“Magic Spot” Conjures Transmission of *Legionella pneumophila*.

by

Zachary D. Dalebroux

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
(Microbiology and Immunology)
in the University of Michigan
2010

Doctoral Committee

Professor Michele S. Swanson, Chair
Professor Victor J. DiRita
Professor Janine R. Maddock
Professor David H. Sherman
Assistant Professor Mary X. O’Riordan

DEDICATION

For Amy, a wonderful wife and true companion.

ACKNOWLEDGEMENTS

I must first acknowledge my mentor and friend Dr. Michele Swanson. Throughout my career Michele has made me feel like a colleague. She has taught me how to write, present, teach, mentor and think critically. Her strength, passion, and loyalty make her a superb leader and excellent friend. Many thanks to my thesis committee, Drs. Vic DiRita, Janine Maddock, Mary O'Riordan, and David Sherman, who have offered advice and encouragement since the day I arrived in Michigan. I must also acknowledge Drs. Vern Carruthers, David Friedman, Harry Mobley, Fred Neidhardt, Alice Telesnitsky and Joel Swanson who have shown special interest in my work and supported me throughout. My coworkers past and present: Zach Abbott, Andrew Bryan, Brenda Byrne, Dr. Jeff Dubuisson, Dr. Rachel Edwards, Dr. Maris Fonseca, Kaoru Harada, Dr. Amrita Joshi, Dr. J.D. Sauer, Dr. Natalie Whitfield, and Brian Yagi, thank you for all the laughter, stories, and discussions we have shared. Without such a cohesive group of colleagues, I would have never made it. Each and every one of you are true friends. Special thanks to my graduate student mentor Dr. Rachel Edwards, who collaborated with me on work described in Chapter II and in the Appendix. Rachel went out of her way to make sure I hit the ground running and directed me toward an exciting project. My undergraduate research assistant Brian Yagi, who assisted with Chapters III and IV. Watching Brian evolve, both as a scientist and a person has been truly rewarding and gratifying. My mentee Zack Abbott, who contributed to Chapter IV, deserves credit for being a great listener and diligent scientist. The sky is truly the limit for him in lab,

and beyond. Brenda Byrne, one of my dearest friends in Michigan. Thank you for sharing your stories, providing a listening ear, and helping me to understand the importance of family. Thank you to my families: The Dalebroux's and the Konkols. Steve, Eileen, Nicole, Dennis, Cheryl, Matt, Kari, and Chris, your support has meant so much. You have taught me an excellent work ethic and enstilled critical family values. I would not be the person I am today without each and every one of you. I must also thank my dog Sasha who never cared how well I did. No matter the outcome she was always excited when I got home. Lastly, I must thank my wife Amy for supporting me through the ups and downs. You deserve this degree as much as I do. You picked up the slack when I was stressed and listened when I needed you most. I appreciate all that you do.

TABLE OF CONTENTS

DEDICATION ..... **ii**

ACKNOWLEDGEMENTS ..... **iii**

LIST OF FIGURES ..... **vii**

LIST OF TABLES ..... **x**

CHAPTER I: INTRODUCTION **1**

"Magic spot" and virulence expression 2

Metabolism of ppGpp 3

Regulatory targets of ppGpp and DksA 6

ppGpp and the *L. pneumophila* life cycle 9

Flagellar biogenesis of *L. pneumophila* 10

Post-transcriptional regulation of *L. pneumophila* transmission 11

Outline of thesis 14

CHAPTER II: SpoT governs *Legionella pneumophila* differentiation in host macrophages. **23**

Summary 23

Introduction 24

Experimental Procedures 28

Results 36

Discussion 48

CHAPTER III: Distinct roles of ppGpp and DksA in *Legionella pneumophila* differentiation. **67**

Summary 67

Introduction 68

Experimental Procedures 70

Results 78

Discussion 90

CHAPTER IV: ppGpp activates two regulatory RNAs to relieve CsrA-mediated repression of *Legionella pneumophila* flagellar biogenesis 115

Summary 115

Introduction 116

Experimental Procedures 120

Results 123

Discussion 130

CHAPTER V: CONCLUSION 145

APPENDIX 156

BIBLIOGRAPHY 191

LIST OF FIGURES

Figure 1.1. Classes and domain architecture of ppGpp synthetases. 17

Figure 1.2. SpoT activity is regulated through ACP interaction..... 18

Figure 1.3. ppGpp and DksA control transcription directly. 19

Figure 1.4. ppGpp and DksA control transcription indirectly through "sigma factor competition"..... 20

Figure 1.5. SpoT governs the *Legionella* life cycle in macrophages. 21

Figure 1.6. ppGpp synthesis leads to derepression of a flagellar gene activator. 22

Figure 2.1. RelA and SpoT contribute to ppGpp accumulation, motility and survival in PE phase. 56

Figure 2.2. *L. pneumophila* requires RelA and SpoT for ppGpp accumulation and *flaA* expression in response to distinct metabolic cues. ……58

Figure 2.3. SpoT mutation abrogates transmission trait expression following perturbations in fatty acid metabolism. 59

Figure 2.4. The stringent response governs *L. pneumophila* transmission traits in broth. 60

Figure 2.5. Induction of either ppGpp synthetase rescues transmission trait expression of *relA spoT* mutants. 61

Figure 2.6. The stringent response is essential for *L. pneumophila* transmission in macrophages. 62

Figure 2.7. SpoT, not RelA, is essential for *L. pneumophila* transmission in macrophages. 63

Figure 2.8. Transmissive *L. pneumophila* requires SpoT to enter the replicative phase. 64

Figure 2.9. SpoT governs *L. pneumophila* differentiation in host macrophages 65

Figure 2.10. Growth patterns of *relA spoT* mutant strains induced to express *spoT*, *spoT-A413E*, or *relA*. 66

Figure 3.1. Kinetics of ppGpp accumulation after p*relA*$^{L.p.}$ induction. 103

Figure 3.2. To survive stationary phase stress, *L. pneumophila* requires DksA and ppGpp..... 104

Figure 3.3. To activate PE phase transmission traits, *L. pneumophila* requires DksA and ppGpp..... 105

Figure 3.4. For optimal growth in *A. castellanii*, *L. pneumophila* requires both DksA and ppGpp..... 106

Figure 3.5. *L. pneumophila* requires ppGpp for macrophage transmission; DksA is less critical. 107

Figure 3.6. Constitutive ppGpp synthesis is sufficient to bypass the *dksA* requirement for cell cycle arrest and pigment production. 108

Figure 3.7. In response to fatty acid stress, DksA activates transmission traits with and independently of ppGpp..... 109

Figure 3.8. Induction of *dksA* is sufficient to bypass the requirement for ppGpp for flagellin-dependent phenotypes. 110

Figure 3.9. *L. pneumophila* uses ppGpp to modulate DksA-dependent gene expression. 111

Figure 3.10. qRT-PCR validation of the *relA*$^{L.p.}$ induction microarray data set. 112

Figure 3.11. qRT-PCR validation of the PE phase *dksA*/WT microarray data set. 113

Figure 3.12. Growth of WT and *dksA* mutant *L. pneumophila* induced to express p*relA*$^{E.c.}$. 114

Figure. 4.1. ppGpp synthesis triggers accumulation of the RsmY and RsmZ non-coding regulatory RNAs. 139

Figure 4.2. *L. pneumophila* requires ppGpp, DksA, RpoS and LetA for PE phase activation of the *rsmZ* promoter..... 140

Figure 4.3. Induction of *rsmZ* is sufficient to bypass ppGpp for macrophage degradation resistance and cytotoxicity..... 141

Figure 4.4. RsmY/Z contribute to PE phase activation of *fliA* and *flaA*..... 142

Figure 4.5. Induction of *csrA* leads to reduced *fliA* mRNA, but increased stability..... 143

Figure 4.6. ppGpp synthesis leads to activation of RsmY/Z and derepression of a *fliA* gene activator. 144

Figure 5.1. ppGpp and DksA control transcriptional activation of RsmY/Z to derepress *fliA*, a transcriptional activator of *flaA*…….155

Figure A.1. Growth inhibition and the premature expression of motility are specific to fatty acid addition. 182

Figure A.2. Fatty acid supplementation of WT *L. pneumophila* induces the early expression of multiple transmissive phase phenotypes. 183

Figure A.3. The LetA/LetS signal transduction system is required for full induction of premature motility..... 184

Figure A.4. Induction of motility by fatty acid addition is independent of *pta ackA2*. . 185

Figure A.5. Alterations in fatty acid biosynthesis induce *L. pneumophila* differentiation……...187

Figure A.6. Perturbations in fatty acid biosynthesis alter *L. pneumophila* acyl-ACP profiles. 188

Figure A.7. *L. pneumophila* employs the stringent response to induce differentiation when fatty acid biosynthesis is altered..... 189

Figure A.8. *L. pneumophila* monitors flux in fatty acid biosynthesis to coordinate differentiation..... 190

LIST OF TABLES

Table 2.1. Bacterial strains, plasmids and primers 55

Table 3.1. Select list of transcripts up-regulated 90 min after relA$^{L.p.}$ induction..... 97

Table 3.2. Flagellar gene expression of PE phase *dksA* mutant *L. pneumophila*. 98

Table 3.3. Bacterial strains and plasmids..... 99

Table 3.4. Select list of transcripts down-regulated 90 min after relA$^{L.p.}$ induction. 100

Table 3.5. List of primers..... 102

Table 4.1. Bacterial strains and plasmids..... 137

Table 4.2. List of primers..... 138

Table A.1. Bacterial strains and plasmids..... 179

Table A.2. Compounds that trigger premature differentiation in *L. pneumophila* 180

Table A.3. Phenotypic response of *letA* and *letS* mutants 3 hours after fatty acid supplementation 181

CHAPTER I

INTRODUCTION

Inhalation of aerosols from man-made water systems containing *Legionella pneumophila* puts immunocompromised individuals at risk of contracting Legionnaire's disease. Environmental legionellae have co-evolved with freshwater protozoa that graze on biofilms. Selective pressure on phagocytosed bacteria to avoid digestion has driven evolution of survival strategies that enable *Legionella* to opportunistically infect human alveolar macrophages. A hallmark virulence trait of *L. pneumophila* is the ability to differentiate between morphologically and phenotypically distinct states within host cells, including replicative and transmissive cell-types (Molofsky & Swanson, 2004, Bruggemann *et al.*, 2006). In some protozoan hosts, transmissive *L. pneumophila* differentiate further into 'mature intracellular forms' suited for environmental persistence (Faulkner *et al.*, 2008).

A variety of bacteria undergo morphological transformation to survive adversity (Zusman *et al.*, 2007, Paredes *et al.*, 2005, Voth & Heinzen, 2007, Abdelrahman & Belland, 2005, Swanson & Hammer, 2000, Molofsky & Swanson, 2004). Rowbotham first observed *L. pneumophila* alternating between two morphologically distinct forms in amoebae, changing their cell shape motility, surface and stores of energy rich polymers (Rowbotham, 1986). Since then, our lab and others have demonstrated that bacterial transformation is influenced by the local nutrient supply (Sauer *et al.*, 2005, Byrne &

Swanson, 1998). Transcriptome studies have shown *L. pneumophila* alters the expression of at least 800 different genes during differentiation (Bruggemann et al., 2006). The extraordinary switch requires activation and repression of elements critical for both morphological states (Molofsky & Swanson, 2004). Here, I demonstrate how *L. pneumophila* controls the level of ppGpp to direct this remarkable process.

"Magic spot" and virulence expression

Over 40 years ago, Cashel and Gallant first visualized guanosine 5'-diphosphate-3'-diphosphate (ppGpp) and guanosine 5'-triphosphate-3'-diphosphate (pppGpp; collectively referred to as ppGpp) by performing two-dimensional thin layer chromatography of radiolabeled nucleotides from amino acid-starved *Escherichia coli*. The appearance of these "magic spots," synthesized from GDP, or GTP, by pyrophosphoryl transfer from ATP, correlated with cessation of ribosomal RNA (rRNA) synthesis, a process referred to as the stringent response (Potrykus & Cashel, 2008). Subsequent research established that bacterial and plant cells which are experiencing nutritional stress synthesize ppGpp to initiate global physiological change. Although new roles for ppGpp continue to be discovered, the alarmone generally functions to promote adaptation and resilience of bacterial cells faced with adversity.

In the heterogeneous environments within mammalian and plant hosts, pathogenic bacteria alter their metabolism and protein repertoire in response to local conditions. Changes in the nutrient supply, alterations in immune responses, or contact with new surfaces can trigger bacterial adaptation. To gain advantage, pathogenic bacteria may activate specialized secretion systems, motility organelles, or adhesins. Such virulence factors may promote survival by equipping microbes to access nutrients, modulate the

host cell biology or immune system, or migrate to more favorable locales. In response to local conditions, pathogens utilize dedicated regulators to change their tactics. The expression and activity of many virulence regulators are integrated into a global response mediated by ppGpp, thereby coupling pathogenesis to metabolic status. As such, control over cellular ppGpp pools is critical for pathogen survival, replication and transmission.

Metabolism of ppGpp

Levels of ppGpp are modulated by two classes of enzymes: monofunctional synthetase-only enzymes and bifunctional synthetase/hydrolase enzymes (Fig. 1.1). As nomenclature of the bifunctional enzymes is not standardized, they can be referred to by their *E. coli* namesakes, RelA (monofunctional), or SpoT (bifunctional), or as RSH (Rel-Spo Homologue) proteins (Magnusson *et al.*, 2005, Braeken *et al.*, 2006, Srivatsan & Wang, 2008, Potrykus & Cashel, 2008). Both the monofunctional RelA- and bifunctional SpoT-like enzymes synthesize ppGpp from either GDP (in the case of guanosine tetraphosphate), or GTP (in the case of guanosine pentaphosphate) and ATP, whereas only the bifunctional enzymes also hydrolyze ppGpp to GDP and pyrophosphate (PPi), or GTP and PPi. Most Gram-negative γ-proteobacteria, like *E. coli*, *Salmonella, Pseudomonas* and *Legionella*, encode both RelA and SpoT (Fig. 1.1). Without SpoT, bacteria cannot degrade RelA-derived ppGpp, and unabated accumulation of the nucleotide disrupts cell cycle control. In these species, SpoT function can be studied in the context of *relA spoT* double mutants (often annotated as $ppGpp^0$ cells), which lack all synthetase activity. Many other pathogenic species encode ppGpp synthetase pathways distinct from the two enzyme RelA/SpoT paradigm. For example, the mycobacteria, the

α-proteobacterial *Brucella* spp., and the ε-proteobacteria each encode a single-bifunctional RSH (Rel/Spo homologue; annotated as Rel, Rsh, and SpoT, respectively), whereas several Gram-positive Firmicutes, such as *Bacillus*, *Listeria*, *Streptococcus* and *Enterococcus*, encode not only a single bifunctional RSH protein (alternately termed Rel or RelA), but also other small RelA-like synthetase fragments (termed RelP and RelQ; Fig. 1.1). Likewise, the γ-proteobacterium *Vibrio cholerae* encodes RelV, another truncated synthetase enzyme (Das *et al.*, 2009). The existence of multiple enzymes devoted to alarmone synthesis and hydrolysis illustrates that bacteria have evolved versatile mechanisms to control ppGpp levels. For a detailed discussion of the versatility of the stringent response in bacterial pathogenesis see *Dalebroux et al*, Microbiol Mol Bio Rev. 2010.

Stimuli eliciting ppGpp accumulation

By regulating the enzyme activities that control synthesis and degradation of ppGpp, bacteria can coordinate global physiological transformations tailored to distinct metabolic stimuli. It has long been established that the synthetase activity of *E. coli* RelA is elicited at the ribosome by uncharged tRNAs that accumulate during amino acid starvation (Potrykus & Cashel, 2008). Likewise, for bacteria harboring only one ppGpp synthetase the bifunctional RSH proteins are activated by amino acid starvation.
In contrast, for bacteria that encode two enzymes, their bifunctional SpoT enzymes respond to a variety of stimuli, including phosphate, carbon, and iron starvation, as well as fatty acid starvation (Potrykus & Cashel, 2008, Battesti & Bouveret, 2009). The monofunctional RelV from *V. cholerae* is allosterically influenced by fatty acid and

carbon starvation, raising the interesting possibility that additional monofunctional stringent response enzymes also sense stresses other than amino acid starvation (Das et al., 2009). In addition to nutritional cues, enzymes that govern ppGpp metabolism can be regulated both transcriptionally and post-translationally when pathogens encounter stress during transmission or infection, such as high osmolarity, extreme pH, or chemical onslaughts (Okada *et al.*, 2002, Wells & Gaynor, 2006, Abranches *et al.*, 2009).

ACP-SpoT interaction and the response to fatty acid starvation

The SpoT response to fatty acid biosynthesis inhibition is mediated by acyl carrier protein (ACP; Fig. 1.2). ACPs transfer acyl groups to enzymes involved in either phospholipid or secondary metabolite biosynthesis. The ACP-SpoT interaction is conserved in bacteria possessing two ppGpp synthetase enzymes (i.e., RelA and SpoT) and appears to be highly specific (Battesti & Bouveret, 2009). In particular, SpoT interactions are restricted to ACP proteins that are encoded within fatty acid biosynthesis operons. SpoT specifically binds to functional ACP proteins, namely those that have been post-translationally modified to carry a fatty acid intermediate (Battesti & Bouveret, 2006). Furthermore, ACP interacts with SpoT, but not RelA. In *E. coli*, the interaction of ACP with SpoT during growth in nutritionally replete media appears to inhibit the ppGpp synthetase domain of SpoT, skewing the balance of ppGpp metabolism by this enzyme toward hydrolysis. Upon fatty acid starvation, in some manner ACP interactions with the TGS domain of SpoT promote accumulation of ppGpp in the cell (Figs. 1.1 and 1.2). In *E. coli*, mutating specific amino acids within the regulatory domain of SpoT (A404E and S587N) abrogates the ACP interaction and eliminates the cellular response

to fatty acid biosynthesis inhibition (Battesti & Bouveret, 2006). Although the mechanistic details remain to be discovered, this physical interaction is influenced by the ratio of unacylated to acylated ACP, enabling bacteria to sense the fatty acid biosynthetic capacity of the cell (Potrykus & Cashel, 2008). Direct physical interaction between SpoT and ACP has been demonstrated for the *E. coli* and *P. aerunginosa* proteins, while genetic evidence suggests a similar interaction exists in *L. pneumophila* (see below)(Battesti & Bouveret, 2009). SpoT-dependent responses can reflect changes in either synthetase or hydrolase activity; thus, the catalytic balance may be a critical point of control.

Regulatory targets of ppGpp and DksA

Cooperative control over RNAP activity

The ppGpp alarmone mediates many of its physiological effects by transcriptional control, either by direct or indirect mechanisms. In *E. coli,* direct deactivation of rRNA operons and direct activation of amino acid biosynthetic operons by ppGpp occurs via an interaction between the nucleotide and RNA polymerase (RNAP) that is not fully understood (Potrykus & Cashel, 2008). DksA, a small protein that binds in the RNAP secondary channel, potentiates the effects of ppGpp on transcription (Fig. 1.3)(Haugen *et al.*, 2008). Whether a given promoter is directly activated or deactivated by ppGpp and DksA is dictated by DNA sequence motifs. Deactivated targets are typically GC-rich between the -10 hexamer and the +1 nucleotide (transcriptional start site), a site known as the discriminator region, whereas activated promoters are typically AT-rich in this position.

Indirect transcriptional control by ppGpp and DksA can occur through a process known as sigma factor competition (Fig. 1.4). In γ-proteobacteria, nearly all direct targets of ppGpp require the vegetative housekeeping sigma factor, σ^{70}. During a stringent response, alarmone inhibition of strong σ^{70}-dependent promoters, such as rRNA promoters, increases the availability of core RNAP for transcription with alternative sigma factors (Bernardo *et al.*, 2006, Szalewska-Palasz *et al.*, 2007, Costanzo *et al.*, 2008, Gummesson *et al.*, 2009). In this manner, ppGpp indirectly promotes alternative sigma factor-dependent gene activation by deactivating transcription of rRNA operons. Together, direct and indirect ppGpp-dependent mechanisms are integrated to mediate the global physiological adaptations of the bacterial cell that comprise the stringent response.

Independent regulation

Recently, the dogma that ppGpp and DksA always collaborate to regulate *E. coli* physiology has been challenged. Phenotypic studies indicate that overproduction of DksA by $ppGpp^0$ bacteria can compensate for the lack of the alarmone (Potrykus & Cashel, 2008). Additionally, ppGpp and DksA have opposite effects on certain *E. coli* traits (Magnusson *et al.*, 2007, Aberg *et al.*, 2009). Furthermore, *in vitro* assays show opposite and independent regulation of some promoters by these two stringent response components (Lyzen *et al.*, 2009, Merrikh *et al.*, 2009b). Also, some regulatory effects that are predicted by phenotypic assays, such as expression of fimbriae and flagella, are not recapitulated when transcriptional regulation by ppGpp and DksA is analyzed *in vitro* at the promoters of critical regulators, like FimB, FlhDC, and FliA (Aberg *et al.*, 2008, Lemke *et al.*, 2009). Accordingly, other factors likely influence regulation by $ppGpp^0$

and *dksA* mutant cells. For example, TraR upregulates transcription from amino acid promoters and downregulates transcription from ribosomal promoters in the absence of ppGpp and DksA (Blankschien *et al.*, 2009). Therefore, additional work is required before these complex regulatory interactions are fully understood.

Interactions with factors other than RNAP

Distinct roles for ppGpp and DksA in the bacterial cell also result from interactions between the alarmone and proteins other than RNAP. For example, in *Bacillus subtilis,* ppGpp prevents DNA replication elongation by inhibiting DNA primase activity (Wang *et al.*, 2007). In *Salmonella enterica* serovar Typhimurium, ppGpp interacts with SlyA, a transcriptional activator of this pathogen's intracellular virulence program, facilitating its dimerization and binding to target promoters (Zhao *et al.*, 2008). In both *E. coli* and *B. subtilus*, ppGpp interacts with Obg, a nucleotide binding protein implicated in a number of physiological processes (Buglino *et al.*, 2002, Persky *et al.*, 2009). Furthermore, the impact of increased alarmone levels on the physiology of some Gram-positive bacteria, like *B. subtilis*, can be elicited through resulting decreases in GTP pools. Fluctuations in GTP levels may affect the activity of global regulators such as CodY (Inaoka & Ochi, 2002). While a portion of cellular GTP is used during ppGpp synthesis, the alarmone also inhibits inosine monophosphate dehydrogenase, an enzyme involved in GTP biosynthesis (Gallant *et al.*, 1971, Lopez *et al.*, 1981). In *B. subtilus*, rRNA promoters initiate with GTP; therefore, ppGpp indirectly controls deactivation of rRNA transcription by reducing GTP pools (Krasny & Gourse, 2004, Krasny *et al.*,

2008). Thus, ppGpp can operate independently of both DksA and RNAP activity, illustrating the alarmone's far-reaching effects.

ppGpp and the *L. pneumophila* life cycle

Regulation of the *L. pneumophila* life cycle in both protist and mammalian host cells requires strict control of ppGpp metabolism (Fig. 1.5). Increased levels of ppGpp cue differentiation of *L. pneumophila* to a motile, coccoid, transmissive form that exhibits increased resistance to stress and the ability to evade lysosomal degradation (Molofsky & Swanson, 2004). Genetic and molecular studies from my thesis support the following model for the *L. pneumophila* life cycle. In the transmissive state, *L. pneumophila* effector proteins are transcribed and translocated into the host cell by the Dot/Icm Type IV secretion system (Nagai *et al.*, 2002, Bruggemann et al., 2006, Rasis & Segal, 2009, Tiaden *et al.*, 2007). In particular, *L. pneumophila* relies on the Dot/Icm system to avoid fusion with the endosomal pathway and to establish a replication niche in a compartment derived from the endoplasmic reticulum (Isberg *et al.*, 2009). To obtain nutrients such as amino acids from the vacuolar lumen, *L. pneumophila* relies on phagosomal transporters (Phts) (Sauer et al., 2005, Chen *et al.*, 2008). When nutrients are abundant, transmissive bacteria hydrolyze ppGpp (Fig. 1.5), resulting in initiation of cell division and repression of transmission factors (Sauer et al., 2005, Molofsky & Swanson, 2003). As a consequence, in mouse macrophages the block to phagosome-lysosome fusion is relieved, and the replication vacuole matures into an acidic lysosomal vacuole (Sturgill-Koszycki & Swanson, 2000). As the replicating bacteria consume nutrients, vacuolar conditions presumably deteriorate and stimulate ppGpp production, prompting the progeny to reenter the transmissive state (Fig. 1.5). For example, elevated ppGpp

triggers expression of factors leading to bacterial cytotoxicity to macrophages, motility, stress resistance and the ability to evade lysosomes, traits which promote transmission of the pathogens from the exhausted host cell and infection of naïve ones. Thus, ppGpp potentiates *L. pneumophila* cell-to-cell transmission.

