. Zheng H, Chatfield CH, Liles MR, Cianciotto NP. 2013. Secreted pyomelanin of *Legionella pneumophila* promotes bacterial iron uptake and growth under iron-limiting conditions. Infect Immun 81:4182-91.

186. White RC, Cianciotto NP. 2019. Assessing the impact, genomics and evolution of type II secretion across a large, medically important genus: the *Legionella* type II secretion paradigm. Microb Genom 5.