Flagellar biogenesis of *L. pneumophila*

At the transition from exponential to stationary phase in broth, *L. pneumophila* undergoes gross morphological transformation and builds a monopolar flagellum. Flagellar assembly also marks differentiation of intracellular bacteria. At the replicative to transmissive phase transition in macrophages and amoebae, the promoter for the primary flagellar subunit *flaA* is activated (Hammer & Swanson, 1999, Bruggemann et al., 2006). Concomitantly, FlaA protein is detectable by immunofluorescence microscopy (Byrne & Swanson, 1998, Molofsky *et al.*, 2005). In freshwater environments, flagellar synthesis in response to deteriorating host conditions likely prepares *L. pneumophila* for migration to a new intracellular niche. Thus, flagellar assembly is a sensitive readout for the transmissive phase of the life cycle.

Flagellar biosynthesis proceeds via a sophisticated transcriptional cascade influenced by ppGpp. Experimental induction of ppGpp synthesis in exponential phase bacteria rapidly activates *flaA* expression and motility of *L. pneumophila* (Hammer & Swanson, 1999). By undefined mechanisms, the alarmone influences the activity of key flagellar gene regulators. Similar to *P. aeruginosa*, *L. pneumophila* flagellar gene transcription occurs in a hierarchy of four classes (Dasgupta *et al.*, 2003, Bruggemann et al., 2006, Heuner, 2007, Albert-Weissenberger *et al.*). The master regulator FleQ and the alternative sigma factors RpoN (σ^{54}) and FliA (σ^{28}) control expression of particular gene

classes. FleQ and RpoN are Class I factors that control activation of Class II genes encoding elements of the flagellar basal body, rod and hook (Jacobi *et al.*, 2004, Albert-Weissenberger et al.). FleQ also contributes modestly to activation of some Class III genes, including FliA (Albert-Weissenberger et al.). FliA is critical for final assembly of the organelle. The sigma factor targets additional Class III elements, including motor components, and all Class IV elements, including the filament FlaA and the capping protein FliD (Fig. 1.6)(Heuner *et al.*, 2002, Bruggemann et al., 2006, Heuner, 2007, Albert-Weissenberger et al.).

The role of FliA is not limited to flagellar regulation. The sigma also controls expression of several genes unrelated to flagellar biogenesis and is critical for *L. pneumophila* to resist degradation in macrophage lysosomes (Molofsky et al., 2005, Bruggemann et al., 2006). Studies in amoebae indicate that FliA is also essential for intracellular replication in particular protozoan hosts (Heuner et al., 2002). Therefore, FliA represents an additional link between the flagellar cascade and virulence of *L. pneumophila*.

Post-transcriptional regulation of *L. pneumophila* transmission

LetA/S, RsmY/Z, and CsrA

From atop a complex regulatory cascade, ppGpp exerts both direct and indirect control over downstream activators and repressors of *L. pneumophila* transmission (Fig. 1.6)(Molofsky & Swanson, 2004). To respond to elevated ppGpp, *L. pneumophila* requires the LetA/LetA two component system (Hammer *et al.*, 2002). The mechanism by which ppGpp activates the LetA/S system remains unknown. The LetA transcriptional activator binds DNA upstream of two non-coding regulatory RNAs,

RsmY and RzmZ, and stimulates their transcription (Sahr *et al.*, 2009, Rasis & Segal, 2009). Both RsmY and RsmZ interact with CsrA, an RNA-binding protein and repressor of *L. pneumophila* transmission, which is predicted to relieve its ability to bind transcripts critical for the transmissive phenotype, such as effectors of the Dot/Icm system (Molofsky & Swanson, 2003, Rasis & Segal, 2009). Genetic studies suggest that during intracellular replication, CsrA negatively regulates particular flagellar gene regulators (Fig. 1.6). Conditional *csrA* null mutant *L. pneumophila* is unable to replicate in host cells and exhibits motility when nutrients are replete (Molofsky & Swanson, 2003). In accordance with these findings, reduced expression of *csrA* leads to accumulation of *fliA* and *flaA* mRNAs (Forsbach-Birk *et al.*, 2004). Conversely, overexpression of *csrA* in stationary phase bacteria represses motility and leads to reduced levels of *fliA* and *flaA* transcripts (Molofsky & Swanson, 2003, Fettes *et al.*, 2001). Therefore, CsrA is a global repressor of the transmissive phenotype and exerts specific control over factors critical for *L. pneumophila* flagellar assembly (Fig. 1.6). Thus, one mechanism by which ppGpp might contribute to flagellar activation is by activating the LetA/S two component system, thereby prompting derepression of flagellar transcripts targeted by CsrA.

RpoS

Another factor purportedly downstream of ppGpp in the *L. pneumophila* virulence cascade is the stationary phase sigma factor, RpoS (σ^{38}) (Fig. 1.6). RpoS is essential for intracellular replication of *L. pneumophila* in *Acanthamoebae castellani* and partially required for growth in macrophages (Hovel-Miner *et al.*, 2009, Bachman & Swanson, 2001). Although RpoS translation efficiency and protein levels have not been monitored

in *L. pneumophila*, the *rpoS* transcript is more abundant in exponential than in stationary phase (Bachman & Swanson, 2004b). As with *E. coli*, *rpoS* expression by *L. pneumophila* is sensitive to ppGpp pools, since transcript levels increase following artificial induction of the alarmone (see below)(Brown *et al.*, 2002). Thus, it appears that ppGpp controls RpoS expression to affect transmission and replication in *L. pneumophila*; however, specifics of the interplay in this complex regulatory mechanism remain to be clarified.

RpoS is also integrated into the *L. pneumophila* CsrA regulatory system. In particular, this sigma factor is required for maximal *rsmY* and *rsmZ* gene expression (Fig. 1.6)(Rasis & Segal, 2009, Hovel-Miner et al., 2009). Therefore, by mechanisms yet to be defined, LetA/S and RpoS cooperate to relieve CsrA-mediated repression of transmissive transcripts. Consistent with this model, LetA/A and RpoS are required for robust accumulation of *fliA* mRNA in stationary phase *L. pneumophila* (Bachman & Swanson, 2004a). However, the basal level of *fliA* transcript in *rpoS* mutant *L. pneumophila* is still sufficient to induce activation of *flaA*, leading to a modest accumulation of *flaA* mRNA and partial motility of mutant bacteria (Bachman & Swanson, 2001, Bachman & Swanson, 2004a). Contrary to this, basal levels of *fliA* are not sufficient for *flaA* expression in *letA/S* mutant bacteria, suggesting LetA/S affects FliA activity independent of RpoS (Fig. 1.6). This incomplete overlap between LetA/S and RpoS suggests dual pathways likely contribute to optimal flagellar expression of *L. pneumophila*. Thus, regulatory interactions between ppGpp, LetA/S, RpoS and CsrA are critical for flagellar assembly and enable *L. pneumophila* to rapidly transition between replicative and transmissive virulence programs in host cells.

Outline of thesis

In this thesis I describe how the ppGpp signaling molecule, or "magic spot," coordinates timely differentiation of *L. pneumophila* and facilitates efficient host-to-host transmission of the bacterium. Rapid morphological transformation of *L. pneumophila* demands regulation on multiple levels. While ppGpp has been traditionally studied as a global regulator of gene transcription in bacteria, *L. pneumophila* has also evolved to use ppGpp to control mRNA stability.

My work extends our knowledge of the role of ppGpp in *L. pneumophila* virulence regulation. Previous work established that the signaling molecule requires the LetA/S two component regulatory system and the alternative sigma factor RpoS to control expression of particular transmission traits (Hammer et al., 2002, Bachman & Swanson, 2001). Recently, these regulators were identified as activators of two non-coding regulatory RNAs, RsmY and RsmZ (Hovel-Miner et al., 2009, Sahr et al., 2009, Rasis & Segal, 2009). RsmY/Z interact with the RNA-binding protein CsrA to relieve its repression of transcripts critical for *L. pneumophila* transmission (Sahr et al., 2009, Rasis & Segal, 2009). By demonstrating how ppGpp pools are balanced during infection, my thesis places the LetA/S-RsmY/Z cascade into the context of the *L. pneumophila* intracellular lifecycle. My investigations led to characterization of an additional component of the cellular response to ppGpp, the transcription factor DksA. Finally, I exploit the *L. pneumophila* flagellar regulon to delineate interactions between ppGpp, DksA and the aforementioned regulators of *L. pneumophila* transmission. My inquiries led to the discovery that for flagellar activation, *L. pneumophila* employs the ppGpp

alarmone to control both transcription initiation and mRNA stability, enabling rapid and robust activation of components such as *flaA*, the gene encoding flagellin.

Specifically, in Chapter II I demonstrate that *L. pneumophila* encodes two enzymes dedicated to ppGpp metabolism: the monofunctional synthetase RelA, and the bifunctional synthetase/hydrolase SpoT. In broth culture, each enzyme responds to distinct metabolic cues to increase the cellular ppGpp pool. In particular, RelA responds to amino acid starvation and SpoT to perturbations in fatty acid biosynthesis, likely through an interaction with acyl carrier protein (ACP). Consistent with its dual enzymatic activities, SpoT is essential for *L. pneumophila* both to initiate replication and to engage cell-to-cell transmission in macrophages. In Chapter III, I define a new component of *L. pneumophila* differentiation, the transcription factor DksA. Specifically, I show that ppGpp and DksA either cooperate, or act independently, to control *L. pneumophila* differentiation. For example, for flagellar assembly, DksA controls basal activation of the flagellar sigma factor *fliA* (σ^{28}) and responds to increasing ppGpp by modulating expression of additional flagellar components. Both are required for differentiation to the transmissive form in broth culture. On the other hand, DksA is dispensable for macrophage-to-macrophage transmission, suggesting ppGpp can act autonomously. In Chapter IV, I provide evidence that ppGpp accumulation stimulates activation of the CsrA-binding regulatory RNAs, RsmY and RsmZ. In particular, initiation of the *rsmZ* promoter requires complex regulatory interactions between ppGpp, DksA, LetA and RpoS. Therefore, multiple signals are integrated at the *rsmZ* promoter to prompt a global change in mRNA stability. In particular, by activating RsmY/Z, ppGpp relieves CsrA repression of *fliA* gene activation, permitting rapid and robust

accumulation of *fliA* message and final assembly of the flagellum. Finally, I highlight the important principles established by my research, and suggest ways to expand upon these findings to further our knowledge of *L. pneumophila* biology and the role of ppGpp in regulating bacterial virulence.

Figure 1.1.

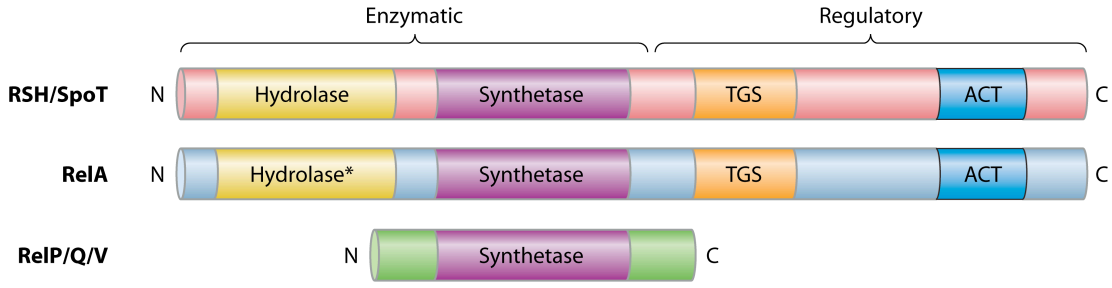

Figure 1.1. Classes and domain architecture of ppGpp synthetases. Bifunctional proteins, represented by RSH/SpoT, contain both synthetase and hydrolase activities in an N-terminal enzymatic domain. RelA proteins are monofunctional, synthetase-only enzymes, as the hydrolase domain contains amino acid changes rendering it inactive ("*"). The small alarmone synthetase (SAS) enzymes (RelP, RelQ, and RelV) are monofunctional and harbour only a synthetase domain. SAS proteins appear related to the *E. coli* RelA synthetase domain, although very limited identity remains at the amino acid level (Lemos *et al.*, 2007, Nanamiya *et al.*, 2008). Activity of RelA and RSH/SpoT proteins may be controlled postranslationally through a C-terminal regulatory domain containing conserved sequences in the TGS and ACT domains (Potrykus & Cashel, 2008). In SpoT, TGS mediates interactions with acyl-carrier protein, whereas the ACT domains contained in RSH and RelA proteins have been suggested to mediate ligand binding. The SAS proteins do not carry such domains and may be regulated at the transcriptional level.

Figure 1.2.

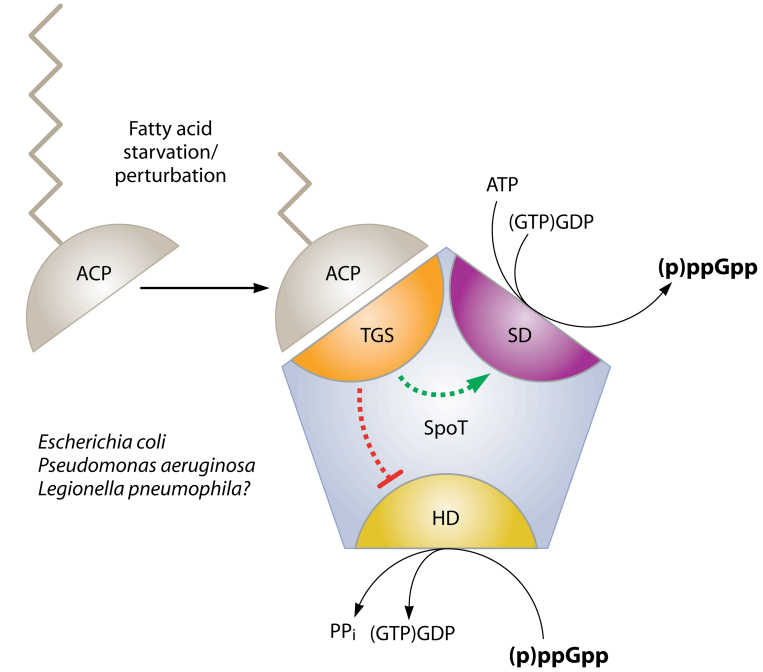

Figure 1.2. SpoT activity is regulated through ACP interaction.
γ-proteobacteria that encode both RelA and SpoT have evolved a SpoT-dependent stringent response to fatty acid starvation that is mediated by an interaction between SpoT and acyl-carrier protein (ACP) (Battesti & Bouveret, 2009, Battesti & Bouveret, 2006). ACP transfers fatty acyl chains to enzymes devoted to phospholipid and secondary metabolite biosynthesis. SpoT interacts with functional acyl-bound ACP at a non-enzymatic region known as the TGS domain. During fatty acid starvation, metabolic signals are transduced through an ACP-SpoT interaction, resulting in an increase in cellular ppGpp pools. It remains to be determined whether, in response to fatty acid stress, the ACP-SpoT interaction specifically modulates the synthetase (SD) or hydrolase (HD) activity of SpoT.

Figure 1.3.

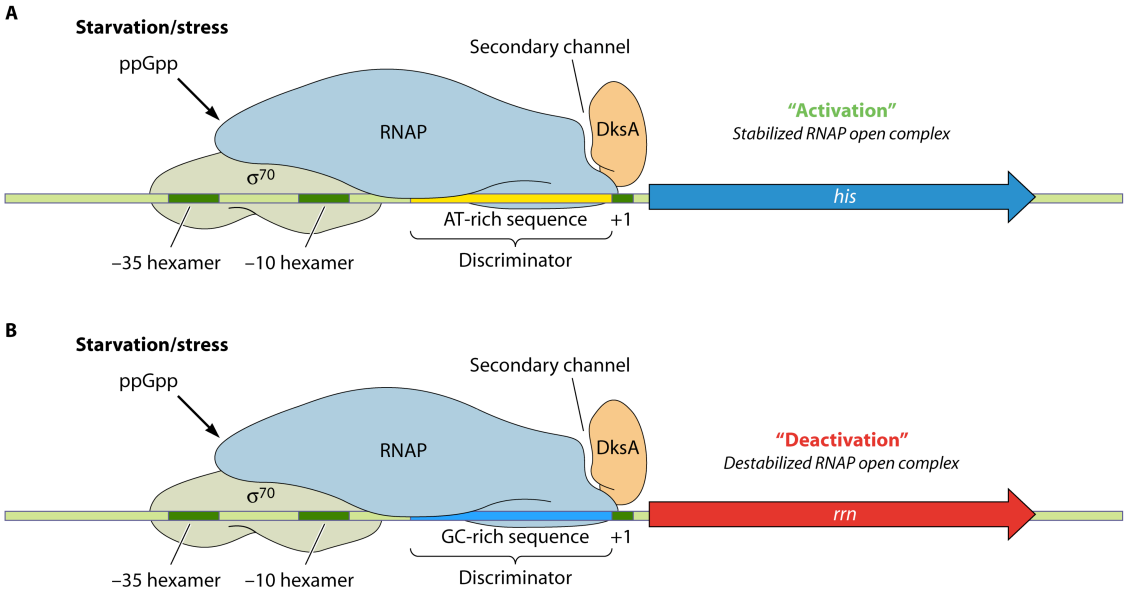

Figure 1.3. ppGpp and DksA control transcription directly.
In response to stress, γ-proteobacteria use ppGpp and DksA to control RNA polymerase (RNAP) activity at particular promoters. Although DksA is known to bind at the secondary channel of RNAP, a binding site for ppGpp has not been confirmed (Haugen et al., 2008). In the presence of DksA and elevated ppGpp, transcription can either be activated or deactivated. Whether transcription is activated or deactivated depends upon intrinsic properties of the promoter. Activated targets such as the *E. coli* promoter for the histidine biosynthetic (*his*) operon typically have an AT-rich DNA sequence between the -10 hexamer and the +1 transcriptional start site, known as the discriminator region (Haugen et al., 2008). Conversely, deactivated targets such as the P1 promoter of ribosomal RNA (*rrn*) operons typically have a GC-rich discriminator sequence. Promoters controlled directly by ppGpp and DksA generally depend on the housekeeping/vegetative sigma factor, σ^{70}.

Figure 1.4.

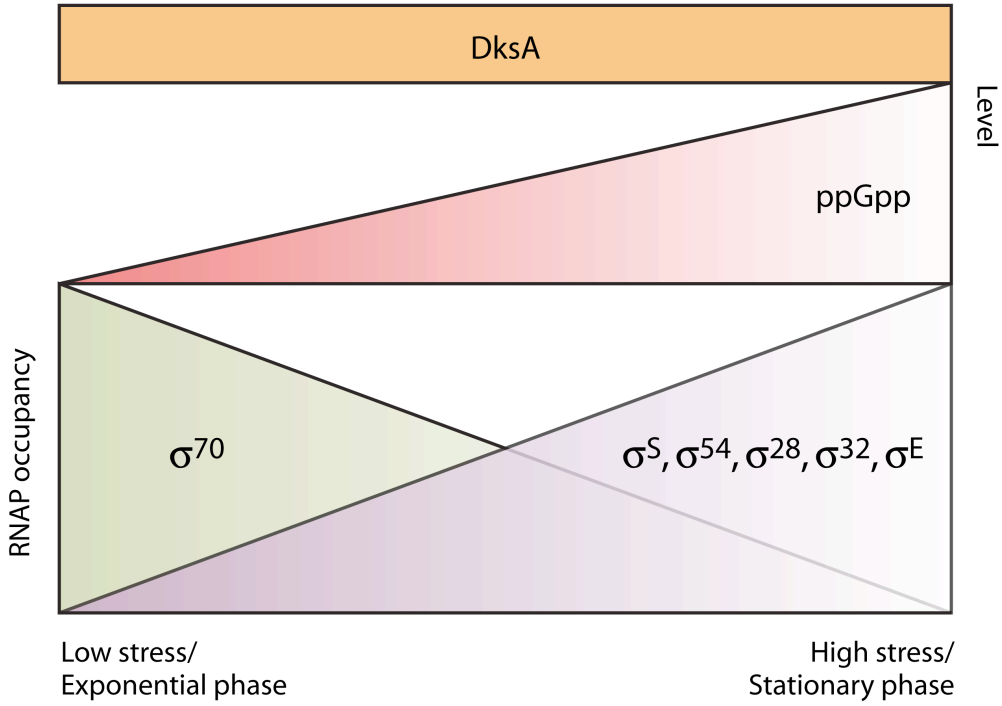

Figure 1.4. ppGpp and DksA control transcription indirectly through "sigma factor competition".
During exponential growth or favorable conditions, ppGpp levels are low in γ-proteobacteria, and transcription from strong σ^{70}-dependent promoters such as those of ribosomal RNA (rRNA) operons is robust. As bacteria exit exponential phase, or during high stress, ppGpp levels accumulate. DksA, whose levels remain constant during growth, cooperates with ppGpp to deactivate transcription of rRNA operons, liberating RNAP to bind alternative sigma factors (σ^{38}, σ^{54}, σ^{28}, σ^{32}, and σ^{E}) (Bernardo et al., 2006, Szalewska-Palasz et al., 2007, Costanzo et al., 2008, Gummesson et al., 2009). As a result, transcription from promoters targeted by these sigma factors increases, inducing specialized stress responses. In this manner, ppGpp and DksA contribute indirectly to bacterial adaptation.

Figure 1.5.

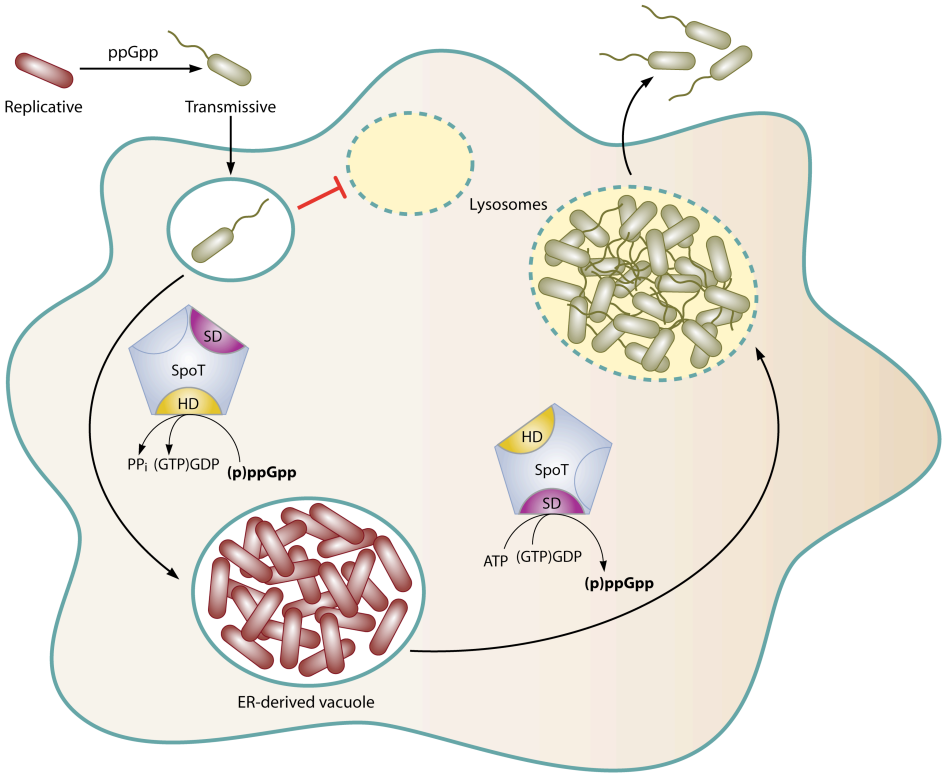

Figure 1.5. SpoT governs the *Legionella* life cycle in macrophages.
During its life cycle, the intracellular pathogen *L. pneumophila* differentiates between two forms, replicative and transmissive. When nutrients become scarce, ppGpp levels increase, coordinating differentiation of replicative bacteria to the highly resilient, motile, transmissive form. After phagocytosis, transmissive bacteria inhibit fusion with degradative lysosomes (small, dashed, empty vacuole). To convert to the replicative form, *L. pneumophila* must sense favorable vacuolar conditions that stimulate the bifunctional SpoT enzyme to reduce alarmone pools via ppGpp hydrolysis (HD). In a vacuole derived from the endoplasmic reticulum (ER), replicative *L. pneumophila* divide exponentially. Gradually the replication vacuole acidifies and acquires lysosomal markers. Deteriorating vacuolar conditions elicit SpoT synthetase (SD) activity, cueing replicative bacteria to differentiate back to the transmissive form. Transmissive *L. pneumophila* resist lysosomal degradation and migrate to a naïve host cell, primed to establish a new infection.

Figure 1.6.

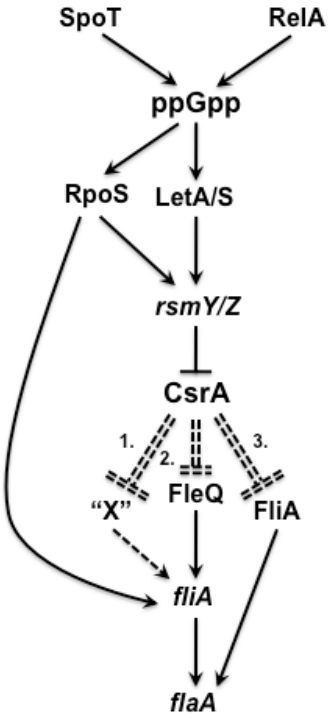

Figure 1.6. ppGpp synthesis leads to derepression of a flagellar gene activator. In response to stress, RelA and SpoT synthesize ppGpp, leading to activation of the alternative sigma factor RpoS and the LetA/S two component regulatory system. LetA/S and RpoS activate transcription of *rsmY* and *rsmZ*, two regulatory RNAs. RsmY/Z bind to CsrA to derepress FliA, the flagellar sigma factor that activates transcription of *flaA*, encoding the filament subunit. By an independent mechanism, RpoS also controls activation of *fliA*. The mechanism by which CsrA represses FliA is unknown. CsrA might: (1) repress an unidentified *fliA* gene activator, (2) repress FleQ, or (3) repress FliA itself. Solid, single lines indicate interactions occurring in the transmissive bacteria, double lines indicate interactions occurring in replicative bacteria, and dashed lines indicate speculative interactions.

CHAPTER II

SpoT governs *Legionella pneumophila* differentiation in host macrophages.

Summary

During its life cycle, *Legionella pneumophila* alternates between a replicative and a transmissive state. To determine their contributions to *L. pneumophila* differentiation, the two-ppGpp synthetases, RelA and SpoT, were disrupted. Synthesis of ppGpp was required for transmission, as *relA spoT* mutants were killed during entry to and exit from macrophages. RelA, which senses amino acid starvation induced by serine hydroxamate, is dispensable in macrophages, as *relA* mutants spread efficiently. SpoT monitors fatty acid biosynthesis (FAB), since following cerulenin treatment, wild-type and *relA* strains expressed the *flaA* transmissive gene, but *relA spoT* mutants did not. As in *E. coli*, the SpoT response to FAB perturbation likely required an interaction with acyl-carrier protein (ACP), as judged by the failure of the *spoT-A413E* allele to rescue transmissive trait expression of *relA spoT* bacteria. Furthermore, SpoT was essential for transmission between macrophages, since secondary infections by *relA spoT* mutants were restored by induction of *spoT*, but not *relA*. To resume replication, ppGpp must be degraded, as mutants lacking *spoT* hydrolase activity failed to convert from the transmissive to the replicative phase in either bacteriological medium or macrophages. Thus, *L.*

pneumophila requires SpoT to monitor FAB and to alternate between replication and transmission in macrophages.

Introduction

To cope with environmental fluctuations such as changes in temperature, osmolarity, and nutrient availability, bacteria modify their physiology. To increase resilience and promote survival in response to deteriorating conditions, some bacteria also undergo morphological adaptations. Examples of microbial differentiation include fruiting body formation by soil dwelling *Myxococcus xanthus* (Zusman et al., 2007), spore formation by species of *Bacillus* and *Clostridium* (Paredes et al., 2005), and alternation between replication and transmission by the intracellular pathogens *Coxiella burnetti* (Voth & Heinzen, 2007), *Chlamydia trachomatis* (Abdelrahman & Belland, 2005), and *Legionella pneumophila* (Swanson & Hammer, 2000, Molofsky & Swanson, 2004). In each case, differentiation is integral to the microbe's resilience and versatility.

L. pneumophila is ubiquitous in aquatic environments where it resides either in biofilms or within freshwater protozoa. When humans inhale aerosols of contaminated water, this opportunistic pathogen can infect alveolar macrophages. Upon phagocytosis, transmissive *L. pneumophila* resist phagosome acidification and maturation and instead become sequestered within a protective, ER-derived vacuole (Shin & Roy, 2008). Favorable metabolic conditions within the vacuole prompt repression of transmissive genes and induction of genes required for protein synthesis and replication (Molofsky & Swanson, 2003, Sauer et al., 2005, Bruggemann et al., 2006). Following multiple rounds of cell division that presumably exhaust host cell nutrients, the bacteria differentiate back to the transmissive form, which can subsequently develop into a "mature intracellular

form" suited for prolonged viability in the environment (Faulkner et al., 2008). In broth cultures, lysosome evasion, Type IV secretion, flagella expression, sodium sensitivity and cytotoxicity are coordinately induced in stationary phase (Byrne & Swanson, 1998, Alli *et al.*, 2000, Molofsky & Swanson, 2004); since each trait is predicted to enhance spread from one cell to another, we refer to this as the transmissive phase. In both broth and amoebae, *L. pneumophila* exhibits a biphasic gene expression pattern that corresponds to the phenotypic switch observed (Bruggemann et al., 2006). Thus, the transition between the exponential (E) phase and the post-exponential (PE) phase in broth cultures reproduces in large part the differentiation from the replicative to the transmissive phase observed in host cells.

In broth cultures, the *L. pneumophila* E to PE phase transition is concomitant with accumulation of guanosine tetraphosphate, or ppGpp (Hammer & Swanson, 1999, Zusman *et al.*, 2002), an alarmone that acts as a general signal of bacterial starvation and stress (Magnusson et al., 2005, Srivatsan & Wang, 2008, Potrykus & Cashel, 2008). The ppGpp alarmone is thought to alter the interaction of RNA polymerase with specific promoters to either activate or inhibit transcription. A regulatory factor termed DksA for *dnaK* suppressor protein, potentiates ppGpp regulation (Magnusson et al., 2005, Srivatsan & Wang, 2008, Potrykus & Cashel, 2008). The global transcriptional changes that ppGpp and DksA instruct are collectively referred to as the stringent response (Magnusson et al., 2005, Srivatsan & Wang, 2008, Potrykus & Cashel, 2008). In *Escherichia coli*, ribosomal and amino acid biosynthetic operons, flagellar and chemotaxis related genes are all under stringent control, as are key enzymes involved in fatty acid biosynthesis (Durfee *et al.*, 2008, Traxler *et al.*, 2008).

Several pathogenic bacteria also exploit ppGpp for virulence gene expression and survival in host cells. Examples include transmission trait expression by *L. pneumophila* (Hammer & Swanson, 1999), type I fimbriae expression by uropathogenic *E. coli* (Aberg *et al.*, 2006), and invasion of intestinal epithelial cells and virulence of *Salmonella* (Thompson *et al.*, 2006, Song *et al.*, 2004, Pizarro-Cerda & Tedin, 2004). Other pathogens, including *Mycobacterium tuberculosis*, *Listeria monocytogenes*, *Pseudomonas aeruginosa*, and *Campylobacter jejuni*, also require ppGpp to regulate particular virulence mechanisms (Primm *et al.*, 2000, Taylor *et al.*, 2002, Erickson *et al.*, 2004)}(Gaynor *et al.*, 2005).

In gram-negative bacteria such as *E. coli* and *Salmonella* spp., the levels of ppGpp in the cell are regulated by the ppGpp synthetase RelA and the bifunctional synthetase/hydrolase SpoT (Magnusson et al., 2005, Srivatsan & Wang, 2008, Potrykus & Cashel, 2008). During amino acid starvation, uncharged tRNAs trigger rapid synthesis of ppGpp by ribosome-bound RelA. During exponential growth, SpoT hydrolyzes ppGpp. Since SpoT hydrolase activity is required to prevent RelA-dependent ppGpp from accumulating unabatedly, *spoT* is essential to bacteria that encode *relA*. When the RelA synthetase is not active, *spoT* is dispensable for replication and its function can be deduced by comparing *relA* mutants to *relA spoT* strains (Magnusson et al., 2005, Srivatsan & Wang, 2008, Potrykus & Cashel, 2008).

SpoT can also generate ppGpp in response to a variety of stresses, including carbon source deprivation, phosphate starvation, iron starvation, and fatty acid biosynthesis inhibition (Magnusson et al., 2005, Srivatsan & Wang, 2008, Potrykus & Cashel, 2008). For *E. coli* to respond to perturbations in fatty acid biosynthesis (FAB),

SpoT interacts with acyl-carrier protein (ACP), a protein critical for fatty acid biosynthesis (Battesti & Bouveret, 2006). Point mutations within the C-terminal TGS domain of SpoT not only disrupt its interaction with ACP, but also alter its activity such that ppGpp synthesis is favored over hydrolysis (Battesti & Bouveret, 2006). Phosphate starvation results in SpoT-dependent ppGpp accumulation that requires SpoT hydrolase activity, not synthetase activity (Spira & Yagil, 1998, Bougdour & Gottesman, 2007). It remains to be determined if other starvation conditions that elicit ppGpp accumulation alter the SpoT-ACP interaction or result from either an inhibition of ppGpp hydrolysis or an increase in synthesis.

L. pneumophila requires RelA both for ppGpp accumulation upon entry into stationary phase and for maximal expression of the primary flagellar subunit *flaA* (Zusman et al., 2002). However, RelA is dispensable for *L. pneumophila* growth in *Acanthamoeba castellanii* or HL-60-human derived macrophages (Zusman et al., 2002, Abu-Zant *et al.*, 2006). SpoT was proposed to be essential to *L. pneumophila* viability, since attempts to disrupt the locus in both WT and *relA* mutant backgrounds were unsuccessful (Zusman et al., 2002).

Here we undertook a comprehensive analysis of the contributions of RelA and SpoT to the *L. pneumophila* life cycle. We report that distinct metabolic cues trigger SpoT and RelA activity and that, during the *L. pneumophila* life cycle in host macrophages, SpoT is critical not only for replicative cells to differentiate to the transmissive state, but also for transmissive cells to re-enter the replicative phase.

Experimental Procedures

Bacterial strains, culture conditions and reagents. *L. pneumophila* strain Lp02 (*thyA hsdR rpsL*; MB110), a virulent thymine auxotroph derived from Philadelphia 1 (Berger & Isberg, 1993), was the parental strain for all the strains analyzed. *L. pneumophila* was cultured at 37°C in 5 ml aliquots of *N*-(2-acetamido)-2-aminoethanesulfonic acid (ACES; Sigma)-buffered yeast extract (AYE) broth with agitation or on ACES-buffered charcoal yeast extract (CYE), both supplemented with 100 μg/ml thymidine (AYET, CYET) when necessary. Bacteria obtained from colonies <5 days old were cultured in broth overnight, then subcultured into fresh AYET prior to experimentation. For all experiments, exponential (E) cultures were defined as having an optical density at 600 nm (OD_{600}) of 0.3 to 2.0; post-exponential (PE) samples were obtained from cultures having an OD_{600} of 3.0 to 4.5 during the period 20 – 30 h after subculture, when viability and cell shape of the strains was similar (Fig. 2.1C). Where indicated, ampicillin (amp; Fisher) was added to a final concentration of 100 μg ml^{-1}, kanamycin (kan; Roche) to 10 μg ml^{-1}, gentamycin (gent; Fisher) to 10 μg ml^{-1}, chloramphenicol (cam; Roche) to 5 μg ml^{-1}, cerulenin (cer; Sigma) to 0.5 μg ml^{-1}, serine hydroxamate (Sigma) to 1 mM, propionic acid to 10 mM, acetic acid to 10 mM, and isopropyl-beta-D-thiogalactopyranoside (IPTG) to the concentrations specified. To determine colony-forming units (CFU), serial dilutions of *L. pneumophila* were plated on CYET and incubated at 37°C for 4-5 days.

relA and relA spoT mutant strain construction. To construct a *relA* insertion mutant, we generated pGEM-*relA* by amplifying the *relA* locus (lpg1457) plus flanking sequence from Lp02 genomic DNA using the primers relA1 and relA2. The kanamycin resistance gene cassette from pUC4K and the gentamycin cassette from PUCGent were obtained from their respective plasmids by *Eco*RI digestion, and pGEM-*relA* was digested with *Sna*BI. The fragments containing the resistance cassettes were blunted with Klenow, gel extracted, and ligated into the blunted pGEM-*relA* plasmid, creating pGEM-*relA::kan* and pGEM-*relA::gent*. After verification by PCR and restriction enzyme digest, the insertion alleles were used to transform Lp02 by natural competence, and the resulting transformants were selected using the appropriate antibiotic (Stone & Kwaik, 1999). Recombination of the desired *relA* insertion alleles onto the Lp02 chromosome was confirmed by PCR, and the resulting *relA::kan* and *relA::gent* mutant *L. pneumophila* were designated MB696 and MB695, respectively.

To construct the *relA spoT* double mutant, we generated pGEM-*spoT* by amplifying the *spoT* locus (lpg2009) plus flanking sequence from Lp02 genomic DNA using the primers spoT1 and spoT2. pGEM-*spoT* was digested with *Hin*dIII, which cuts at sites ~98-bp and ~588-bp 3' to the translational start, releasing an ~490-bp fragment encoding the consensus HD motif required for hydrolase activity, as well as a significant portion of the predicted synthetase coding regions at the N-terminus of SpoT. The deletion leaves intact 32 amino acids at the extreme N-terminus; as well as, amino acids 195-715 at the C-terminus. By analogy to deletion analysis conducted with the *E. coli spoT* gene (Gentry and Cashel, 1996), our deletion should have eliminated both enzymatic activities of SpoT. The linear fragment containing the *spoT* deletion was

blunted, and a kanamycin cassette was ligated to the blunted fragment, generating pGEM-*spoT::kan*. After verification by PCR and restriction enzyme digest, the deletion/insertion alleles were used to transform *relA::gent* mutant *L. pneumophila* MB695 by natural competence, and transformants were selected using the appropriate antibiotic (Stone & Kwaik, 1999). Recombination of the desired *spoT* deletion/insertion allele into the *relA::gent* mutant chromosome was confirmed by PCR. The resulting *relA::gent spoT::kan* double mutant *L. pneumophila* was designated MB697. MB355 contains the p*flaA*gfp plasmid that encodes thymidylate synthetase as a selectable marker and a transcriptional fusion of the *flaA* promoter to *gfp* (Hammer & Swanson, 1999, Hammer et al., 2002). MB696 and MB697 were transformed with the p*flaA*gfp reporter plasmid to create MB684 and MB685, respectively.

Inducible relA and spoT expression. To generate strains in which expression of either *relA* or *spoT* could be induced, we cloned promoterless fragments of *relA* and *spoT* into pMMB206-Δmob, a plasmid containing a $P_{taclacUV5}$ IPTG inducible promoter and a chloramphenicol resistance cassette. To construct pGEM-*relA*i, the *relA* locus was amplified from Lp02 genomic DNA using the primers relAi1 and relAi2, each containing a *Sal*I restriction site. The fragment was excised from pGEM-*relA*i using *Sal*I and ligated into pMMB206-Δmob at the *Sal*I site within the MCS immediately 3' of the $P_{taclacUV5}$ promoter, generating p*relA*i. Insertion and orientation were confirmed by both PCR and restriction enzyme digest. p*relA*i was used to transform MB697, and transformants were selected on chloramphenicol, creating MB687 for inducible *relA* expression. To construct pGEM-*spoT*i, the *spoT* locus was amplified from Lp02 genomic DNA using the

primers spoTi1, which contains a *Sal*I restriction site, and spoTi2, which contains a *Hin*dIII site. pGEM-*spoT*i and pMMB206-Δmob were each digested with *Sal*I and *Hin*dIII and then ligated, generating p*spoT*i. Insertion and orientation of *spoT* in the MCS of pMMB206-Δmob was confirmed by PCR and restriction enzyme digest. p*spoT*i was used to transform MB697, generating MB688 for inducible *spoT* expression.

Inducible spoT-A413E expression. pGEM-*spoT* was used as a template for *spoT-A413E* mutant allele construction. To generate site-directed mutations in *spoT*, Stratagene's QuickChange® II XL Site-Directed Mutagenesis Kit was used. To synthesize *spoT-A413E*, pGEM-*spoT* plasmid DNA was amplified with P*fuUltra* HF DNA polymerase (Stratagene) and the complementary primers spoTA413E1 and spoTA413E2. Parental DNA was digested with *Dpn*I, and DH5α transformants were selected on ampicillin. To verify the GCC to GAA codon change, candidate plasmids were sequenced and then designated pGEM-*spoT-A413E*. pGEM-*spoT-A413E* was digested with *Sal*I and *Hin*dIII, and the locus cloned into pMMB206-Δmob, as described for the WT *spoT* allele, generating p*spoT-A413E*. MB697 was transformed with p*spoT-A413E*, and transformants were selected on chloramphenicol, generating MB689 for inducible *spoT-A413E* expression.

Detection of ppGpp. Accumulation of the ppGpp in the PE phase and in response to amino acid starvation and fatty acid biosynthesis inhibition was detected by thin-layer chromatography (TLC) as described (Cashel & Gallant, 1969, Cashel, 1994, Hammer & Swanson, 1999). Briefly, to detect PE phase ppGpp production in WT and mutant *L.*

pneumophila, 100 μCi/ml carrier-free [^{32}P]-phosphoric acid (ICN Pharmaceuticals) was added in late-E phase, and bacteria were cultured at 37°C on a roller drum to the PE phase, or approximately 6 h. To detect ppGpp accumulation following amino acid starvation and fatty acid biosynthesis inhibition, E phase cultures were diluted to an OD_{600} = 0.25 and labelled with approximately 100 μCi/ml of carrier-free [^{32}P]-phosphoric acid for 6 h, or two generation times, at 37°C on a roller drum. After incorporation of the radioactive label, cultures were supplemented with a carrier control, 1 mM serine hydroxamate, or 0.5 μg/ml cerulenin. Cultures were incubated for an additional 1.5 h at 37°C. To extract the nucleotides, 50 μl aliquots were removed from each culture, added to 13 M formic acid, and then incubated on ice for 15 min. Samples were subjected to two freeze-thaw cycles and stored at -80°C until chromatographed. Formic acid extracts (35 μL in the case of the treated cultures and 25 μL in the case of the PE samples) were applied to a PEI-cellulose TLC plates (20 × 20;Sorbent) and developed with 1.5 M KH_2PO_4, pH 3.4 as described (Cashel & Gallant, 1969, Cashel, 1994, Hammer & Swanson, 1999). TLC plates were exposed to Kodak BioMax MR Film (18 x 24 cm) for 72 hours before development. To monitor growth in these experiments, OD_{600} were determined for non-radioactive cultures grown under identical conditions.

Fluorometry. To monitor expression of the flagellin promoter in WT and mutant cultures, *L. pneumophila* containing the reporter plasmid p*flaAgfp* were cultured in AYE media. Overnight cultures in mid-E phase OD_{600} = 1.0-1.75 were back diluted to early-E phase OD_{600} = 0.50-0.85 (T = 0). At the times indicated, the cell density of each culture was measured as OD_{600}. To analyze similar bacterial concentrations, aliquots were

collected by centrifugation, and the cells were normalized to OD_{600} = 0.01 in 1 × PBS. An aliquot of each sample (200 µL) was transferred to black 96-well plates (Costar), and the relative fluorescence intensity was measured using a Synergy™ HT microplate reader and 485 nm excitation, 530 nm emission, sensitivity of 50. A similar protocol was used to monitor *flaA* promoter activity in cultures supplemented with 1 mM serine hydroxamate and 0.5 µg/ml cerulenin. Overnight cultures in mid-E phase OD_{600} of 1.0-1.75 were back diluted to early-E phase OD_{600} of 0.50-0.85 (T = 0), at which time WT and mutant bacteria were treated with either serine hydroxamate or cerulenin. *L. pneumophila* cultures supplemented with water or DMSO served as negative and vehicle controls, respectively. Optical density and relative fluorescence were measured as described above.

Sodium sensitivity. To calculate the percentage of *L. pneumophila* that were sensitive to sodium, PE bacteria or E cultures were plated onto CYET and CYET containing 100 mM NaCl. After a 6-day incubation at 37°C, CFUs were enumerated, and the percentage of sodium sensitive microbes calculated as described (Byrne & Swanson, 1998).

Macrophage cultures. Macrophages were isolated from femurs of female A/J mice (Jackson Laboratory) and cultured in RPMI-1640 containing 10% heat-inactivated fetal bovine serum (RPMI/FBS; Gibco BRL) as described previously (Swanson & Isberg, 1995). Following a 7-day incubation in L-cell supernatant-conditioned media, macrophages were plated at either 5×10^4 per well for cytotoxicity, or 2.5×10^5 per well for degradation assays, infectivity assays and intracellular growth curves.

Lysosomal degradation. The percentage of intracellular *L. pneumophila* that remain intact after a 2 h macrophage infection was quantified by fluorescence microscopy (Bachman & Swanson, 2001). Briefly, macrophages were plated at 2.5×10^5 onto coverslips in 24 well plates. Then, E or PE phase microbes were added to macrophage monolayers at an MOI ~ 1. The cells were centrifuged at 400 × g for 10 min at 4°C and then incubated for 2 h at 37°C. Extracellular bacteria were removed by washing the monolayers three times with RPMI/FBS, and the macrophages were fixed, permeabilized and stained for *L. pneumophila* as described (Molofsky et al., 2005). Except for longer incubation times (18 and 24 h), identical procedures were used to image and score infected macrophages at later time points of the primary infection period (Fig. 2.6B and C)

Infectivity and intracellular growth. Infectivity is a gauge of the ability of *L. pneumophila* to bind, enter, and survive inside macrophages during a 2 h incubation, as previously described (Byrne & Swanson, 1998, Molofsky & Swanson, 2003). In brief, macrophages were infected with strains at an MOI of ~1, with centrifugation at 400 x g for 10 min. Cells were incubated for 2 h at 37°C, washed three times with fresh RPMI, and lysed in a 1:1 solution of RPMI and saponin, then intracellular CFU were enumerated. Results are expressed as the percentage of the inoculated CFU recovered from lysed macrophages at 2 h post-infection. To gauge intracellular growth, the pooled macrophage supernatant and lysate were plated for CFU at various times post-infection, as described elsewhere (Molofsky & Swanson, 2003). Results for intracellular growth

are expressed as either total CFU recovered or relative CFU. In the latter, CFU recovered at 24, 48 and 72 h are divided by the number of CFU at 2 h. Culturing conditions for WT and mutant *L. pneumophila* varied depending upon the experimental question addressed. For experiments assaying infectivity and intracellular survival of WT, *relA* and *relA spoT* bacteria lacking expression vectors, cultures were grown to E or PE phase prior to infection. To test the contribution of *relA* and *spoT* to the replicative to transmissive phase transition in broth (Fig. 2.5), overnight cultures in mid-E phase OD_{600} of 1.0-1.75 were back diluted to early-E phase OD_{600} of 0.50-0.85. Cultures were incubated for approximately 3 h at 37°C to an OD_{600} of 1.0-1.75; then, 200 μM IPTG was added and maintained until PE phase. To assess the role of *relA* and *spoT* in the transmissive to replicative phase transition within host cells (Fig. 2.8A), bacteria were cultured in broth with 200 μM IPTG to PE phase, and 200 μM IPTG was maintained throughout macrophage infection. A similar protocol was used to test the transmissive to replicative transition in bacteriological medium by plating bacteria that had been induced with 200 μM IPTG and cultured into PE phase onto CYET with 200 μM IPTG (Fig. 2.8B).

Cytotoxicity. To determine contact-dependent cytotoxicity of *L. pneumophila* following macrophage infection, bacteria were added to macrophage monolayers at the indicated MOI. After centrifugation at 400 × g for 10 min at 4°C (Molofsky et al., 2005), the cells were incubated for 1 h at 37°C. For quantification of macrophage viability, RPMI/FBS containing 10% alamarBlue™ (Trek Diagnostic Systems) was added to the monolayers for 6-12 h, and the reduction of the colorimetric dye was measured spectrophoretically as described (Molofsky et al., 2005). Protocols for culturing WT and mutant microbes

varied depending upon the experimental question. To determine the effect *relA* and *spoT* induction on cytoxicity of *relA spoT* mutant bacteria (Fig. 2.5A), microbes were cultured with 200 μM IPTG to PE phase in broth prior to the assay, as described in *Infectivity and intracellular growth*. To determine if expression of WT and mutant *spoT* was sufficient to rescue the cytotoxicity defect of *relA spoT* mutant bacteria in which fatty acid biosynthesis had been perturbed (Fig. 2.3), oovernight cultures in mid-E phase OD_{600} of 1.0-1.75 were back diluted to early-E phase OD_{600} of 0.45-0.60 and treated with either 25 or 50 μM IPTG for 1 h in the case of the 25 μM induction and 2 h for the 50 μM induction. After incubation on a roller at 37°C, mutant cultures were split and either mock-treated or treated with propionic acid, acetic acid, or cerulenin. Cultures were incubated at 37°C for an additional 3 h prior to assaying cytotoxicity.

Results

In the absence of relA, spoT is not essential in L. pneumophila.

To rigorously test the hypothesis that the stringent response controls *L. pneumophila* virulence expression, we constructed and analyzed *relA* and *relA spoT* mutant strains. In the stationary phase, *L. pneumophila* requires RelA to produce pyomelanin, a secreted pigmented molecule important for iron metabolism (Zusman et al., 2002, Chatfield & Cianciotto, 2007). Therefore, to verify that *relA* was disrupted, we assayed the extracellular pigment of stationary phase cultures. As expected, our *relA* insertion mutant was completely defective for pyomelanin production (data not shown).

As observed for other gram-negative bacteria, we and others were unable to generate *spoT* null mutant strains of *L. pneumophila,* and attempts to construct a conditional *spoT* mutant were also unsuccessful (Zusman et al., 2002). Therefore, to

analyze the contribution of SpoT to the *L. pneumophila* life cycle, we constructed a *relA spoT* double mutant in which 136 amino acids near the N-terminus, including the consensus His-Asp (HD) motif required for ppGpp hydrolysis (Magnusson et al., 2005, Srivatsan & Wang, 2008, Potrykus & Cashel, 2008) and a significant portion of the putative ppGpp synthetase domain were replaced by a kanamycin resistance cassette (Gentry & Cashel, 1996). As the cultures entered late PE phase, the *relA spoT* double mutant cells adopted a hyperfilamentous cellular morphology (data not shown), a phenotype reminiscent of that described for *relA spoT* mutants of *E. coli* (Xiao *et al.*, 1991, Traxler et al., 2008). Other than hyperfilamentation in late PE phase, in rich medium the growth kinetics of E phase *relA spoT* mutant cells was identical to WT bacteria, as judged by optical density and CFU (Fig. 2.1C). Therefore, when *relA* is absent, *spoT* is not essential for *L. pneumophila* to replicate in rich bacteriological medium. Why previous attempts to isolate a *spoT* null mutant were unsuccessful is difficult to assess since the allele construction was not described (Zusman et al., 2002). However, *spoT* mutations that eliminate hydrolase but not synthetase activity would be lethal.

RelA and SpoT contribute to ppGpp accumulation, motility and survival in stationary phase.

To assess in greater detail the contributions of RelA and SpoT to *L. pneumophila* biology, we tested the ability of *relA* and *relA spoT* mutants to accumulate ppGpp in stationary phase when cultured in rich broth containing yeast extract. After radiolabeling the *L. pneumophila* guanosine nucleotide pools for 6 h (two doubling times), ppGpp

levels were analyzed by thin-layer chromatography. WT *L. pneumophila* cultured into PE phase accumulated a pool of ppGpp, much of which was dependent upon *relA* (Fig. 2.1A). The *relA* mutants also accumulated a small but appreciable pool of ppGpp that was not observed in the *relA spoT* double mutant (Fig. 2.1A). Thus, when *L. pneumophila* are cultured in rich broth to the PE phase, RelA synthetase activity accounts for the majority of ppGpp, whereas SpoT synthetase activity contributes modestly. Since *L. pneumophila* obtains carbon and energy from amino acids, not sugars (Tesh & Miller, 1981, Tesh *et al.*, 1983), the predominant role for RelA in these yeast extract broth cultures is not surprising.

To test the biological consequences of the small amount of *spoT*-dependent ppGpp that accumulates in PE phase, we assayed *flaA* expression and motility, two hallmarks of stationary phase *L. pneumophila*. The *relA* and *relA spoT* mutants were transformed with a plasmid containing a *flaA-gfp* transcriptional fusion that enables promoter activity of *flaA*, the primary flagellar subunit of *L. pneumophila*, to be analyzed by fluorometry (Hammer & Swanson, 1999). As expected, the *flaA* promoter was inactive during exponential (E) phase in WT, *relA* and *relA spoT* cultures (Hammer & Swanson, 1999). Upon entry into PE phase, both WT and *relA* mutant *L. pneumophila* activated the *flaA* promoter; however, in *relA* mutant cultures the *flaA* promoter activity was decreased relative to WT, consistent with a previous report (Zusman et al., 2002). In contrast, *relA spoT* mutant cultures failed to activate *flaA* expression upon entry into PE phase, indicating that residual *flaA* promoter activity observed in *relA* mutants is dependent upon SpoT (Fig. 2.1B). Consistent with their *flaA* expression, the *relA* mutants also exhibited a 30-50% decrease in the fraction of motile bacteria per field

relative to WT cultures, while *relA spoT* mutants failed to express motility at all PE phase time points analyzed (data not shown). Therefore, both RelA and SpoT contribute to *flaA* expression by PE *L. pneumophila*, and the small *spoT*-dependent ppGpp pool that accumulates in PE phase (Fig. 2.1A) is nevertheless sufficient to activate partial *flaA* expression and motility by *L. pneumophila*.

To investigate the importance of the stringent response to survival in the PE phase, we assessed the culture density and viability of WT, *relA* and *relA spoT* cultures over time. Both parameters were similar for all strains between 2 and 30 h. However, by 44 h, or ~20 h after entry into PE phase, *relA spoT* cells appeared hyperfilamentous relative to WT and *relA*, which had each adopted the more coccoid-like morphology that is characteristic of PE phase *L. pneumophila* (Molofsky & Swanson, 2004). Concomitant with filamentation, by 44 h *relA spoT* cultures began to exhibit a loss in CFU relative to WT and *relA* (Fig. 2.1C). This decrease in viability of *relA spoT* cells was even more pronounced after 66 h. Therefore, as reported for other bacteria, including *C. jejuni* and *Helicobacter pylori* (Gaynor et al., 2005, Mouery *et al.*, 2006), *L. pneumophila* requires the stringent response for differentiation and survival in stationary phase.

RelA-dependent ppGpp synthesis is required for flaA expression following amino acid starvation.

To verify that, like *E. coli*, *L. pneumophila* relies on RelA to initiate the stringent response following amino acid starvation, we subjected WT and mutant cultures to treatment with serine hydroxamate. An analog of the amino acid serine, serine hydroxamate inhibits the attachment of serine to transfer RNA, and it has been used

extensively to characterize in *E. coli* the mechanism by which RelA senses accumulation of uncharged tRNAs at the ribosome (Tosa & Pizer, 1971, Magnusson et al., 2005). Unlike mock-treated cultures, WT cells subjected to serine hydroxamate treatment accumulated an appreciable pool of ppGpp (Fig. 2.2A). In contrast, neither *relA* nor *relA spoT* mutant *L. pneumophila* appeared to respond to serine hydroxamate treatment.

To determine the physiological relevance of the ppGpp pool detected, we asked if the *relA*-dependent ppGpp that accumulated after serine hydroxamate treatment was sufficient to activate *flaA* expression. Indeed, the pattern of *flaA* expression in serine hydroxamate-treated cultures (Fig. 2.2B, top panel) mimicked that observed for ppGpp production (Fig. 2.2A). Whereas WT cultures activated *flaA* as early as 3 h post treatment, neither *relA* nor *relA spoT* treated with serine hydroxamate activated *flaA* throughout the time course analyzed (Fig. 2.2B, top panel). Mock-treated cultures activated *flaA* with kinetics similar to that of control PE phase cultures (Fig. 2.1B). Serine hydroxamate treatment did partially inhibit growth of all strains relative to mock-treated control samples (Fig. 2.2B, bottom panel), an effect that occurred independently of appreciable ppGpp accumulation (Fig. 2.2A). Together these observations verify that, in response to amino acid starvation, RelA synthesizes adequate ppGpp to activate *flaA* in *L. pneumophila*.

SpoT-dependent ppGpp accumulation activates flaA when fatty acid biosynthesis is inhibited.

In *E. coli*, RelA responds to amino acid starvation, whereas SpoT responds to carbon, phosphate, and iron starvation and fatty acid biosynthesis inhibition (Magnusson

et al., 2005). Therefore, we tested whether *L. pneumophila* RelA and SpoT also respond to distinct metabolic cues. For this purpose we used the antibiotic cerulenin, a specific inhibitor of fatty acid biosynthesis in bacteria that acts on the 3-ketoacyl-[acyl carrier-protein (ACP)] synthases I and II, FabB and FabF (Seyfzadeh *et al.*, 1993). Appreciable amounts of ppGpp were detected in WT and *relA* cultures treated with cerulenin, whereas *relA spoT* mutant *L. pneumophila* failed to accumulate detectable ppGpp under these conditions (Fig. 2.2C). The response was specific to the antibiotic, since cultures receiving the DMSO vehicle did not exhibit ppGpp.

To investigate the phenotypic consequences of the modest SpoT-dependent ppGpp pool, we assayed the ability of WT and mutant cultures to activate *flaA* following cerulenin treatment. As observed after the serine hydroxamate treatment, the pattern of *flaA* expression reflected the size of the ppGpp pool in each strain (Fig. 2.2D, top panel). Cerulenin-treated WT and *relA* mutant *L. pneumophila* activated *flaA*, but *relA spoT* cells did not (Fig. 2.2D, top panel). As observed for cultures treated with serine hydroxamate, cerulenin treatment also inhibited growth independently of ppGpp accumulation in the strain (Fig. 2.2D, bottom panel). Control cultures treated with DMSO were not inhibited for growth and activated fluorescence only upon entry into PE phase. Thus, *L. pneumophila* is equipped to differentiate in response to amino acid starvation via RelA, and inhibition of fatty acid biosynthesis using SpoT.

Amino acid substitution in SpoT confers enzyme activity that is blind to fatty acid signals.

In addition to their response to fatty acid biosynthesis inhibition (Fig. 2.2), *L. pneumophila* that are exposed to excess short chain fatty acids (SCFA) also activate

transmissive phenotypes in a SpoT-dependent manner (Edwards *et al.*, 2009). Like cerulenin treatment, addition of either 10 mM acetic or propionic acid to E phase *L. pneumophila* triggers expression of several transmission traits, including lysosome evasion, *flaA* expression and motility, as well as cytotoxicity (Edwards et al., 2009). In *E. coli*, SpoT monitors and responds to perturbations in fatty acid metabolism through a specific interaction with ACP (Battesti & Bouveret, 2006). Accordingly, we tested the hypothesis that a SpoT-ACP interaction is essential for *L. pneumophila* to activate transmissive phenotypes when fatty acid biosynthesis is perturbed by excess SCFA. To do so, we designed plasmids for the inducible expression of either WT *spoT* or *spoT-A413E*, an allele encoding an enzyme predicted to be defective for ACP interaction (Battesti & Bouveret, 2006). Within the ~60 amino acid TGS domain in which the critical alanine residue resides, *L. pneumophila* SpoT exhibits 67% identity and 86% similarity to the *E. coli* protein, and the seven amino acids C-terminal to the alanine residue are identical. In particular, we asked whether expression of plasmid-borne *spoT* or *spoT-A413E* was sufficient to restore flagellin expression by *relA spoT* bacteria exposed to 10 mM SCFA. Since similar origins of replication and antibiotic resistance markers for the relevant plasmids complicated use of the *flaA* reporter, we instead measured cytotoxicity, a phenotype dependent upon flagellin as well as Type IV secretion (Molofsky & Swanson, 2004).

WT or mutant *spoT* expression was first induced for 1 h with 25 μM IPTG before the cells were cultured for 3 h with 10 mM SCFA and 25 mM IPTG. When exposed to propionic acid, WT *spoT* restored *relA spoT* mutant cytotoxicity toward macrophages, but *spoT-A413E* did not (Fig. 2.3A). Likewise, only WT *spoT* rescued *relA spoT*

cytotoxicity after acetic acid or cerulenin addition (data not shown). The plasmid-encoded *spoT-A413E* allele was functional: When the IPTG concentration was doubled to 50 μM, *relA spoT* mutants carrying the *spoT-A413E* plasmid were even more cytotoxic than those harboring either WT *spoT* or the empty vector (Fig. 2.3B). Furthermore, the enhanced cytotoxicity occurred independently of SCFA treatment. Thus, the *spoT-A413E* mutation appears to render the enzyme insensitive to perturbations in fatty acid metabolism and also increases the ppGpp pools, presumably by disrupting the balance between ppGpp synthesis and hydrolysis (Battesti & Bouveret, 2006).

The stringent response activates transmissive traits.

L. pneumophila coordinates entry into stationary phase with expression of not only motility but also virulence-associated phenotypes like sodium sensitivity, cytotoxicity, and lysosome avoidance (Byrne & Swanson, 1998). Accordingly, to assess the contributions of RelA and SpoT to virulence trait expression in PE *L. pneumophila*, we assayed WT and mutant bacteria for sodium sensitivity and lysosome evasion. As predicted, unlike WT, *relA spoT* mutants remained sodium resistant even when cultured into PE phase (Fig. 2.4A). PE phase *relA* mutant *L. pneumophila*, which accumulate low amounts of ppGpp, activate *flaA* and become motile (Fig. 2.1), also become more sensitive to sodium. Likewise, whereas PE WT and *relA* resisted phagosome-lysosome fusion and remained intact following macrophage phagocytosis, E phase WT and PE phase *relA spoT* were degraded (Fig. 2.4B). Therefore, even the modest SpoT-dependent pool of ppGpp is sufficient to induce significant sodium-sensitivity and to protect *L. pneumophila* from lysosomal degradation.

Induction of either ppGpp synthetase is sufficient to restore the transmissive phenotype of relA spoT mutants in broth.

Since *L. pneumophila* requires ppGpp for *flaA* expression, motility, sodium sensitivity and lysosome avoidance (Fig. 2.1 and 2.4) we predicted that, during PE phase starvation, induction of either synthetase would equip replicative *relA spoT* mutants to enter the transmissive phase. Indeed, IPTG induction of plasmid-borne *relA*, *spoT*, or *spoT-A413E* suppressed both the flagellar and the cell-remodeling defect of *relA spoT* mutant bacteria, as judged by the motility of coccoid bacteria. In contrast, *relA spoT* bacteria carrying only the vector remained amotile and elongated (data not shown). Furthermore, when either *relA*, *spoT* or *spoT-A413E* was induced, *relA spoT L. pneumophila* killed more macrophages than either *relA spoT* mutant bacteria carrying vector alone or E phase WT (Fig. 2.5A). Induction of *relA*, *spoT* and *spoT-A413E* also increased the infectivity of *relA spoT* bacteria: When harboring plasmids encoding either ppGpp synthetase and cultured with IPTG to the PE phase, significantly more *relA spoT* mutants were infectious than those carrying the empty vector (Fig. 2.5B). Thus, expression of either *relA*, *spoT* or *spoT-A413E* was sufficient to trigger replicative phase *relA spoT* mutants to enter the transmissive phase. These results not only verify that the phenotypic defects of *relA spoT* mutants were not due to secondary site mutations or polar effects, but also indicate that, regardless of its source, ppGpp coordinates the replicative to transmissive phase transition in broth.

The stringent response is essential during macrophage infection.

As in broth, *L. pneumophila* cycles between replicative and transmissive phases in host cells (Byrne & Swanson, 1998, Hammer & Swanson, 1999, Bruggemann et al., 2006, Alli et al., 2000). Since ppGpp is essential for activation of the transmission phenotype *in vitro* (Figs. 2.1A, 2.4, and 2.5), we postulated that RelA and SpoT are also required for transmission between host cells.

As observed previously, *relA* was dispensable for *L. pneumophila* growth and survival in macrophages (Zusman et al., 2002, Abu-Zant et al., 2006). In contrast, *relA spoT* mutants infected with a decreased efficiency relative to PE phase WT, a phenotype similar to WT in E phase (Fig. 2.6A; 2 h time point). Those *relA spoT* and E phase WT bacteria that survived the initial attack moved on to replicate efficiently between 2 and 24 h. However, after 24 h, E phase WT CFU increased to values equivalent to PE phase WT, but *relA spoT* mutants exhibited no further increase in CFU over the remainder of the period analyzed (Fig. 2.6A).

Since 24 h corresponds to the period of flagellar expression and initiation of secondary infection (Byrne & Swanson, 1998), we postulated that *relA spoT* mutants were being killed either during escape from an exhausted host or upon entry into a naïve one. To distinguish between these two hypotheses, we performed immunofluorescence microscopy. At 16 h, both WT and *relA spoT* bacteria occupied replication vacuoles (data not shown); however, between 18 and 24 h, a unique class of infected macrophages emerged in *relA spoT* cultures. Intact bacteria were no longer observed; instead, degraded *L. pneumophila* particles were dispersed throughout the macrophage (Fig. 2.6B). In addition, there was scant evidence of secondary infection by *relA spoT* mutants at time points beyond 18 h (data not shown). Thus, the majority of mutant bacteria were

apparently destroyed following the replicative growth period, prior to escape. In the rare macrophages that contained one or two bacteria at time points beyond 18 h, the *relA spoT* mutants were also degraded, likely due to their inability to avoid phagosome-lysosome fusion (Fig. 2.6B). At 18 h, the percentage of infected macrophages that harbored either single or multiple degraded *relA spoT* mutant bacteria was nearly 80%, approximately three times greater than the ~25% of infected macrophages containing degraded WT bacteria (Fig. 2.6C). Therefore, the stringent response is required for *L. pneumophila* transmission between macrophages, and SpoT is a critical component of its developmental switch.

SpoT is essential for transmission between macrophages.

To investigate further the contributions of RelA and SpoT to differentiation of replicative bacteria to the transmissive state in macrophages, we tested whether induction of either *relA* or *spoT* was sufficient to rescue the intracellular *relA spoT* mutants. The *relA spoT* mutant *L. pneumophila* were cultured in the absence of IPTG to early PE phase and then added to macrophages. After ~16 h, IPTG was added to the cultures and maintained for the remainder of the infection. Like *relA spoT* bacteria harboring empty vector, double mutants in which *relA* had been induced exhibited a ~20 fold increase in CFU between 2 and 24 h, but no appreciable increase beyond 24 h (Fig. 2.7). Likewise, constitutive expression of *relA* by *relA spoT* mutants in rich broth was also incompatible with replication in broth, as judged by measuring both the optical density and CFU of the cultures (Fig. 2.10C). In contrast, when *spoT* was induced, *relA spoT* mutants replicated similarly to wild type in macrophages (Fig. 2.7) and in broth (Fig. 2.1A). In mouse

macrophages, the CFU increase observed after 24 h reflects secondary and tertiary infections; therefore, SpoT activity is critical for *L. pneumophila* transmission between host cells.

To re-enter the replicative phase in macrophages, L. pneumophila requires SpoT.

To investigate whether SpoT synthetase and/or hydrolase activity is critical for the life cycle of *L. pneumophila* in macrophage cultures, we tested whether induction of *relA*, *spoT* or *spoT-A413E* equipped transmissive bacteria to differentiate into the replicative phase in both macrophages and on solid medium. Broth grown cultures of *relA spoT* bacteria carrying inducible *relA*, *spoT*, or *spoT-A413E* were cultured with IPTG into PE phase to activate transmission traits. Next, transmissive bacteria were either added to macrophages in the presence of IPTG (Fig. 2.8A), or plated onto rich medium with or without IPTG (Fig. 2.8B). During macrophage infection with the *relA spoT* strains, induction of WT *spoT* resulted in a pattern of intracellular growth identical to that of PE phase WT bacteria (Fig. 2.8A). In contrast, induction of neither *relA* nor *spoT-A413E* equipped *relA spoT L. pneumophila* to replicate intracellularly (Fig. 2.8A). Similar patterns were observed when transmissive bacteria were plated onto bacteriological medium. All the strains formed colonies when transferred to medium without IPTG (Fig. 2.8B, left panel). On medium containing IPTG, mutant *relA spoT L. pneumophila* that expressed either WT *spoT* or carried the empty vector readily formed colonies (Fig. 2.8B, right panel). In contrast, consistent with their intracellular growth defect, *relA spoT* mutants harboring either inducible *relA* or *spoT-A413E* failed to replicate when IPTG was maintained in the medium (Fig. 2.8B, right panel). These

genetic data suggest that the enzyme encoded by *spoT-A413E* favors ppGpp accumulation, more closely resembling RelA. Together, these results indicate that SpoT hydrolase activity is required for transmissive cells to re-enter the replicative phase either during macrophage infection or on bacteriological medium.

Discussion

To endure starvation and other stresses, many bacteria rely on the stringent response pathway to alter their transcriptional profiles and enhance their fitness. When nutrients become scarce in broth, *L. pneumophila* generates ppGpp to coordinate its transition from a replicative to a transmissive form (Hammer & Swanson, 1999, Zusman et al., 2002). Although *L. pneumophila* utilizes RelA to produce ppGpp upon entry into the PE phase in yeast extract broth, this enzyme is dispensable for replication in either amoeba or human macrophages (Zusman et al., 2002, Abu-Zant et al., 2006). Like several other microbes, *L. pneumophila* also encodes the bifunctional and essential enzyme SpoT, which can either synthesize or hydrolyze ppGpp depending on local cues. Analysis of broth cultures indicates that the two ppGpp synthetases confer versatility to *L. pneumophila*, enabling conversion to the transmissive state when either protein or fatty acid synthesis is compromised (Fig. 2.2). However, in macrophages only SpoT is required, and its role is bipartite (Figs. 2.6A and 2.8A). After establishing its protective vacuole, intracellular *L. pneumophila* relies on SpoT to hydrolyze ppGpp to reduce the alarmone pool and switch from the transmissive to the replicative form. When conditions deteriorate, the intracellular progeny elicit SpoT synthetase activity to coordinate their conversion to the transmissive state and escape from the exhausted host (Fig. 2.9).

In broth, *L. pneumophila* requires ppGpp from either RelA or SpoT to initiate the replicative to transmissive transition (Fig. 2.9). When *L. pneumophila* is cultured in broth, a large proportion of its ppGpp pool is generated by RelA (Fig. 2.1A). The more minor contribution by SpoT can be observed as the residual ppGpp detected in *relA* mutants, since only two ppGpp synthetases are apparent in the *L. pneumophila* genome, and no alarmone is detectable in *relA spoT* double mutant cells (Fig. 2.1A). Like *E. coli* SpoT, the *L. pneumophila* enzyme may be a weak synthetase (Seyfzadeh et al., 1993). Alternatively, the low levels of SpoT-dependent ppGpp observed may be a consequence of experimental conditions: Its fastidious character precludes use of a defined medium where competing inorganic phosphate levels can be minimized. Here *L. pneumophila* was cultured in a rich media that contains amino acids as the sole energy source, which not only reduces the efficiency of the radiolabeling of the phosphate pool, but also favors RelA-dependent ppGpp production. Nevertheless, the weak ppGpp signal generated by SpoT in this medium is sufficient to induce with timing similar to WT *L. pneumophila* not only the *flaA* promoter, but also motility, sodium sensitivity, and evasion of lysosomes (*relA* mutant; Figs. 2.1B, 2.4, and data not shown).

In addition to triggering its panel of transmission traits, ppGpp from either synthetase is also critical for *L. pneumophila* to survive the PE phase in broth cultures (Fig. 2.1C). In *E. coli*, ppGpp-dependent control of fatty acid and phospholipid metabolism allows the bacterium to selectively modify its membranes during stress. For example, ppGpp inhibits fatty acid and phospholipid biosynthesis through repression of *plsB* (sn-glycerol-3-phosphate acyltransferase), resulting in accumulation of long-chain acyl-ACPs and cell shortening (Heath *et al.*, 1994). Indeed, the *E. coli* stringent response

both activates and represses several genes in the fatty acid and phospholipid biosynthetic pathways, as judged by transcriptional profile analysis (Durfee et al., 2008). Accordingly, we predict that the stringent response pathway induces synthesis of specific membrane constituents that are critical for *L. pneumophila* cells to survive periods of stress, including that encountered during entry and exit from host phagocytes. Taken together, our phenotypic and biochemical analysis of WT, *relA,* and *relA spoT* bacteria establish that synthesis of ppGpp from either enzyme is sufficient to coordinate *L. pneumophila* differentiation into the transmissive form and to promote survival in detrimental environments.

To alternate between transmissive and replicative forms in macrophages, *L. pneumophila* uses the SpoT enzyme to tightly regulate ppGpp synthesis and degradation. As reported previously (Zusman et al., 2002, Abu-Zant et al., 2006), the RelA enzyme itself is dispensable for *L. pneumophila* to survive phagocytosis by macrophages and to replicate intracellularly (Fig. 2.6A). On the other hand, the alarmone ppGpp is critical to induce the virulence factors that equip *L. pneumophila* to establish its protected intracellular niche, since mutants that lack both *relA* and *spoT* survive poorly within macrophages (Fig. 2.6). For the replicative to transmissive transition in broth, the source of ppGpp is inconsequential, since expression of either RelA or SpoT largely bypasses the transmissive trait defects of *relA spoT* double mutant bacteria (Fig. 2.5A and B). Once replication ensues in the macrophage, ppGpp is no longer required, since the few *relA spoT* mutant bacteria that do survive the initial infection replicate efficiently during the subsequent 24 h primary infection period (Fig. 2.6A). When vacuolar conditions deteriorate and *L. pneumophila* must transit to a new host, SpoT is required, since

induction of SpoT but not RelA bypasses the growth defect of *relA spoT* mutant bacteria beyond the 24 h time point (Fig. 2.7). During the transmission period, the synthetase activity of SpoT is sufficient to promote escape and entry into a new host (*relA* mutant bacteria; Fig. 2.6A), whereas its hydrolase is absolutely required for newly engulfed bacteria to initiate replication (Fig. 2.8A). Thus, SpoT governs differentiation of *L. pneumophila* at two critical nodes of its life cycle in macrophage cultures.

L. pneumophila has evolved two ppGpp synthetases capable of responding to distinct metabolic inputs during growth *in vitro* (Fig. 2.2); however, during intercellular transmission only SpoT is essential (Figs. 2.6A and 2.7). Therefore, the stress signal that promotes replicative bacteria to convert to the transmissive form in macrophages must be sensed by SpoT. In *E. coli*, RelA responds to amino acid limitation, whereas SpoT activates the stringent response pathway during all other types of stresses and starvation (Magnusson et al., 2005, Srivatsan & Wang, 2008, Potrykus & Cashel, 2008). Amino acid depletion also stimulates *L. pneumophila* to produce ppGpp and induce differentiation by a RelA-dependent pathway (Figs. 2.2A and B). On the other hand, to respond to perturbations in fatty acid biosynthesis (FAB), *L. pneumophila* relies on SpoT (Fig. 2.2C and D). Since SpoT alone is sufficient to induce transmission in macrophages (Figs. 2.6A and 2.7), we postulate that the ability of SpoT to sense variations in fatty acid metabolism equips *L. pneumophila* to respond to fluctuations in the lipid supply during its intracellular life cycle. Whether SpoT also equips *L. pneumophila* to respond to other metabolic stimuli that are critical within host cells remains to be explored experimentally.

Our genetic data suggest that, as in *E. coli,* the *L. pneumophila* response to perturbations in FAB requires SpoT interaction with acyl-carrier protein (ACP); by some

mechanism, this interaction regulates SpoT enzyme activity (Battesti & Bouveret, 2006). In particular, Battesti and Bouveret identified a single amino acid substitution in SpoT that abrogates a physical interaction with ACP and renders the mutant bacteria blind to fatty acid starvation (Battesti & Bouveret, 2006). Likewise, the analogous *spoT-A413E* mutant *L. pneumophila* fail to trigger cytotoxicity in response to excess short chain fatty acids (Fig. 2.3A). In addition, interaction between SpoT and ACP affects the ability of SpoT to reduce the ppGpp pool, as judged by three phenotypic assays. *L. pneumophila* treated to express strongly the *spoT-A413E* allele apparently contain excessive ppGpp, as the cells are more cytotoxic than bacteria expressing WT *spoT* (Fig. 2.3B), and they survive yet fail to replicate when cultured either in macrophages or on rich medium (Fig. 2.8A and B). Based on the capacity of the analogous *E. coli spoT-A404E* mutant to adapt to histidine starvation, Battesti and Bouveret also deduced that excess ppGpp accumulates when the SpoT-ACP interaction is disrupted (Battesti & Bouveret, 2006). We note two caveats to our experimental approaches. The contribution of a physical interaction between SpoT and ACP to the *L. pneumophila* fatty acid response is based on a precedent established in *E. coli* and our analogous genetic analysis, which remains to be verified by direct biochemical assays. Second, the simple interpretation that increased concentrations of IPTG used to induce either the *spoT* or the *spoT-A413E* allele increases the amount of the respective proteins (Fig. 2.3) has not been verified. Our attempts to monitor SpoT protein levels by Western analysis using a previously described polyclonal antibody specific to the *E. coli* enzyme (Gentry & Cashel, 1996) were not successful.

Given our genetic data and the published genetics and biochemistry (Battesti & Bouveret, 2006), several working models remain in play. When FAB is perturbed, an

unidentified signaling ligand either accumulates or is depleted, thereby altering ACP in a manner that is sensed by SpoT. Consequently, ACP could either repress SpoT-dependent ppGpp hydrolysis, or activate SpoT-dependent ppGpp synthesis. Biochemical studies can distinguish between these possibilities. In any case, when FAB is perturbed, SpoT-dependent ppGpp accumulates and replicative *L. pneumophila* transition to the transmissive form.

In addition to monitoring FAB, SpoT activity is also critical for transmissive bacteria to reenter the replicative state when nutrients are abundant, likely due to the need to hydrolyze the alarmone (Fig. 2.8A and B). The intracellular phenotype of *L. pneumophila* that generate excess ppGpp (i. e., *relA spoT* mutant bacteria that constitutively express either *relA* or the mutant allele *spoT-A413E*), is similar to that of *phtA* mutant *L. pneumophila,* which lack the high-affinity transporter for threonine (Sauer et al., 2005). In each case, the mutant bacteria persist as single, intact, rods within their vacuoles, while WT microbes replicate robustly (Sauer et al., 2005). Without the capacity to acquire threonine, as an essential amino acid, *L. pneumophila* may become locked in a state of ppGpp synthesis. Thus, the local nutrient supply dictates when transmissive bacteria activate replicative functions. To resume multiplying, *L. pneumophila* must activate the SpoT hydrolase to reduce its ppGpp pool (Fig. 2.9B).

The intracellular pathogen *Mycobacterium tuberculosis* encodes a single-bifunctional Rel-Spo homologue (RSH), Rel, required for long-term survival *in vitro* (Primm et al., 2000) and long-term persistence in mice (Dahl *et al.*, 2003, Karakousis *et al.*, 2004). The ability of this pathogen to differentiate to a persister state may contribute to the failure of frontline anti-microbial agents (Warner & Mizrahi, 2006). Thus,

understanding the biochemistry of the stringent response enzymes provides an avenue to analyze the life cycle of a variety of intracellular pathogens and, more importantly, to develop new strategies and therapeutics to eradicate them.

Encoding both RelA and SpoT confers versatility to *L. pneumophila* and likely enables the bacterium to survive a variety of assaults encountered in the extracellular environment. Although the precise stimuli that cue *L. pneumophila* differentiation *in vivo* remain to be determined, our evidence points to a SpoT-dependent signal. Furthermore, the bifunctional SpoT enzyme plays two critical and distinct roles during the intracellular life cycle. By acting as a receiver of metabolic cues, SpoT coordinates a rapid response when either favorable or unfavorable environmental circumstances are encountered. Thus the stringent response pathway is fundamental to the virulence of *L. pneumophila,* an opportunistic pathogen that must not only replicate within but also transit between macrophages.

Table 2.1. Bacterial strains, plasmids and primers

Strain	Relevant genotype/phenotype	Reference
E.coli		
DH5α	F-endA1 *hsdR17* (r- m+) *supE44 thi-1 recA1 gyrA* (Nal^r) *relA1* Δ(*lacZYA-argF*)$_{U169}$Φ80dLacZΔM15*λpir*RK6	Laboratory collection
L. pneumophila		
MB110	Lp02 wild-type, Str^R, Thy-, HsdR-	(Berger & Isberg, 1993)
MB695	Lp02 *relA::gent* mutant	This work
MB696	Lp02 *relA::kan* mutant	This work
MB697	Lp02 *relA::gent spoT::kan* double mutant	This work
MB698	Lp02 p206-empty, vector control strain	This work
MB687	Lp02 *relA::gent spoT::kan* double mutant p*relA*i	This work
MB688	Lp02 *relA::gent spoT::kan* double mutant p*spoT*i	This work
MB689	Lp02 *relA::gent spoT::kan* double mutant p*spoT-A413E*	This work
MB355	Lp02 p*flaA*gfp td(Δ)I	(Hammer & Swanson, 1999)
MB684	Lp02 *relA::kan* mutant p*flaA*gfp td(Δ)I	This work
MB685	Lp02 *relA::gent spoT::kan* double mutant p*flaA*gfp td(Δ)I	This work
Plasmids		
pGEMT-Easy	MCS within coding region of B-lactamase α-fragment linearized with single-T overhangs, Amp^R	Promega
pGEM-Gent	pGEMT-Easy with 1.7 kb gentamycin cassette clone MCS source of 1.7kb gentamycin resistance cassette, Amp^R, $Gent^R$	(Molofsky & Swanson, 2003)
pGEM-*relA*	pGEMT-Easy with 3.2kb PCR amplified *relA* chromosomal region ligated into T overhangs, Amp^R	This work
pGEM-*relA*i	pGEMT-Easy with 2.7kb PCR amplified *relA* chromosomal region ligated into T overhangs, Amp^R	This work
pGEM-*relA::gent*	pGEM-*relA* with 1.7kb gentamycin cassette from pUCGent bluntly ligated into the *Sna*BI site ~1.0kb from transcriptional start site of *relA* ORF Amp^R, $Gent^R$	This work
pGEM-*relA::kan*	pGEM-*relA* with 1.3kb kanamycin cassette from pUC4K bluntly ligated into the *Sna*BI site ~1.0kb from transcriptional start site of *relA* ORF Amp^R, Kan^R	This work
pGEM-*spoT*	pGEMT-Easy with 2.9kb PCR amplified *spoT* chromosomal region ligated into T overhangs, Amp^R	This work
pGEM-*spoT*i	pGEMT-Easy with 2.5kb PCR amplified *spoT* chromosomal region ligated into T overhangs, Amp^R	This work
pGEM-*spoT::kan*	pGEM-*spoT* with 489bp *Hin*dIII/*Hin*dIII SpoT ORF fragment deleted and a 1.3kb kanamycin cassette from pUC4K bluntly ligated between the HindIII sites, Amp^R, Kan^R	This work
pGEMT-*spoT-A413E*	pGEM-*spoT*i with a GCC to GAA change, Amp^R	This work
pMMB206-Δmob	pMMB66EH derivative, Δmob, $lacI^q$,$P_{taclacUV5}$, Cam^R	(Morales *et al.*, 1991)
p*relA*i	pMMB206-Δmob with 2.7kb *Sal*I fragment from pGEM-*relA*i ligated at the *Sal*I site in the MCS, collinear with the $P_{taclacUV5}$ promoter, $lacI^q$, Inducible RelA expression, Cam^R	This work
p*spoT*i	pMMB206-Δmob with 2.5kb *SalI*/*Hin*dIII fragment from pGEM-*spoT*i ligated between the SalI/HindIII sites in the MCS, collinear with the $P_{taclacUV5}$ promoter, $lacI^q$, Inducible SpoT expression, Cam^R	This work
p*spoT-A413E*	pMMB206-Δmob with 2.5kb *SalI*/*Hin*dIII fragment from pGEM-*spoT-A413E* ligated between the *SalI*/*Hin*dIII sites in the MCS, collinear with the $P_{taclacUV5}$ promoter, $lacI^q$, Inducible SpoT-A413E expression, Cam^R	This work
p*flaA*gfp	150bp *flaA* promoter fragment fused to GFPmut3 in pKB5 with P_{tac} and $lacI^q$ removed, td(Δ)i	(Hammer & Swanson, 1999)

Primers	Sequence	Wild-type Amplicon Size
relA1 fwd.	5'-CGTGCTAGACTTATTTTGTGGACTG-3'	
relA2 rvs.	5'-ATTGACCTCTGTGATATACTGTTAG-3'	3.1 kb
relAi1 fwd.	5'-GTCGACATGCCCGAGTCTATT-3'	
relAi2 rvs.	5'-GTCGACATTGACCTCTGTGATA-3'	2.7kb
spoT1 fwd.	5'-GTCGACAATTGAAAAACAGGTAAA-3'	
spoT2 rvs.	5'-GTCGACTTCGATTGCCGCTC-3"	2.9kb
spoTi1 fwd.	5'-GTCGACCGAGGCATAAAACC-3'	
spoTi2 rvs.	5'-AAGCTTTTCGATTGCCGCTC-3'	2.5kb
spoTA413E1 fwd.	5'-GGAGTTGCCGAAAGGAGAAACTCCTGTGGATTTTGC-3'	
spoTA413E2 rvs.	5'-GCAAAATCCACAGGAGTTTCTCCTTTCGGCAACTCC-3'	5.5kb

Figure 2.1.

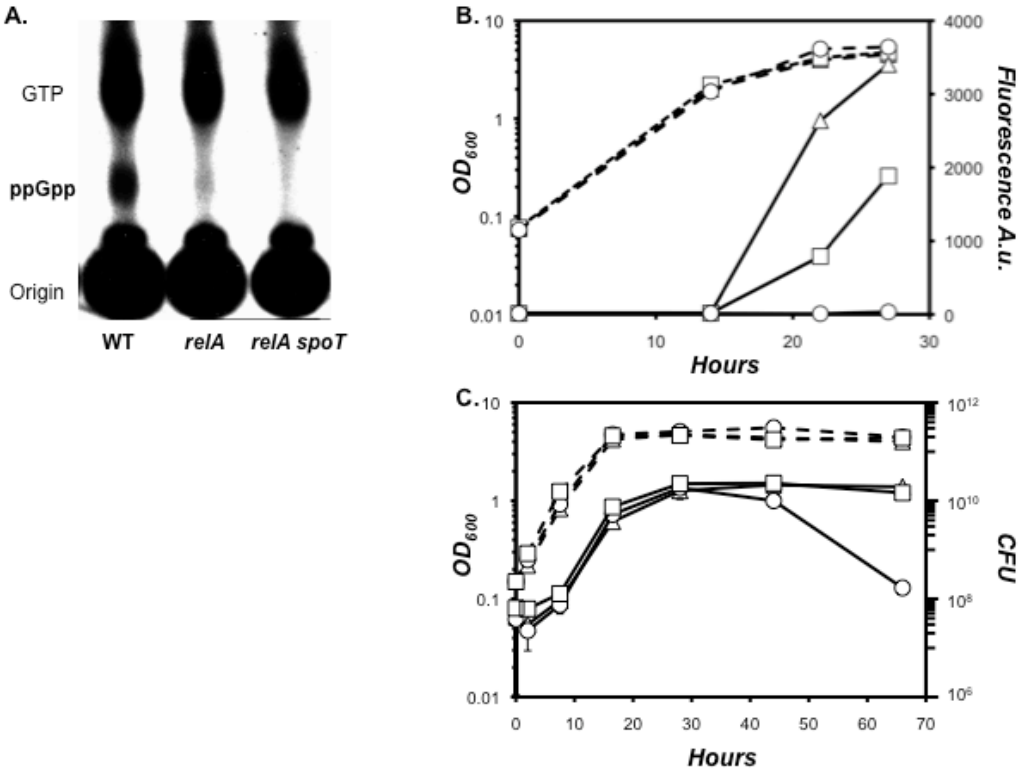

Figure 2.1. RelA and SpoT contribute to ppGpp accumulation, motility and survival in PE phase.
A. To evaluate ppGpp accumulation in PE phase, mid-E phase AYET broth cultures of WT, *relA*, and *relA spoT* bacteria were incubated for 6 h with ^{32}P phosphoric acid (~two generation times), a period sufficient for the cells to enter the PE phase. At this time, cell extracts were prepared, and nucleotides were separated by PEI-TLC. The autoradiogram shown represents one of two separate experiments. **B.** To monitor *flaA* expression (solid lines), WT (triangles), *relA* (squares) and *relA spoT* (circles) strains transformed with p*flaAgfp* were cultured in broth, then at the times indicated bacterial density was quantified by measuring OD_{600} (dashed lines), and Green Fluorescence Protein (GFP) accumulation was quantified by fluorometry (open symbols, solid lines). Shown is a growth curve beginning in early-E phase that is representative of multiple cultures in >5 independent experiments. **C.** To evaluate survival of PE phase stress, early-E phase cultures of WT (triangles), *relA* (squares), and *relA spoT* (circles) bacteria were diluted to an OD_{600} of 0.1, then at the times indicated bacterial density was quantified by measuring OD_{600} (dashed lines), and viability was assessed by enumerating colony forming units (CFU) ml^{-1} on CYET (solid lines). Shown are mean CFU ± SE from duplicate samples, and the data represent one of three independent experiments. The difference in the mean CFU values calculated in three independent experiments for WT and *relA spoT* mutant bacteria at 66 h was statistically significant by a two-tailed Student *t* test ($P < 0.01$).

Figure 2.2. *L. pneumophila* requires RelA and SpoT for ppGpp accumulation and *flaA* expression in response to distinct metabolic cues.
A. To analyze ppGpp accumulation in response to amino acid starvation, early-E phase AYET broth cultures were labeled with ^{32}P phosphoric acid for two generations before addition of either 1 mM serine hydroxamate (+), or water (-). After an additional 1.5 h incubation period, cell extracts were prepared, and nucleotides were separated by PEI-TLC. The autoradiogram represents one of two independent experiments. **B.** To quantify *flaA* expression in response to amino acid starvation, mid-E phase AYET cultures of WT (triangles), *relA* (squares), *relA spoT* (circles) strains harboring p*flaAgfp* were diluted to early-E phase before addition of either 1 mM serine hydroxamate (solid lines), or water (dashed lines), then cultures were sampled at 3 h intervals until WT and *relA* reference samples entered PE phase (OD_{600}, bottom panel) and exhibited *flaA* promoter activity fluorescence (9 h; top panel). These data represent one of two independent experiments. In the second experiment, the actual fluorescence values at 9 h post serine hydroxamate addition were WT, 1098 A.u.; *relA*, 22 A.u.; and *relA spoT*, 11 A.u. **C.** To analyze ppGpp accumulation in response to inhibition of fatty acid biosynthesis, mid-E phase AYET cultures of WT (triangles), *relA* (squares), *relA spoT* (circles) were incubated for 1 h with cerulenin at 0.5 ug ml^{-1} (+) or DMSO (-), then cell extracts were prepared and analyzed by PEI-TLC. Shown is one autoradiogram representing one of three independent experiments. **D.** To quantify *flaA* expression in response to fatty acid biosynthesis inhibition, mid-E phase cultures of WT (triangles), *relA* (squares), or *relA spoT* (circles) bacteria were treated with cerulenin (solid lines) or DMSO (dashed lines), then samples were collected at 3 h intervals to measure bacterial density (bottom panel) and fluorescence (top panel) until WT and *relA* reference cultures entered PE phase and exhibited fluorescence (12 h). These data represent one of two independent experiments. In the second experiment, the actual fluorescence values at 12 h post cerulenin addition were WT, 1617 A.u.; *relA*, 1082 A.u.; and *relA spoT*, 21 A.u.

Figure 2.2.

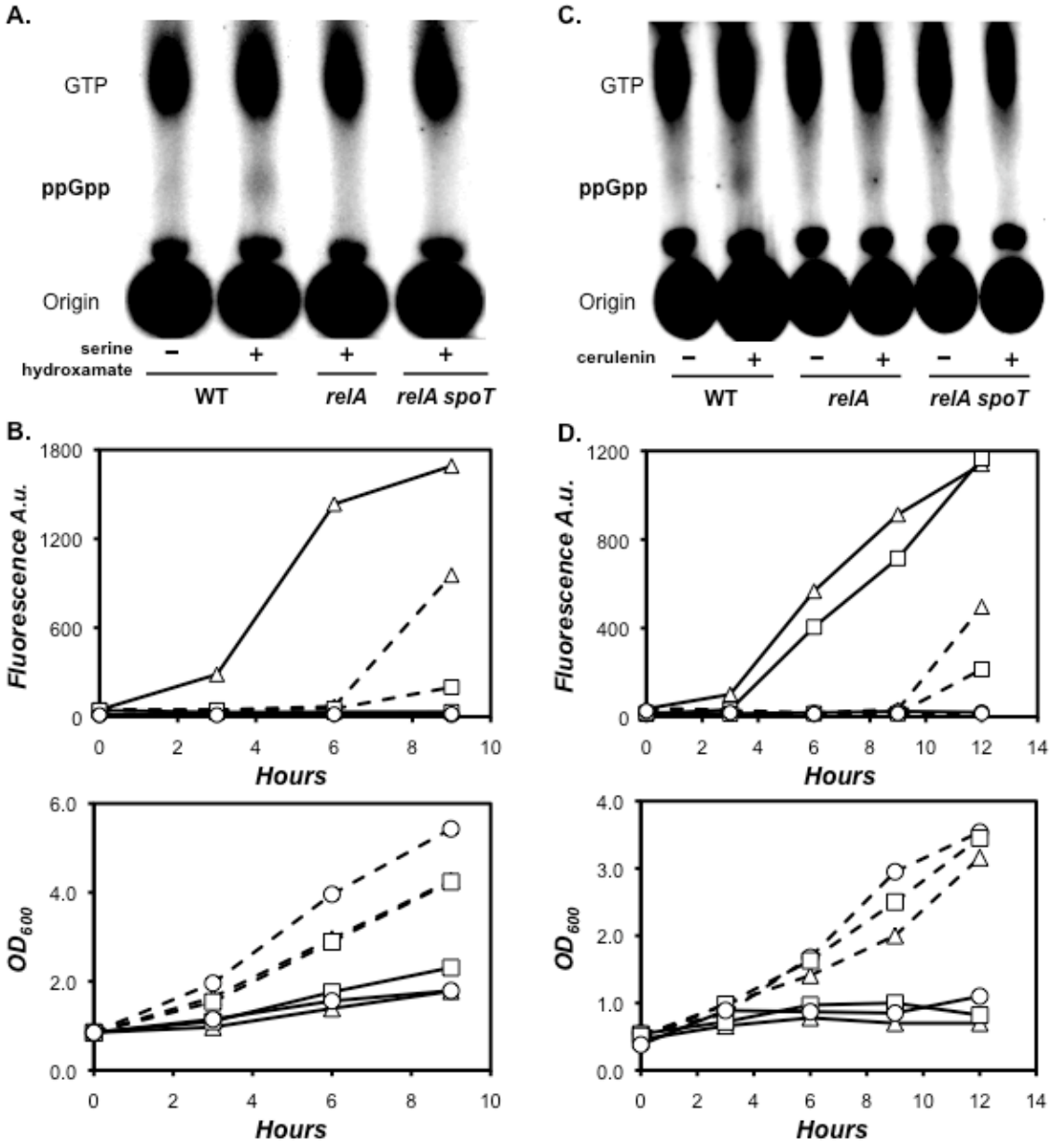
A.
GTP
ppGpp
Origin
serine hydroxamate
– + + +
WT
relA
relA spoT
C.
GTP
ppGpp
Origin
cerulenin
– + – + – +
WT
relA
relA spoT
B.
Fluorescence A.u.
1800
1200
600
0
0 2 4 6 8 10
Hours
OD600
6.0
4.0
2.0
0.0
0 2 4 6 8 10
Hours
D.
Fluorescence A.u.
1200
800
400
0
0 2 4 6 8 10 12 14
Hours
OD600
4.0
3.0
2.0
1.0
0.0
0 2 4 6 8 10 12 14
Hours

Figure 2.3.

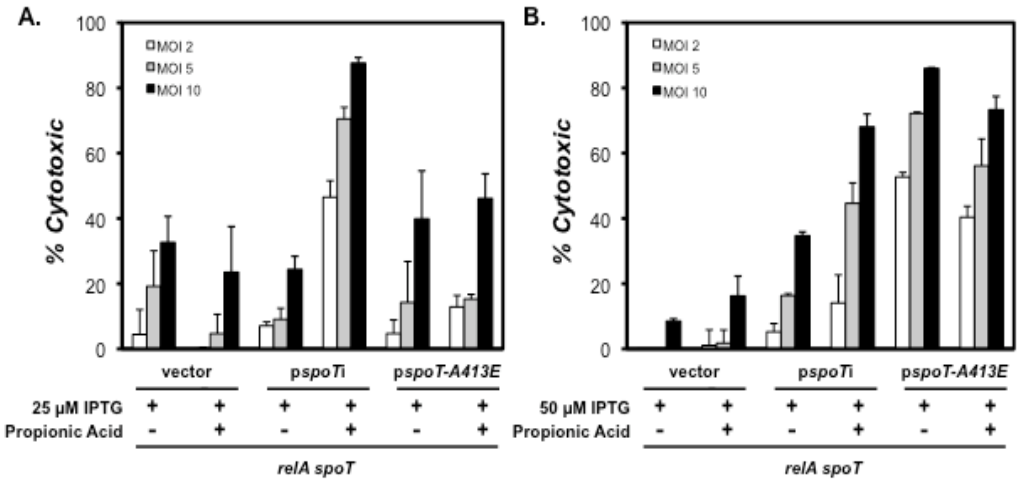

Figure 2.3. SpoT mutation abrogates transmission trait expression following perturbations in fatty acid metabolism.

A. E phase cells normalized to an OD_{600} of 0.5-0.6 were incubated with 25 μM IPTG for 1 h. Then cultures were split and 10 mM propionic acid was added to one half, while the other half was mock-treated. After 3 h of incubation, triplicate wells of macrophages were infected at various multiplicities of infection (MOI) with treated or mock treated *relA spoT* mutant bacteria harboring vector backbone, p*spoT*i, or p*spoT-A413E*. After 1 h incubation with the macrophages, cytotoxicity was measured as the ability of viable macrophages to reduce the colorimetric dye Alamar blue. **B.** As described in A, macrophages were infected with propionic acid-, or mock-treated *relA spoT* mutant bacteria harboring the plasmids shown. To induce high levels of expression, bacteria were incubated with 50 μM IPTG for 2 h, twice the amount and period used for A. Then cultures were split and either treated with propionic acid or mock-treated. After 3 h of treatment, macrophages were infected in triplicate, and cytotoxicity was measured. The values plotted represent the mean ± standard error for triplicate samples determined in one of three similar experiments.

Figure 2.4.

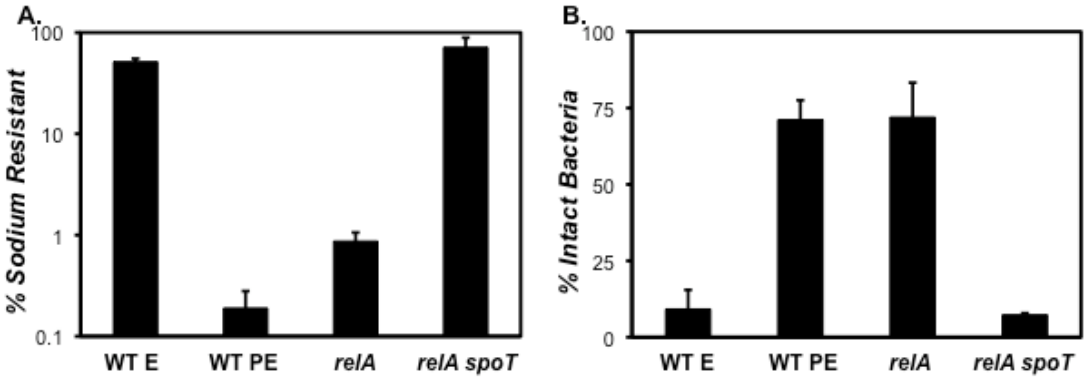

Figure 2.4. The stringent response governs *L. pneumophila* transmission traits in broth.
A. To quantify sodium resistance, E or PE phase cultures of the strains shown were plated on medium with or without 100 mM NaCl, then efficiency of colony formation was calculated as [(CFU on CYET + 100 mM NaCl)/(CFU on CYET)] x 100. Shown are mean percentages ± SE from duplicate samples, and the data represent one of three independent experiments. **B.** The ability of bacteria to bind, enter, and survive ingestion by macrophages was quantified by fluorescence microscopy by scoring the percent of bacteria that were intact 2 h after infection. Infected macrophages were double-labeled with the DNA stain DAPI to visualize macrophage nuclei and intact bacteria and with *L. pneumophila*-specific antibody to visualize both intact and degraded *L. pneumophila*. A total of 100 intracellular bacteria were counted per coverslip. Shown are mean percentages ± SE from duplicate coverslips, and the data represent one of three independent experiments.

Figure 2.5.

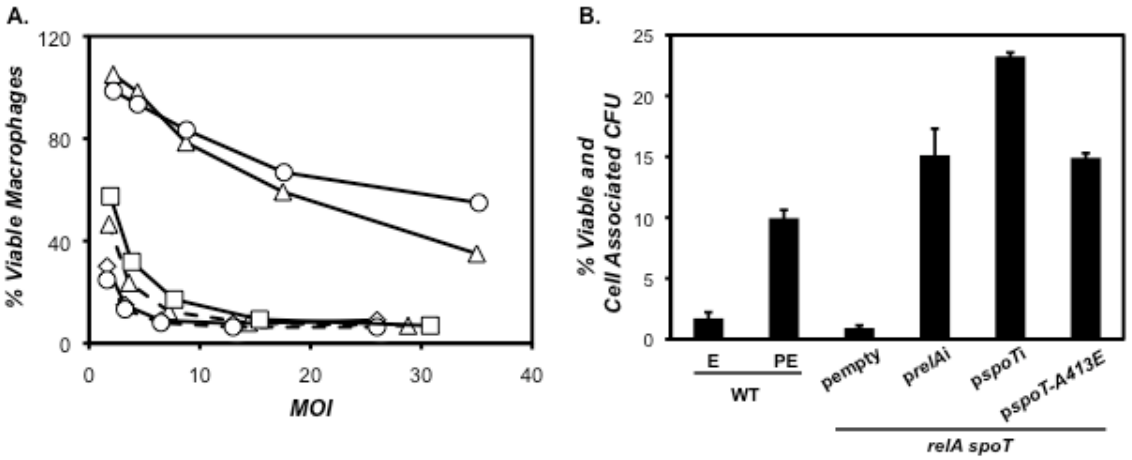

Figure 2.5. Induction of either ppGpp synthetase rescues transmission trait expression of *relA spoT* mutants.
A. To determine the contribution of RelA and SpoT to *L. pneumophila* cytotoxicity, *relA spoT* mutants transformed with vector (circles), p*relA*i (squares), p*spoT*i (diamonds), or p*spoT-A413E* (circles, dashed line) were cultured from mid-E phase to PE phase with 200 μM IPTG. Bacteria were added to triplicate wells of macrophages at the MOI shown. WT cells carrying vector and cultured to either E phase (triangles), or PE phase (triangles, dashed line) were added as controls. After 1 h incubation, cytotoxicity was measured. The values plotted represent the mean ± standard error for triplicate samples determined in one of three similar experiments. **B.** Macrophages were infected at an MOI of ~1.0 for 2 h with either E or PE phase WT carrying vector , *relA spoT* mutants transformed with vector, p*relA*i, p*spoT*i, or p*spoT-A413E* cultured from mid-E phase to PE phase with 200 μM IPTG, as described above. Shown are mean percent of cell-associated CFU ± SE from duplicate wells in one of three independent experiments.

Figure 2.6.

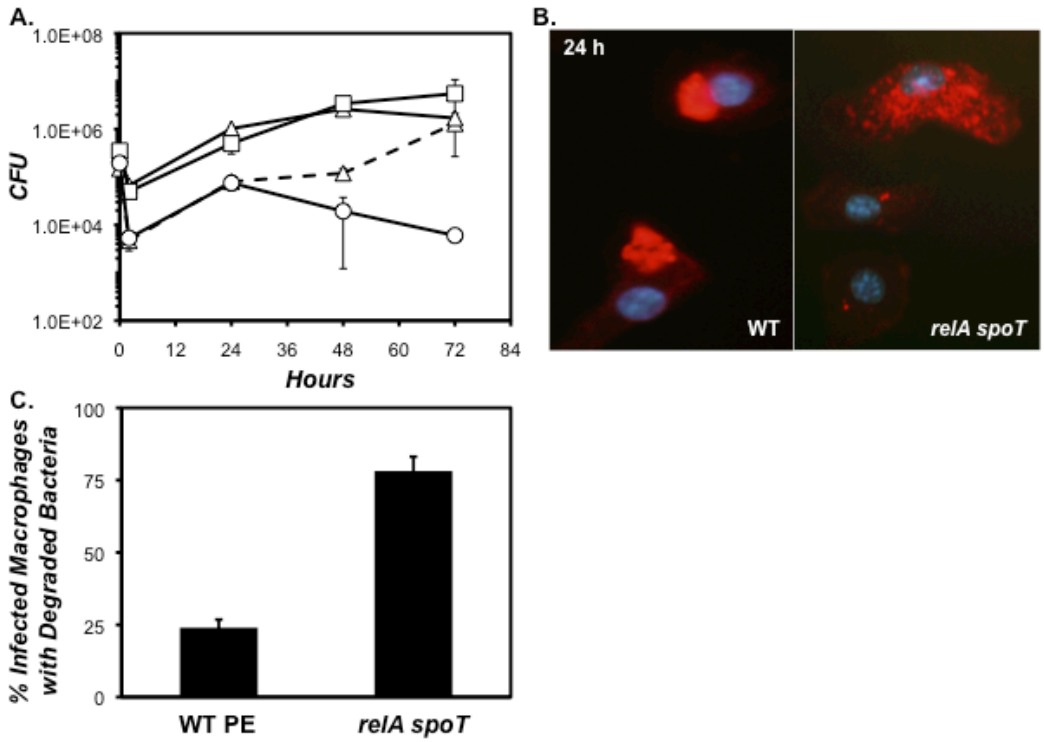

Figure 2.6. The stringent response is essential for *L. pneumophila* transmission in macrophages.
A. Macrophages were infected at an MOI of ~1.0 with E phase WT (triangles, dashed line), PE phase WT (triangles, solid line), PE phase *relA* (squares) or PE phase *relA spoT* mutants (circles) for the periods shown, and then the number of viable bacteria per well was determined. Shown are mean CFU ± SE from duplicate samples in one of three independent experiments. The difference in the mean CFU values calculated from three independent experiments for WT and *relA spoT* mutant bacteria at 72 h was statistically significant by a two-tailed Student *t* test ($P < 0.02$). B. Macrophages were infected at an MOI of ~1.0 with PE-phase WT or PE-phase *relA spoT* mutant bacteria and coverslips were fixed at 24 h. Cells were labeled blue with DAPI and red with anti-*L. pneumophila* antibody, and fluorescence microscopy was performed. The image of the *relA spoT* mutant infected macrophages represents the categories of degradation scored in C. **C.** Macrophages were infected as in B, coverslips were fixed at 18 h, and fluorescence microscopy was performed as described above. A total of 100 infected macrophages were scored on duplicate coverslips. Shown are the mean percentages of macrophages containing single, or multiple degraded bacteria from duplicate samples in one of two independent experiments.

Figure 2.7.

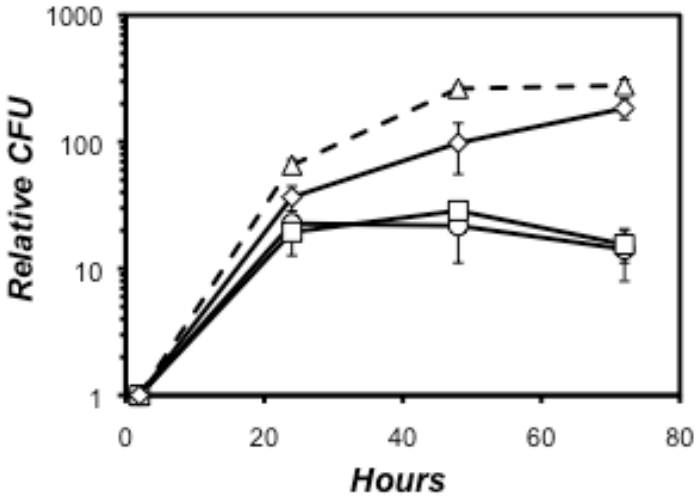

Figure 2.7. SpoT, not RelA, is essential for *L. pneumophila* transmission in macrophages.
Macrophages were infected at an MOI ~ 1.0 with E phase WT (triangles, dashed line) and PE phase *relA spoT* harboring: vector (circles), *prelA*i (squares), or *pspoT*i (diamonds). At 16 h post infection, 200 μM IPTG was added to the wells and maintained throughout the remainder of experiment. Relative CFU was calculated by dividing the CFU values obtained at 24, 48 and 72 h by the 2 h value. Shown are mean fold increase in CFU ± SE from duplicate wells in one of three independent experiments.

Figure 2.8.

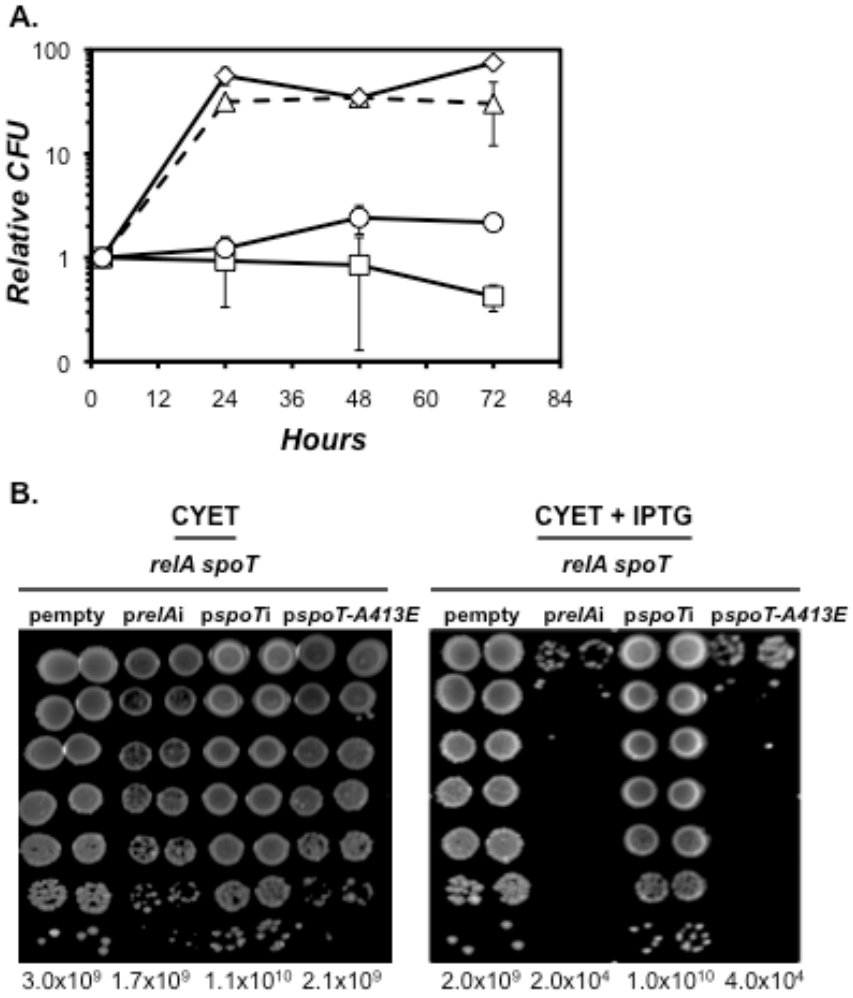

Figure 2.8. Transmissive *L. pneumophila* requires SpoT to enter the replicative phase.

A. To determine if SpoT hydrolase activity contributes to the transmissive to replicative phase transition within host cells, *relA spoT* mutant *L. pneumophila* carrying either p*relA*i (squares), p*spoT*i (diamonds), or p*spoT-A413E* (circles) were induced with 200 μM IPTG in mid-E phase, cultured into PE phase (4-5 h), and then used to infect macrophages at an MOI of ~1. During the 72 h infection, 200 μM IPTG was maintained. WT carrying empty vector cultured into PE phase (triangles, dashed line) served as a positive control in this assay. Relative CFU was as described above. Shown are mean fold increase in CFU ± SE from duplicate wells in one of three independent experiments. **B.** As in A, *relA spoT* mutant derivatives of *L. pneumophila* were cultured in mid-E phase to PE phase (4-5 h) in the presence of 200 μM IPTG. Then, to mimic the induction conditions used in the macrophage experiment, bacteria were plated onto CYET with or without 200 μM IPTG. CFU (below) was calculated as the number of bacteria from the induction in broth capable of initiating replication to form a colony in the presence, or absence of IPTG. Shown are images of CYET agar plates of one of three independent experiments.

Figure 2.9.

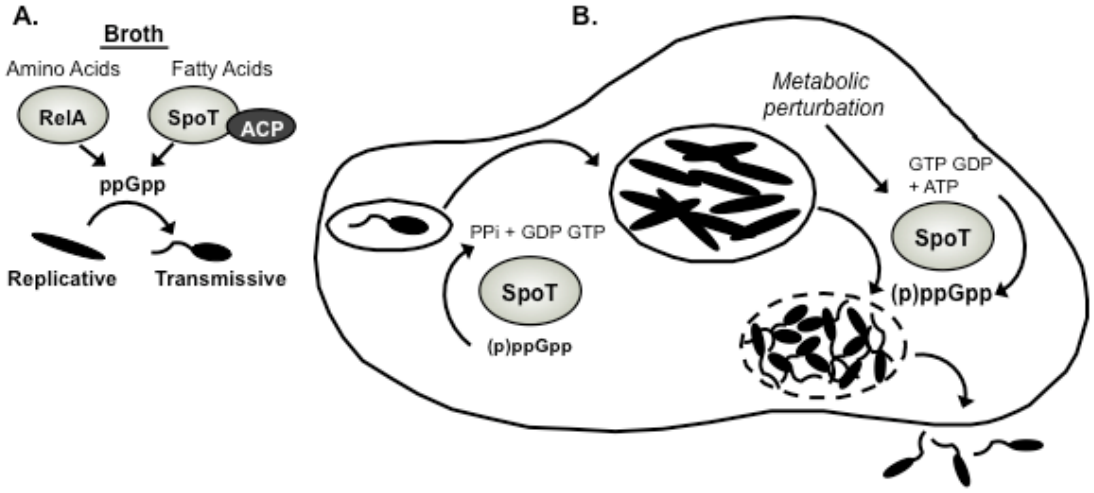

Figure 2.9. SpoT governs *L. pneumophila* differentiation in host macrophages
A. To coordinate exit from the replicative state and entry into the transmissive state in broth, *L. pneumophila* utilizes its two ppGpp synthetases, RelA and SpoT. Each enzyme responds to distinct metabolic cues and SpoT monitors fatty acid biosynthesis likely through an interaction with acyl-carrier protein (ACP). **B.** During the intracellular life cycle of *L. pneumophila*, the bifunctional enzyme SpoT plays a dual role. When transmissive bacteria have evaded initial host cell defenses and gain access to nutrients, SpoT-dependent ppGpp hydrolysis enables transformation into the replicative form. Following robust replication, nutrients become exhausted and SpoT synthetase activity is elicited. Accumulation of SpoT-dependent ppGpp initiates global metabolic change as replicative traits are repressed and the bacterium converts back to the transmissive form. Transmissive bacteria are equipped to exit the depleted host cell and prepared to infect a naïve one.

Figure 2.10.

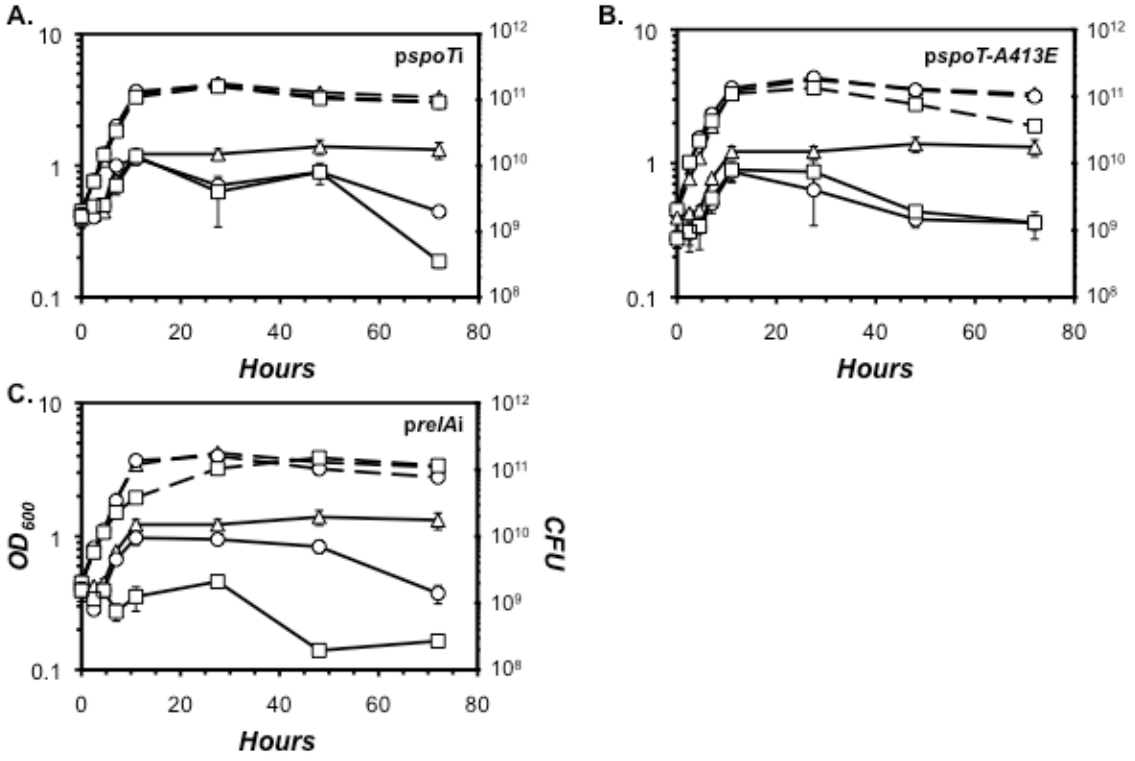

Figure 2.10. Growth patterns of *relA spoT* mutant strains induced to express *spoT*, *spoT-A413E*, or *relA*.
When *relA spoT* mutant bacteria are induced to express either p*spoT-A413E* or p*relA*i they fail to replicate in macrophages or on rich medium that contains IPTG (Fig. 2.8). To determine the impact of p*spoT-A413E* or p*relA*i on growth of *relA spoT* mutant bacteria in broth, we monitored OD_{600} and CFU following IPTG treatment for a time period equivalent to that applied in macrophages (Fig. 2.8A). **A.** Early E phase cultures were diluted to an OD_{600} of 0.45, cultured for 4.5 h to OD_{600} of 1.1-1.5, divided, and then either induced with 200 μM IPTG or left untreated. OD_{600} (dashed lines) and CFU (solid lines) were monitored throughout for cultures of WT pempty + IPTG (triangles), *relA spoT* p*spoT*i uninduced (circles), and *relA spoT* p*spoT*i + IPTG (squares). **B.** The experimental conditions in B and C were identical to and were assessed in parallel to those in A. Represented are WT pempty + IPTG (triangles), *relA spoT* p*spoT-A413E* uninduced (circles), and *relA spoT* p*spoT-A413E* + IPTG (squares). **C.** Represented are WT pempty + IPTG (triangles), *relA spoT* p*relA*i uninduced (circles) and *relA spoT* p*relA*i + IPTG (squares). The experiment was performed once for the extended induction period shown (~ 68 h). In addition, the OD_{600} was monitored following induction for > 3 h in more than three independent experiments, including those represented in Figs. 2.5 and 2.8. In those experiments, the growth patterns of induced strains were identical to those depicted here. Mean CFU ± SE was calculated from serial dilutions plated in duplicate.

CHAPTER III

Distinct roles of ppGpp and DksA in *Legionella pneumophila* differentiation.

Summary

To transit between hosts, intracellular *Legionella pneumophila* transform into a motile, infectious, transmissive state. Here we exploit the pathogen's life cycle to examine how guanosine tetraphosphate (ppGpp) and DksA cooperate to govern bacterial differentiation. Transcriptional profiling revealed that during transmission alarmone accumulation increases the mRNA for flagellar and Type IV-secretion components, secreted host effectors, and regulators, and decreases transcripts for translation, membrane modification and ATP synthesis machinery. DksA is critical for differentiation, since mutants are defective for stationary phase survival, flagellar gene activation, lysosome avoidance, and macrophage cytotoxicity. The roles of ppGpp and DksA depend on the context. For macrophage transmission, ppGpp is essential, whereas DksA is dispensable, indicating ppGpp can act autonomously. In broth, DksA promotes differentiation when ppGpp levels increase, or during fatty acid stress, as judged by *flaA* expression and evasion of degradation by macrophages. For flagella morphogenesis, DksA is required for basal *fliA* (σ^{28}) promoter activity. When alarmone levels increase, DksA cooperates with ppGpp to generate a pulse of Class II rod RNA or to amplify the

Class III sigma factor and Class IV flagellin RNAs. Thus, DksA responds to the level of ppGpp and other stress signals to coordinate *L. pneumophila* differentiation.

Introduction

During infection, pathogens respond to local cues by altering their metabolism and virulence factor production. To coordinate physiological adaptation with virulence mechanisms, many pathogens employ two factors known to be critical for stationary phase resilience of most bacterial species, guanosine tetraphosphate (ppGpp) and DksA. Components of the stringent response regulate processes as diverse as *Mycobacterium tuberculosis* persistence (Dahl et al., 2003, Stallings *et al.*, 2009), *Salmonella enterica* invasion (Pizarro-Cerda & Tedin, 2004, Thompson et al., 2006), *Shigella flexneri* intercellular spread (Sharma & Payne, 2006), and *Legionella pneumophila* transmission (Hammer & Swanson, 1999, Dalebroux *et al.*, 2009).

L. pneumophila resides in aquatic reservoirs within biofilms or protozoa. When humans inhale contaminated aerosols, *L. pneumophila* infects alveolar macrophages. In host cells, the bacteria differentiate between two forms, replicative and transmissive (Molofsky & Swanson, 2004, Sauer et al., 2005, Bruggemann et al., 2006). Upon phagocytosis, transmissive bacteria utilize the Dot/Icm Type IV secretion system to avoid lysosomes and traffic to a vacuole derived from the endoplasmic reticulum (Isberg et al., 2009). In this compartment, bacteria that sense favorable conditions repress transmissive functions and activate genes needed for protein synthesis and replication (Sauer et al., 2005, Bruggemann et al., 2006). Eventually conditions deteriorate, cueing bacteria to synthesize ppGpp. The alarmone triggers differentiation to the motile, transmissive form, which resist degradation, lyse the exhausted host cell and are equipped to infect naive

host cells (Molofsky & Swanson, 2004, Dalebroux et al., 2009). Under certain conditions, transmissive *L. pneumophila* develop into 'mature intracellular forms', which are fit to persist in the environment (Faulkner et al., 2008).

In most gamma-proteobacteria, including *L. pneumophila*, ppGpp levels are controlled by the synthetase RelA and the bifunctional synthetase/hydrolase SpoT. RelA monitors amino acid availability through its association with the ribosome, whereas SpoT responds to a variety of stimuli including fatty acid starvation, which requires direct interaction with acyl-carrier protein (Potrykus & Cashel, 2008, Battesti & Bouveret, 2009, Edwards et al., 2009, Dalebroux et al., 2009). Synthesis of ppGpp by exponential (E), replicative phase broth-grown *L. pneumophila* triggers differentiation to the post-exponential (PE), transmissive form (Hammer & Swanson, 1999, Dalebroux et al., 2009). For transmission between macrophages, ppGpp synthesized from SpoT is sufficient (Dalebroux et al., 2009). When conditions are favorable in host cells or in media, transmissive bacteria require SpoT to hydrolyze ppGpp and initiate replication. Thus, *L. pneumophila* modulates ppGpp levels to coordinate timely differentiation.

Many of the physiological effects of ppGpp are mediated through interactions with RNA polymerase (RNAP) in cooperation with the RNAP secondary channel interacting protein DksA (Haugen et al., 2008, Potrykus & Cashel, 2008). Whether ppGpp and DksA co-exert positive or negative regulation depends upon intrinsic properties of the promoters. While deactivating ribosomal RNA operons (rRNA), ppGpp and DksA activate amino acid biosynthetic operons and alternative metabolic pathways. Direct co-positive regulation has also been observed during *in vitro* studies of promoters of critical virulence regulators (Nakanishi *et al.*, 2006, Aberg et al., 2008, Sharma &

Payne, 2006). Recently, DksA and ppGpp were shown to directly inhibit transcription of the *E. coli* σ^{70}-dependent promoters of critical flagellar gene regulators, *flhDC* and *fliA* (σ^{28}) to repress flagellar synthesis during starvation (Lemke et al., 2009).

Indirect transcriptional control reflects the impact of ppGpp and DksA on RNAP availability. During *E. coli* growth, nearly half the cellular RNAP is localized to rRNA operons by the σ^{70} vegetative sigma factor (Bremer, 1996). Upon nutrient limitation, ppGpp and DksA deactivate transcription from these loci, increasing the amount of core RNAP available to alternative sigma factors. These specialized subunits then direct polymerase to promoters of genes involved in particular stress responses (Bernardo et al., 2006, Szalewska-Palasz et al., 2007, Costanzo et al., 2008, Gummesson et al., 2009). Therefore, ppGpp and DksA indirectly control transcription by alternative sigma factors.

Recent evidence from *E. coli* suggests that DksA is more than a cofactor for ppGpp-dependent transcriptional control. Overproduction of DksA in ppGpp^0 bacteria can compensate for lack of alarmone, indicating that DksA can act independently of ppGpp (Potrykus & Cashel, 2008). Additionally, phenotypic and *in vitro* assays show that ppGpp and DksA oppositely regulate some processes and promoters (Lyzen et al., 2009). Therefore, we exploited *L. pneumophila* differentiation and its flagellar cascade to investigate the functional relationship between ppGpp and DksA.

Experimental Procedures

Bacterial strains and culture. *L. pneumophila* strain Lp02 (*thyA hsdR rpsL*; MB110), a virulent thymine auxotroph derived from Philadelphia 1 (Berger & Isberg, 1993), was the parental strain for all the strains analyzed. *L. pneumophila* was cultured at 37°C with

agitation in 5 ml of *N*-(2-acetamido)-2-aminoethanesulfonic acid (ACES; Sigma)-buffered yeast extract (AYE) broth or on ACES-buffered charcoal yeast extract (CYE), supplemented with 100 μg/ml thymidine (AYET, CYET) when necessary. Bacteria from colonies <5 days old were cultured in broth overnight, then subcultured into fresh AYET prior to experiments. Exponential (E) cultures had an optical density at 600 nm (OD_{600}) of 0.3 to 2.0; post-exponential (PE) cultures had an OD_{600} of 3.0 to 4.5, a period when the viability of the strains was similar (Fig. 3.2). Where indicated, ampicillin (amp; Fisher) was added to a final concentration of 100 μg ml^{-1}; gentamycin (gent; Fisher) to 10 μg ml^{-1}; chloramphenicol (cam; Roche) to 5 μg ml^{-1} for *L. pneumophila* and 10 ug ml^{-1} for *E. coli*; propionic acid to 10 mM; and, isopropyl-beta-D-thiogalactopyranoside (IPTG) to the concentrations specified. To determine colony-forming units (CFU), serial dilutions of *L. pneumophila* were plated on CYET and incubated at 37°C for 4-5 days.

dksA mutant construction. To construct the *dksA* mutant, a deletion insertion allele was first generated by recombineering in *E. coli* (Yu *et al.*, 2000, Datsenko & Wanner, 2000). pGEM-*dksA* was generated by amplifying the *dksA* region (*lpg2338*) using primers dksA1 and 2 (Table 3.5). The FRT::*cat*::FRT cassette was amplified from pKD3 using primers dksA-pKD3a and dksA-pKD3b. To generate pGEM-Δ*dksA*::FRT::*cat*::FRT, pGEM-*dksA* and the linear PCR product were co-electroporated into DY330, prepared as described (Yu et al., 2000), recombinants were selected on LB-cam, and the insertion verified by PCR. Plasmid DNA from DY330 recombinants was used to transform DH5α, and transformants were verified by PCR. Lp02 was transformed with the Δ*dksA*::FRT::*cat*::FRT allele by natural competence (Stone & Kwaik, 1999).

Chromosomal recombination was confirmed by PCR, and the resulting Δ*dksA*::FRT::*cat*::FRT mutant *L. pneumophila* was designated MB699 (Table 3.3).

Inducible dksA expression. To generate strains in which expression of *dksA* could be induced, a promoterless fragment of *dksA* was cloned into either pMMB206-Δmob, a broad host range vector containing a $P_{taclacUV5}$ IPTG-inducible promoter with a cam cassette (p206-*cat*), or the same vector with a gent cassette (p206-*gent*). To construct pGEM-*dksA*i, the *dksA* locus was amplified from Lp02 using primers dksAi1 and i2. The fragment was excised from pGEM-*dksA*i and ligated into p206-*cat* or p206-*gent* immediately 3' of the $P_{taclacUV5}$ promoter, generating p*dksA*i-*cat* and p*dksA*i-*gent*, respectively. Insertion was confirmed by PCR. For complementation experiments, MB699 transformed with p*dksA*i-*gent* were selected on gent, creating MB701 for inducible *dksA* expression (Table 3.3). To induce *dksA* expression by ppGpp^0 mutant *L. pneumophila*, MB697 transformed with p*dksA*i-*cat* were selected on cam, creating MB700 for inducible *dksA* expression in the absence of ppGpp.

fliA-gfp promoter fusion. A fragment containing 304 bp 5' of the *fliA* RBS (K. Heuner, personal communication) and encoding the putative σ^{70} promoter and transcriptional start was amplified using primers fliAP1 and P2. The fragment was ligated into pKB5 directly 5'of *gfp* as described (Hammer & Swanson, 1999). This plasmid was used to transform MB110, MB697 and MB699 (Table 3.3), generating MB733, MB734 and MB735, respectively.

Fluorometry. To monitor expression of the flagellin promoter, E phase cultures of MB355, MB685, and MB732 were diluted to OD_{600} (0.75-0.85) and treated with 10 mM propionic acid or water (t = 0; Fig. 3.7A). At the times indicated, the cell density of each culture was measured as OD_{600} and fluorescence was quantified as described (Edwards et al., 2009). To quantify *fliA* promoter activity, E phase cultures of MB733, MB734 and MB735 were diluted to OD_{600} = 1.0 (t = 0) and cultured to stationary phase. Fluorescence was detected as described above except that cultures were normalized to OD_{600} = 3.0.

Detection of ppGpp. Accumulation of ppGpp was detected by thin-layer chromatography (TLC) as described (Dalebroux et al., 2009). Briefly, E phase AYET broth cultures of $ppGpp^0$ mutant bacteria carrying either p*empty* or p$relA^{L.p}$ were diluted to OD_{600} = 0.25 and labelled with ~ 100 μCi/ml of carrier-free [^{32}P]-phosphoric acid at 37°C for 6 h (~ 2 generations). After labelling, the E phase cultures were treated with 500 μM IPTG and sampled at 30, 60, and 90 min post-induction.

Flagellin western analysis. After culture to OD_{600} = 1.0 in 50 ml, mutant bacteria were treated with 200 μM IPTG; untreated WT bacteria were the positive control. After culture to PE phase (~ 9 h indicated by * in Fig. 3.6A), cell pellets were harvested. Since induction of p$relA^{E.c.}$ Immediately inhibited growth of *dksA* mutants, *dksA* p*empty* cultures were normalized to the *dksA* p$relA^{E.c.}$ culture density prior to pelleting. Bacteria were lysed with Qproteome™ kit (Qiagen), and equivalent volumes of $ppGpp^0$ and *dksA* mutant *L. pneumophila* lysates were denatured and separated on a SDS-10% polyacrylamide gel. To avoid signal overload, WT cell lysates were diluted 1:100 in PBS

prior to denaturing. Flagellin was detected using a 1:50 dilution of monoclonal antibody 2A5 (Molofsky et al., 2005).

Microarrays. To study the effect of ppGpp induction on *L. pneumophila* gene expression (Table 3.1), ppGpp^0 mutants carrying either p*empty*, or p*relA*$^{L.p.}$, were grown in AYET to E phase OD_{600} = 1.4 before treatment with 500 μM IPTG. After 45 min (when the p*empty* and p*relA*$^{L.p.}$ cultures had increased to OD_{600} = 1.90 and 1.70, respectively) and 90 min (OD_{600} = 2.45 and 2.10 for p*empty* and p*relA*$^{L.p.}$, respectively), cells were harvested, and total RNA was extracted as described (Milohanic *et al.*, 2003). To test the contribution of DksA to PE phase expression of *L. pneumophila* flagellar genes (Table 3.2), WT and *dksA* mutant bacteria were normalized to an OD_{600} = 1.6 and cultured until 95-100% of the WT culture was motile. RNA was prepared from two independent cultures, and each RNA sample was hybridized twice with dye swap to the microarrays. The design of microarrays was based on all predicted genes of the genomes of *L. pneumophila* Paris, Lens and Philadelphia (Bruggemann et al., 2006) in addition to the ncRNAs *rsmY* and *rsmZ* (Sahr et al., 2009). Reverse transcription, labeling and hybridization were carried out as described (Sahr et al., 2009).

Data normalization and differential analysis were conducted as described (Sahr et al., 2009). If not stated otherwise, only differentially expressed genes with 2-fold changes were taken into consideration. Complete data sets are available at http://genoscript.pasteur.fr in a MIAME compliance public database maintained at the Institut Pasteur and were submitted to the ArrayExpress database maintained at http://www.ebi.ac.uk/microarray-as/ae/ under the Acc. No. A-MEXP-1804.

Quantitative Real-time PCR. To validate the microarray data sets (Tables 3.1, 3.2 and 3.S1), bacteria were cultured as described for *Microarray analysis*. To assess whether *dksA* contributes to ppGpp-dependent transcript accumulation (Fig. 3.9A), WT and *dksA* mutant bacteria carrying p*relA*$^{E.c.}$ were cultured to E phase OD_{600} (0.7-0.8) and treated with 500 μM IPTG. Bacteria were harvested at 0, 90 and 300 min post-IPTG. By 300 min, WT p*relA*$^{E.c.}$ bacteria had transformed to the transmissive state and were fully motile. To isolate RNA for Real-time PCR (RT-PCR), *L. pneumophila* cell pellets were lysed in TRIzol (Invitrogen), extracted with chloroform/isoamyl alchohol (24/1) and precipitated. Total nucleic acid concentration was assessed, and total nucleic acid was treated with Turbo DNA Free DNase (Ambion) to digest residual genomic DNA (gDNA). RNA integrity was assessed with an Agilent 2100 Bioanalyzer, and cDNA was synthesized using Superscript® II Reverse Transcriptase (RT) (Invitrogen) and random hexamers (New England Biolabs). To control for residual gDNA contamination, a no-RT cDNA synthesis reaction was run for each sample. Prior to qRT-PCR, cDNA was diluted to ~ 300 pg/ul (assuming a 100% RT yield) and ~ 1.5 ng of cDNA was added to the reaction mixture containing Brilliant II SYBR® Green Q-PCR Master Mix (Stratagene), a reference dye, and primers for each target at a final concentration of 300 nM, and reactions were run in triplicate. Non-template control reactions and dissociation curves were run for each primer pair. The *lpg2096* locus served as an internal reference, as it showed no change in expression by microarray under any conditions or strains tested in these or other studies (data not shown). qRT-PCR was performed using an MX3000P instrument (Stratagene). For validation of the p*relA*$^{E.c.}$ (Tables 3.1 and 3.S1) and

dksA/WT PE phase (Table 3.2) microarray data sets, *letE*, *rpoS*, *flgB*, *fliA*, *flaA*, *fliD*, *ndk*, *lag-1* and *lpg0260* (Table 3.5) were targeted (Figs. 3.10 and 3.11). To test the contribution of *dksA* to ppGpp dependent expression of flagellar genes (Fig. 3.9), *flgB*, *fliA* and *flaA* were targeted. All primers were designed to amplify 100-200 bp segments of cDNA.

Comparisons of the transcriptional profiles of PE phase WT and ppGpp^0 mutants and of *dksA* and ppGpp^0 mutants *L. pneumophila* were also sought. However, since these ppGpp^0 mutant microarray data sets failed a series of independent qRT-PCR and promoter fusion validation tests, the data were not analyzed further.

Intracellular growth in A. castellanii. Three-day-old cultures of amoebae were washed in infection buffer (PYG 712 medium without tryptone, glucose, or yeast extract) and adjusted to 5 x 10^5 to 1.0 x 10^6 cells ml^{-1}. PE phase AYE broth grown *L. pneumophila* harboring a plasmid conferring thymine prototrophy (p*flaAgfp*) were diluted in buffer and mixed with *A. castellanii* at an MOI of ~ 0.05. After allowing invasion for 1 h at 37°C, adherent amoebae were washed three times with infection buffer, and flasks were returned to 37°C. At the times shown, 300 μl aliquots from each flask were centrifuged and vortexed to lyse intact amoebae, and CFU were enumerated by plating dilutions on CYE agar. Each infection was carried out in duplicate.

Macrophage cultures. Macrophages were isolated from femurs of female A/J mice (Jackson Laboratory) and cultured in RPMI-1640 containing 10% heat-inactivated fetal bovine serum (RPMI/FBS; Gibco BRL) as described (Swanson & Isberg, 1995).

Following a 7-day incubation in L-cell supernatant-conditioned media, macrophages were plated at 5×10^4 per well for cytotoxicity assays or 2.5×10^5 per well for lysosomal degradation assays, infectivity assays and intracellular growth curves.

Infection and growth in macrophages. *L. pneumophila* binding, entry, and survival inside macrophages during a 2 h incubation was measured as described (Dalebroux et al., 2009). To complement the *dksA* mutant infectivity defect, plasmid carrying bacteria were induced with 25 μM IPTG in early E phase and cultured to PE phase prior to infection (Fig. 3.3A). To quantify intracellular growth, each pooled macrophage supernatant and lysate was plated for CFU at various times post-infection as described (Bachman & Swanson, 2001).

Degradation in macrophages. The percentage of intracellular *L. pneumophila* that remain intact after a 2 h infection was quantified by fluorescence microscopy (Bachman & Swanson, 2001). Except for longer incubation times (18 h) and MOI adjustments, identical procedures were used to image and score infected macrophages at later time points of the primary infection period (Fig. 3.5B).

Cytotoxicity. To measure contact-dependent cytotoxicity, *L. pneumophila* were added to macrophages at the indicated MOI, and cytotoxicity was measured spectrophoretically as described (Molofsky et al., 2005). To determine the contribution of *dksA* to PE phase activation of *L. pneumophila* cytotoxicity (Fig. 3.3C), WT p*empty* was cultured to E or PE phase, $ppGpp^0$ p*empty* to PE phase, and *dksA* mutants from E to PE phase with 25 μM

IPTG prior to infection. To determine if induction of *dksA* expression or constitutive ppGpp synthesis could restore ppGpp^0 and *dksA* mutant cytotoxicity (Fig. 3.8), WT, ppGpp^0, and *dksA* bacteria carrying p*empty*, p*dksA*, or p*relA*$^{E.c.}$ were cultured from E to PE phase (~ 9 h, indicated by * in Fig. 3.6A) in the presence of 200 μM IPTG prior to infection.

Sodium sensitivity. The percentage of *L. pneumophila* that were sensitive to sodium was determined by enumerating colony formation on CYET and CYET containing 100 mM NaCl as described (Byrne & Swanson, 1998).

Results

ppGpp induces rapid accumulation of virulence transcripts

Although ppGpp is known to induce *L. pneumophila* transmission traits, genes regulated by the alarmone have not been identified. To begin to define the ppGpp regulon, we developed a genetic system to synchronize ppGpp accumulation by *L. pneumophila*. When treated with IPTG, a *relA spoT* double mutant strain (ppGpp^0) that carries an inducible allele of *relA* is locked in the transmissive state and exhibits heightened virulence (Dalebroux et al., 2009). Although replication is stunted, viability is not compromised. By 60 min after IPTG addition, a pool of ppGpp was evident in ppGpp^0 p*relA*$^{L.p.}$ cell extracts, but not in ppGpp^0 p*empty* controls; by 90 min, this pool had increased (Fig. 3.1).

To identify ppGpp-sensitive transcripts, cDNA prepared from ppGpp^0 p*relA*$^{L.p.}$ and ppGpp^0 p*empty* bacteria harvested at 45 and 90 min post-induction was hybridized to a comprehensive *L. pneumophila* microarray (Bruggemann et al., 2006), and relative

transcript levels were calculated. This experimental design will identify loci whose expression is affected by cell growth, together with genes for which ppGpp accumulation activates or represses transcription initiation or transcript stability. For brevity, we will use "up-" and "down-regulation" to describe the relative differences between the two cultures, but kinetic studies of the corresponding promoters and transcripts will be required to identify the mechanism(s) that contribute.

Microarray results were validated by qRT-PCR analysis of six loci, and a strong correlation was apparent ($R^2 = 0.98$; Fig. 3.10). Although statistically significant differences were observed at 45 min, most were less than two-fold, our criterion for ppGpp-regulated transcripts (http://www.ebi.ac.uk/microarray-as/ae/). Therefore, the 90 min data set was analyzed further. Many transcripts identified as ppGpp-sensitive at 90 min (Tables 3.1 and 3.S1) were also differentially expressed at 45 min. By 90 min these differences had increased, suggesting that as time with ppGpp increased, existing differences were amplified.

Consistent with the *E. coli* stringent response to amino acid starvation (Durfee et al., 2008, Traxler et al., 2008), by 90 min of exposure to ppGpp, the transcripts for several ribosomal proteins were less abundant (Table 3.4). RNAs encoding components of the ATP synthase complex also decreased. Strikingly, RNAs for enzymes involved in lipopolysaccharide (LPS) modification and phospholipid biosynthesis were less abundant, including *plsB*, a known mediator of ppGpp-dependent regulation of fatty acid and phospholipid biosynthesis in *E. coli* (Heath et al., 1994). Thus, *L. pneumophila* employs ppGpp for stringent control over its membrane machinery and energy intensive processes like protein synthesis.

RNAs for several virulence factor loci accumulated within 90 min of *relA* induction. Consistent with their motility and toxicity toward macrophages (Dalebroux et al., 2009), ppGpp^0 p*relA*$^{L.p.}$ bacteria contained significantly more transcripts for structural and regulatory elements of the flagellar apparatus (Table 3.1). Additionally, the amount of RNAs encoding membrane, channel and other components of the Dot/Icm Type IV secretion system increased. Consistent with transcriptional activation and secretion of Type IV effectors in PE phase (Tiaden et al., 2007, Rasis & Segal, 2009, Nagai et al., 2002), we observed an increase in the *legC3*, *ylfA*, *sdeD* and *lepA* Dot/Icm effector RNAs. Transcripts encoding virulence regulators also accumulated, including the alternative sigma factor *rpoS* (Hovel-Miner et al., 2009, Bachman & Swanson, 2001), the transmission trait enhancer *letE* (Bachman & Swanson, 2004b), and the regulatory RNA *rsmZ* (Sahr et al., 2009, Rasis & Segal, 2009). In addition, the macrophage infectivity potentiator *mip* (Cianciotto & Fields, 1992), the catalase peroxidase *katB* (Bandyopadhyay *et al.*, 2003), and the enhancer of macrophage uptake *enhC* (Liu *et al.*, 2008) were up-regulated. Together these data demonstrate that *L. pneumophila* employs ppGpp to down-regulate protein synthesis machinery and up-regulate factors dedicated to survival and transmission in host cells.

L. pneumophila encodes a DksA homologue required for stationary phase morphogenesis and survival

L. pneumophila encodes a 158 amino acid DksA-like protein (*lpg2338*) that is 72% identical to that of *E. coli* K-12 (Fig. 3.2A). Conserved in DksA$_{Lpn}$ are two aspartate residues known to comprise the acidic tip of DksA$_{Eco}$ and other related RNAP

secondary interacting proteins. The amino acid sequences in the two α-helical regions immediately adjacent to this pair of aspartates are also highly conserved, suggesting that $DksA_{Lpn}$ adopts a similar coiled-coil fold (Perederina *et al.*, 2004). Also present in $DksA_{Lpn}$ are four cysteine residues known in $DksA_{Eco}$ to comprise a zinc-finger motif. Obvious sequence dissimilarities lie at the extreme N-terminus, where the *E. coli* protein adopts a globular fold (Perederina et al., 2004). Therefore, *L. pneumophila* encodes a DksA protein with several but not all features of its *E. coli* counterpart.

To assess the contribution of DksA to differentiation, a *dksA* deletion insertion mutant was constructed and analyzed. Since *L. pneumophila* requires the ppGpp alarmone to survive in stationary phase (Dalebroux et al., 2009), we first tested if DksA also contributes to persistence in broth. From 3 - 24 h, both culture density and viability were similar for WT, $ppGpp^0$, and *dksA* mutant bacteria (Fig. 3.2B). However, beginning in early stationary phase (~ 22 h), $ppGpp^0$ and *dksA* mutant bacteria began to filament; by 48 h, or ~ 28 h into stationary phase, the mutants exhibited several defects. WT bacteria had become coccoid and motile (data not shown), two characteristics of transmissive cells (Molofsky & Swanson, 2004), whereas the $ppGpp^0$ and *dksA* mutants remained amotile and had become more filamentous. Filamentation of *dksA* mutant bacteria was modest relative to $ppGpp^0$ bacteria, which typically elongated to ~ 50 X the length of PE phase WT *L. pneumophila* (data not shown). After extended culture in stationary phase, $ppGpp^0$ and *dksA* mutant bacteria lost viability: By 72 h, their CFU values had declined ~2 logs below the WT yield (Fig. 3.2B). The *dksA* mutant survival, morphogenesis, and motility defects were each restored when expression of plasmid-borne *dksA* was induced continuously from early-E phase to the late stationary phase with 25 μM isopropyl-beta-

D-thiogalactopyranoside (IPTG; Fig. 3.2B; data not shown). Therefore, DksA and ppGpp each contribute to survival, morphology, and motility of stationary phase *L. pneumophila*.

To test the simple model that *L. pneumophila* require DksA to generate ppGpp, we performed thin layer chromatography. Similar to PE phase WT bacteria, *dksA* mutant *L. pneumophila* accumulated ppGpp, and the size of the pool increased with the length of the PE phase (data not shown). Therefore, the stationary phase defects of *L. pneumophila dksA* mutants could not be attributed to a deficiency in alarmone accumulation.

DksA contributes to PE phase virulence

Concomitant with its morphogenesis, stationary phase *L. pneumophila* activate several virulence phenotypes, including infectivity, lysosome evasion, cytotoxicity, and sensitivity to sodium, a phenotype associated with Type IV secretion (Byrne & Swanson, 1998, Vogel *et al.*, 1996). Since ppGpp activated several loci implicated in particular transmission traits, we investigated whether DksA also contributes.

L. pneumophila required both DksA and ppGpp for PE phase activation of a panel of virulence phenotypes. Like E phase WT bacteria, PE phase *dksA* and ppGpp^0 mutants were poorly infectious (Fig. 3.3A), frequently degraded within 2 h of infection (Fig. 3.3B), non-cytotoxic to macrophages (Fig. 3.3C), and sodium resistant (Fig. 3.3D). Induction of plasmid-borne *dksA* restored macrophage infectivity and cytotoxicity of the mutants (Fig. 3.3A and C). Thus, in broth, *L. pneumophila* employs both DksA and ppGpp to activate transmission traits.

DksA and ppGpp promote growth in amoebae

In the environment, *L. pneumophila* parasitizes numerous species of protozoa, including *Acanthamoeba castellanii*. Optimal growth in *A. castellanii* required both DksA and ppGpp. During the 4-day incubation, WT bacteria replicated robustly, increasing their CFU ~ 10^4 fold by 48 h and exhibiting a growth rate constant (μ) of 0.19 h^{-1} between 1 and 48 h (Fig. 3.4). The yield of *dksA* and ppGpp^0 mutants was less than that of WT at each time analyzed (Fig. 3.4). Bacteria that lacked *dksA* were better equipped for intracellular replication than those that could not generate ppGpp (μ = 0.10 h^{-1} and 0.06 h^{-1}, respectively, between 1 and 48 h). By 96 h, the yield of *dksA* CFU approached the WT level, while ppGpp^0 mutant values were 1-2 logs lower (Fig. 3.4). Thus, for optimal growth in *A. castellanii*, *L. pneumophila* requires both DksA and ppGpp, but the alarmone is more critical.

L. pneumophila requires ppGpp but not DksA for transmission between macrophages

As in broth, *L. pneumophila* alternates between replicative and transmissive forms in amoebae and macrophages (Byrne & Swanson, 1998, Hammer & Swanson, 1999, Alli et al., 2000, Molofsky & Swanson, 2003, Sturgill-Koszycki & Swanson, 2000, Sauer et al., 2005, Bruggemann et al., 2006). Whereas ppGpp is dispensable for intracellular replication, the alarmone is essential for intracellular progeny to survive transmission from one macrophage to another (Dalebroux et al., 2009). Therefore, we analyzed the contribution of DksA to transmission in murine macrophages.

As reported in Figure 3.3A, DksA and ppGpp were essential for efficient infection by PE phase *L. pneumophila*. Both mutants behaved like E phase WT bacteria, yielding

less CFU at 2 h than PE phase WT (Fig. 3.5A). As expected, neither DksA nor ppGpp were required during the primary replication period: From 2 - 24 h, their growth curves were nearly indistinguishable, each exhibiting an ~ 1 log increase in CFU. Between 18 and 24 h, when replicating WT bacteria differentiate to the transmissive form (Byrne & Swanson, 1998, Hammer & Swanson, 1999, Molofsky & Swanson, 2003, Sturgill-Koszycki & Swanson, 2000, Sauer et al., 2005, Bruggemann et al., 2006), DksA was dispensable, but ppGpp was not. The *dksA* mutant CFU continued to increase until 48 h, when their numbers were similar to WT. In contrast, the yield of $ppGpp^0$ mutants failed to increase throughout the remainder of the infection (Fig. 3.5A).

At the end of the primary replication period in macrophages, *L. pneumophila* that lack ppGpp are vulnerable to lysosomal degradation (Dalebroux et al., 2009). Consistent with their lower yield between 48 and 72 h, $ppGpp^0$ mutants were more frequently degraded at 18 h than were *dksA* mutants, as judged by immunofluorescence microscopy (Fig. 3.5B). To assess the ability of transmitted *L. pneumophila* to resist lysosomal degradation, macrophages that contained only a single intact or degraded bacterium at 24 h were scored in two independent experiments. By this criterion, $ppGpp^0$ mutant *L. pneumophila* were more susceptible to lysosomal degradation during host-to-host transmission than either *dksA* mutant or WT *L. pneumophila*, since 73% ± 8% of infected macrophages harbored a single degraded $ppGpp^0$ bacterium, whereas 53% ± 5%, 51% ± 5%, and 49% ± 1% of infected macrophages contained a single degraded WT E, WT PE, or *dksA* bacterium, respectively. Together, the CFU and morphological data indicate that, whereas DksA is dispensable, *L. pneumophila* requires the alarmone ppGpp for efficient transmission to a new macrophage.

Constitutive ppGpp synthesis by L. pneumophila can halt replication and stimulate pigmentation independently of DksA

The distinct phenotypes of *dksA* and ppGpp^0 mutant bacteria during growth in *A. castellanii* (Fig. 3.4) and transmission in macrophages (Fig. 3.5) motivated us to investigate whether ppGpp or DksA can activate *L. pneumophila* transmission traits independently of each other. For this purpose, *dksA* mutant *L. pneumophila* were transformed with p*relA*$^{E.c.}$, which encodes an inducible *E. coli* K-12 RelA with a C-terminal truncation that prevents its interaction with the ribosome, resulting in constitutive synthetase activity (Hammer & Swanson, 1999). In parallel, ppGpp^0 bacteria were transformed with p*dksA*.

Induction of *dksA* did not impact replication of E phase *L. pneumophila*, since the culture densities of WT, ppGpp^0 p*empty*, and ppGpp^0 p*dksA* bacteria were similar after IPTG addition (Fig. 3.6A). In contrast, replication of *dksA* p*relA*$^{E.c.}$ cells halted shortly after IPTG addition, and their OD_{600} values subsequently remained lower than WT and *dksA* p*empty* control bacteria (Fig. 3.6A). Therefore, when expression of *relA*$^{E.c.}$ is highly induced, ppGpp can arrest the *L. pneumophila* cell cycle independently of DksA.

In late stationary phase, *L. pneumophila* produce a secreted pyomelanin pigment by a pathway induced by RelA (Chatfield & Cianciotto, 2007, Zusman et al., 2002). Pigmentation required both DksA and ppGpp, as pyomelanin was not detected after a ~ 44 h treatment of *dksA* and ppGpp^0 mutant bacteria that carried only the vector (Fig. 3.6B). Nevertheless, DksA was not essential to this response, since ectopic expression of *relA*$^{E.c.}$ by *dksA* mutants was sufficient to generate as much pyomelanin as WT cultures.

Nor could DksA function independently of ppGpp to activate this pathway, since ppGpp^0 p*dksA* cultures failed to pigment. Therefore, ppGpp can bypass the *dksA* requirement for pigment production, but the opposite is not true.

DksA controls the propionic acid response cooperatively and independently of ppGpp

When treated with 10 mM propionic acid, E phase *L. pneumophila* rapidly transition to the transmissive state, a response that is specific to carboxylic acids rather than pH (Edwards et al., 2009). To assess the contribution of DksA to *L. pneumophila's* stringent response to fatty acid addition, we used a transcriptional reporter of transmission. The plasmid p*flaAgfp* encodes the *flaA* promoter fused to the gene encoding green fluorescent protein (Hammer & Swanson, 1999).

Neither ppGpp nor DksA is required for growth inhibition by propionic acid, since WT, ppGpp^0 and *dksA* mutant cultures responded similarly (Fig. 3.7A, top). However, unlike WT bacteria, which exhibited heightened fluorescence 3 h post-propionic acid treatment, *dksA* mutant bacteria failed to fluoresce throughout the time course analyzed (Fig. 3.7A, bottom). Consistent with their lack of motility in the PE phase, *dksA* mutant control cultures did not activate *flaA* expression even when the cells reached stationary phase, while WT bacteria did (9 h; Fig. 3.7A, bottom). Therefore, *L. pneumophila* require DksA to induce the *flaA* promoter when the stringent response is triggered by propionic acid or at the transition to PE phase (Hammer & Swanson, 1999, Edwards et al., 2009).

To verify its role in the *L. pneumophila* response to fatty acid perturbation, we analyzed the contribution of DksA to evasion of lysosomal degradation. After a 3 h

propionic acid treatment, ~ 50% of the infected macrophages contained intact WT bacteria, whereas < 10% harbored intact *dksA* mutant bacteria (Fig. 3.7B). Therefore, *L. pneumophila* also require DksA to induce resistance to macrophage degradation when the stringent response is triggered by propionic acid.

Unlike DksA, ppGpp was only partially required for activation of the *flaA-gfp* transmission reporter. We repeatedly observed modest promoter activation 6 - 9 h after propionic acid addition to ppGpp^0 cultures (Fig. 3.7A, bottom). In addition, ~ 20% of alarmone deficient bacteria avoided degradation, another intermediate response (Fig. 3.7B). Importantly, DksA accounted for the ppGpp-independent *flaA* promoter activity and degradation evasion, since *dksA* mutants treated with propionic acid did not increase their fluorescence (Fig. 3.7A, bottom) or remain intact in macrophages (Fig. 3.7B). Thus, *L. pneumophila* transmission trait activation in response to excess fatty acids is mediated by a DksA activity that is enhanced by ppGpp.

DksA induction leads to ppGpp-independent flagellar biosynthesis

To continue to investigate the capacity of DksA to act independently, we asked whether this transcription factor could control flagellar biogenesis in the absence of ppGpp. Using the strains characterized in Figure 3.6, we measured whether induction of *dksA* expression could rescue the flagellar synthesis defect of ppGpp^0 mutant *L. pneumophila*.

Once control bacteria entered stationary phase (~ 9 h post-IPTG; Fig. 3.6A), we assayed levels of flagellin and macrophage cytotoxicity, a flagellin-dependent phenotype (Molofsky *et al.*, 2006). Induction of plasmid-borne *dksA* increased both the cell-

associated flagellin and cytotoxicity of ppGpp^0 bacteria (Fig. 3.8). Likewise, *dksA* induction restored motility of ~ 20 - 35% of the ppGpp^0 bacteria. Full bypass of ppGpp by DksA was not achieved, as flagellin levels, macrophage killing and motility of ppGpp^0 mutant bacteria were still lower than PE phase WT (Fig. 3.8). In contrast, *relA*$^{E.c.}$ induction did not restore flagellin synthesis, cytotoxicity or motility to *dksA* mutants (Fig. 3.8; data not shown). Therefore, when expressed from a plasmid, *dksA* can activate flagellin-dependent phenotypes independently of ppGpp. Furthermore, this contribution by DksA cannot be bypassed by constitutive ppGpp synthesis.

DksA contributes to the flagellar regulon

As an independent approach to analyze the contribution by DksA to the flagellar gene regulon, the transcriptional profiles of PE phase WT and *dksA* mutant *L. pneumophila* were compared (Table 3.2). Consistent with a role for DksA in the down-regulation of ribosomal genes in PE phase, several ribosomal transcripts were elevated in *dksA* mutants relative to WT bacteria (http://www.ebi.ac.uk/microarray-as/ae/). As in *P. aeruginosa*, *L. pneumophila* flagellar gene transcription occurs in a hierarchy of four classes (Dasgupta et al., 2003, Bruggemann et al., 2006, Heuner, 2007). DksA was especially important for PE phase accumulation of late flagellar transcripts, including those encoding structural elements critical for final assembly and motor components essential for flagellar rotation (Table 3.2). In addition, Class II rod and hook transcripts and the Class III sigma factor *fliA* as well as each of its downstream flagellar gene targets required DksA for up-regulation (Table 3.2)(Albert-Weissenberger et al.). DksA also contributed to an increase in RNA for RpoN (σ^{54}), a factor shown to play a modest role in

PE phase up-regulation of a few Class II flagellar genes, including (Albert-Weissenberger et al.). Thus, *L. pneumophila* employs the DksA protein to increase the level of flagellar transcripts in the stationary phase of growth.

ppGpp modulates DksA-dependent expression of flagellar genes

To investigate how DksA contributes to expression of flagellar genes stimulated by alarmone, we induced synchronous ppGpp synthesis by WT and *dksA* mutant bacteria, and then analyzed expression of genes representing three tiers of the flagellar regulon 0, 90 and 300 min later. By 300 min, 95-100% of WT cells were motile, verifying functional flagella. Furthermore, the growth of both WT and *dksA* mutant *L. pneumophila* was rapidly inhibited (Fig. 3.12), suggesting that the strains accumulated ppGpp similarly (Schreiber *et al.*, 1991). Therefore, the fold-increase relative to t = 0 was calculated for three representative transcripts: *flgB*, a Class II gene encoding a flagellar basal-body rod protein; *fliA*, a putative a Class III gene encoding σ^{28}, the sigma factor that activates *flaA* (Heuner et al., 2002); and *flaA*, a Class IV gene encoding flagellin.

By 90 min, *flgB*, *fliA* and *flaA* RNA levels had increased in WT bacteria, revealing that soon after ppGpp synthesis, each tier of the flagellar gene cascade is active (Fig. 3.9A). Between 90 and 300 min, *fliA* and *flaA* levels increased further in WT bacteria, whereas *flgB* levels declined. Therefore, as alarmone accumulates (Hammer & Swanson, 1999), expression of the Class III *fliA* and Class IV *flaA* loci is continuously induced, whereas expression of the Class II *flgB* gene is induced, and then repressed.

Rapid ppGpp-mediated up-regulation required DksA, as no increase was observed for these loci at 90 min post-induction in *dksA* mutants (Fig. 3.9A). The DksA contribution to *flaA* RNA accumulation could be partially bypassed by $relA^{E.c.}$ induction, as *dksA* mutants showed modest ~ 3-fold increase in *flaA* transcript at 300 min. However, when their *flaA* levels were compared directly, *dksA* mutants contained ~ 140 fold less *flaA* mRNA than WT bacteria (data not shown).

Consistent with *fliA* mRNA accumulation patterns, ppGpp and DksA were both required for activation of the *fliA* promoter at the E to PE phase transition, as judged by a *fliA-gfp* reporter (Fig. 3.9B). Additionally, DksA contributed to basal *fliA* promoter activity in E phase, since WT and $ppGpp^0$ mutant fluorescence values were consistently 2-3 fold higher than those of *dksA* bacteria. Likewise, in the uninduced qRT-PCR samples, WT bacteria contained ~ 3 fold more *fliA* mRNA than *dksA* mutants (data not shown). Therefore, *L. pneumophila* requires the DksA transcription factor to activate the flagellar regulon, whereas the alarmone modulates this activation, by either amplifying or repressing flagellar gene expression over time.

Discussion

When their capacity to synthesize either proteins or fatty acids is compromised, replicating *L. pneumophila* rely on the signaling molecule ppGpp to orchestrate differentiation to a transmissive, infectious form (Edwards et al., 2009). In the process, intracellular *L. pneumophila* alter the level of ~ 800 transcripts (Bruggemann et al., 2006). Here we demonstrate that ppGpp and the transcription factor DksA can either cooperate or act independently to coordinate this developmental program.

Composed of over 40 genes whose expression occurs in four distinct phases, the flagellar transcriptional cascade provides a sensitive read-out for analyzing the impact of ppGpp and DksA during *L. pneumophila* differentiation. Several factors regulate the hierarchy, including the master regulator FleQ (Class II and III), the response regulator FleR and the alternative sigma factors σ^{54} (Class II) and σ^{28} (Bruggemann et al., 2006, Heuner, 2007). The alarmone rapidly increases the level of *fleQ*, *fleR* and several Class II flagellar transcripts, including *flgB* (Table 3.1).

Kinetic analysis of *flgB* expression revealed the versatility of ppGpp and DksA control. In response to ppGpp, DksA is essential for *flgB* expression, but the amount and/or duration of the ppGpp stimulus governs the level of *flgB* RNA. As ppGpp accumulates, RNA for the Class II, σ^{54}-dependent gene target *flgB* first accumulates and then decreases (Fig. 9A). In *S. enterica*, only six subunits of FlgB polymerize with other rod proteins prior to hook formation (Chevance & Hughes, 2008). Thus, the pulse of *flgB* RNA orchestrated by ppGpp and DksA may contribute to proper stoichiometry of rod and hook subunits.

In contrast to *flgB*, ppGpp and DksA continuously activate *fliA* (σ^{28}) and *flaA* during differentiation (Fig. 9A), consistent with induction of *fliA* in the transmissive phase (Bruggemann et al., 2006). As alarmone increases (Hammer & Swanson, 1999), so do *fliA* and *flaA* RNA levels, suggesting functional FliA accumulates. FliA activates Class IV flagellar genes such as *flaA* and *fliD*, in addition to genes unrelated to flagellar biogenesis (Heuner et al., 2002, Molofsky et al., 2005, Bruggemann et al., 2006). Continuous elevated activation of *flaA* by FliA is fitting, since the typical bacterial flagellum is comprised of ~ 20,000 filament subunits (Chevance & Hughes, 2008).

Phenotypic analyses also indicate that ppGpp modulates activation initiated by DksA. In response to fatty acid stress, DksA controls *L. pneumophila flaA* promoter activation and evasion of macrophage degradation independently of ppGpp (Fig. 3.7). When plasmid-borne *dksA* expression is induced, $ppGpp^0$ bacteria initiate flagellar biosynthesis, motility and cytotoxicity to macrophages (Fig. 3.8). Furthermore, DksA mediates basal *fliA* promoter activity during E phase (Fig. 3.9B). Likewise, in both exponential and stationary phase *E.coli*, DksA regulates flagellar biogenesis directly, in this case by inhibiting transcription from a σ^{70}-dependent *fliA* promoter (Lemke et al., 2009). Furthermore, when over-expressed, DksA suppresses several *E. coli* $ppGpp^0$ mutant phenotypes, including amino acid auxotrophy, autoaggregation, motility and RpoS accumulation (Potrykus & Cashel, 2008). DksA can also regulate *E. coli* gene expression independently and even oppositely of ppGpp (Magnusson et al., 2007, Aberg et al., 2008, Aberg et al., 2009, Lyzen et al., 2009, Merrikh et al., 2009b). Perhaps at the promoters of certain flagellar genes or their regulators (FleQ, σ^{54}, FleR, and σ^{28}), DksA controls RNAP activity independently of ppGpp.

It is noteworthy that *E. coli* and *L. pneumophila* each enlist DksA and ppGpp to regulate flagellar genes, but to the opposite effect. Commensal enteric *E. coli* utilize DksA and ppGpp to deactivate ribosome and flagella synthesis simultaneously (Lemke et al., 2009), whereas aquatic intracellular *L. pneumophila* have co-opted the stringent response to down-regulate ribosomal transcripts while activating flagellar genes, which promote transmission to a new replication niche. Judging from BLASTP analyses, *L. pneumophila* also lack the canonical chemotaxis components that equip *E. coli* to swim toward nutrients (data not shown). Because *L. pneumophila* is constrained within a host

vacuole during replication, this pathogen presumably exploits whatever nutrients are available. Also, *E. coli* is equipped with numerous peritrichous flagella, whereas *L. pneumophila* synthesize a monopolar flagellum. Regardless of the distinct costs and benefits that motility confers to *E. coli* and *L. pneumophila* in their natural reservoirs, mechanistic studies can now exploit this dichotomy. Perhaps sequence differences observed in the N-terminal globular domain of the two DksA proteins (Fig. 3.2A) account for their distinct mode of regulation. Alternatively, the promoter architecture of key regulators like *fliA* may dictate the impact of each stress response.

To express certain transmission traits fully, *L. pneumophila* requires that DksA cooperate with ppGpp. For example, DksA only partially activates the *flaA* promoter and evasion of lysosomal degradation by $ppGpp^0$ mutants in response to propionic acid (Fig. 3.7). The alarmone is also essential for activation of transmission phenotypes in response to acetate (data not shown) or entry into stationary phase (Figs. 3.3 and 3.7A). Moreover, *dksA* induction fails to rescue pigmentation (Fig. 3.6B), sodium sensitivity, infectivity and stationary phase survival defects of $ppGpp^0$ mutants (data not shown). Whether ppGpp affects DksA protein levels has not been tested in *L. pneumophila,* but *dksA* RNA levels are similar during the replicative and transmissive phases in broth and amoebae (Bruggemann et al., 2006). The genetic studies presented here establish that the capacity of DksA and ppGpp to act independently or cooperatively during *L. pneumophila* differentiation is context-dependent.

Analysis of DksA and ppGpp also revealed that the requirements for *L. pneumophila* to transit from broth to macrophage and from macrophage-to-macrophage are distinct. Like DksA, the regulators LetA, LetS, LetE and FliA each activate

transmission phenotypes in broth, yet are dispensable for transmission from one macrophage to another (Fig. 3.3) (Hammer et al., 2002). In contrast, ppGpp-dependent factors are critical for *L. pneumophila* transmission from lysosomal vacuoles of permissive mouse macrophages (Dalebroux et al., 2009, Sturgill-Koszycki & Swanson, 2000). Perhaps in host cells, ppGpp induces *L. pneumophila* pathways that promote infection of *A. castellanii* (Fig. 3.4) and spread between macrophages (Fig. 3.5) but are poorly expressed in broth culture. In fact, of the cohort of genes upregulated during transmission in amoebae, only ~ 77% are also elevated during PE phase in broth (Bruggemann et al., 2006). It is also possible that *L. pneumophila* regulators control parallel, or perhaps even redundant pathways in particular host environments. Indeed, although dispensable for macrophage transmission, the FliA sigma factor and the LetA response regulator are required for bacterial growth in particular amoebae (Heuner et al, 2002; Gal Mor & Segal, 2003). Also, some bacteria use ppGpp to control cellular processes through physical interactions with factors other than RNAP (Wang et al., 2007, Zhao et al., 2008).

Synthesis of ppGpp by *L. pneumophila* rapidly alters the level of numerous transcripts. As in *E. coli* (Durfee et al., 2008, Traxler et al., 2008), ppGpp immediately down-regulates the protein synthesis machinery of *L. pneumophila*. Indeed, of the targets showing decreased RNA levels, nearly half encode ribosomal proteins and translation machinery (Table 3.4). While the impact of ppGpp on RNAP has been the focus of much research, the alarmone also induces growth arrest and may alter transcript stability, factors which will also affect the level of transcripts in a cell. Mechanistic studies of

particular promoters and transcripts can now identify how ppGpp alters the *L. pneumophila* transcriptional profile during its life cycle.

In the transmissive state, *L. pneumophila* activates its Dot/Icm Type IV secretion system; this virulence mechanism is also coordinated in part by ppGpp. For example, genes encoding secreted substrates are activated in the PE phase (Nagai et al., 2002, Bruggemann et al., 2006, Rasis & Segal, 2009, Tiaden et al., 2007), and effectors such as LepA and LepB contribute to non-lytic release from amoebae (Chen *et al.*, 2004). Consistent with these findings, RNAs for three secreted coiled-coil domain containing host cell effectors rapidly accumulate, including LepA (Table 3.1). Transcripts encoding membrane and channel components of the Dot/Icm system are also responsive to ppGpp. However, since many of these proteins are constitutively expressed during E and PE phase in broth (J. P. Vogel, personal communication), basal levels of ppGpp may mediate their expression, or more complex post-transcriptional mechanisms contribute.

L. pneumophila differentiation also entails a variety of metabolic changes. In the transmissive form, the pathogen activates several phospholipases, some of which are secreted into host cells via the Type II secretion (Lsp) and Type IV Dot/Icm secretion systems (Banerji *et al.*, 2008). The level of a phospholipase (*lpg2837*) RNA immediately increases in response to ppGpp (Table 3.1). Additionally, transcripts for central metabolic and fatty acid activating enzymes like *pta* are up-regulated by ppGpp. Alarmone accumulation also leads to accumulation of an RNA encoding an uncharacterized type I polyketide synthase (Gokhale *et al.*, 2007). During differentiation to the transmissive form, *L. pneumophila* modifies its surface and sheds vesicles that inhibit phagosome-lysosome fusion (Fernandez-Moreira *et al.*, 2006). Concomitant with

this process, several LPS modification and phospholipid biosynthesis enzymes are down-regulated by ppGpp (Table 3.4). These alterations in LPS structure and phospholipid content may be critical for transmission of progeny, since mutants that lack ppGpp are degraded at the end of the replication period (Fig 3.5B). Therefore, regulation of a number of lipid pathways by ppGpp is another component of *L. pneumophila* differentiation.

L. pneumophila also employs ppGpp and DksA to recruit other regulatory factors to orchestrate its cellular differentiation. In response to alarmone accumulation, *L. pneumophila* up-regulates transcripts for the stationary phase sigma factor RpoS (σ^{38}) and the non-coding regulatory RNA RsmZ (Table 3.1). On the other hand, the amount of RsmY RNA did not increase in response to ppGpp. Therefore, our study supports the prediction that the RsmY and RsmZ regulatory RNAs exhibit varying degrees of sensitivity to ppGpp, possibly at the level of transcription initiation or RNA stability.

The *L. pneumophila* flagellar cascade illustrates how stringent response factors can cooperate to govern bacterial life cycles. The interplay between DksA and ppGpp is complex, as their roles are distinct at different loci. With the identification of candidate genes by microarray analysis, detailed kinetic studies in which ppGpp synthesis by WT and *dksA* bacteria is synchronized can reveal how ppGpp and DksA coordinate complex developmental processes.

Table 3.1. Select list of transcripts up-regulated 90 min after relA$^{L.p.}$ induction.

Gene Name / Function	Gene.ID	Annotation	ppGpp^0 p*relA*$^{L.p.}$/p*empty*
Flagellar Genes			
fleQ	*lpg0853*	Master regulator of flagellar gene transcription (Class I)	2.01
fliH	*lpg1758*	Export and assembly (Class IIa)	2.41
fliG	*lpg1759*	Motor switch protein (Class IIa)	3.88
fleR	*lpg1762*	Two-component response regulator (Class IIa)	3.54
fleS	*lpg1763*	Two-component sensor histidine kinase (Class IIa)	2.58
fliN	*lpg1791*	Motor switch protein (Class IIa)	5.25
flgB	*lpg1216*	Proximal rod protein (Class IIb)	4.32
flgC	*lpg1217*	Proximal rod protein (Class IIb)	4.03
flgG	*lpg1221*	Distal rod protein (Class IIb)	4.81
flgH	*lpg1222*	L-ring protein precursor (Class IIb)	4.42
flgJ	*lpg1224*	Peptidoglycan-hydrolyzing protein (Class IIb)	2.87
fleN	*lpg1783*	Regulator of flagellar synthesis (Class IIb)	3.01
flhF	*lpg1784*	Biosynthesis regulator GTP-binding protein (Class IIb)	3.87
Dot/Icm Structural Genes			
icmR	*lpg0443*	Chaperone for IcmQ; a pore forming molecule	2.46
icmL/dotI	*lpg0449*	Inner membrane protein	2.23
icmK/dotH	*lpg0450*	Might be an outer membrane channel	2.18
icmE/dotG	*lpg0451*	Major component of a channel	1.96
icmG/dotF	*lpg0452*	Interacts with substrates; predicted channel component	2.14
icmC/dotE	*lpg0453*	Predicted inner-membrane protein, Similar to DotV	2.31
icmD/dotP	*lpg0454*	Predicted inner-membrane protein	3.43
icmJ/dotN	*lpg0455*	Probable ATPase component	2.10
Dot/Icm Secreted Effectors			
legC3	*lpg1701*	Coiled-coil domain, disrupts vacuolar trafficking, IcmSW-dependent	2.11
ylfA	*lpg2298*	Coiled-coil domain, disrupts vacuolar trafficking,	3.33
sdeD	*lpg2509*	Substrate of the Dot/Icm system	1.98
lepA	*lpg2793*	SNAREs and coiled-coil domain; bacterial egress	3.99
Regulatory Factors			
lpg0433	*lpg0433*	Putative transcriptional regulator	1.99
letE	*lpg0537*	Transmission trait enhancer protein	3.60
rpoS	*lpg1284*	RNA polymerase sigma factor (sigma-38)	3.11
relA	*lpg1457*	ppGpp synthetase	16.16
lpg2732	*lpg2732*	Putative response regulator	2.28
rsmZ		non-coding, CsrA binding, regulatory RNA	4.18
Known Virulence Factors			
mip	*lpg0789*	Macrophage infectivity potentiator	2.06
katB	*lpg2389*	Catalase-peroxidase	3.03
enhC	*lpg2639*	Enhanced entry protein	2.73
Fatty Acid / Carbon Metabolism			
plsC	*lpg0551*	similar to 1-acyl-sn-glycerol-3-phosphate acyltransferase	2.51
phaB1	*lpg0560*	Similar to acetoacetyl-CoA reductase	2.09
yfcX	*lpg1596*	similar to alpha subunit of fatty-acid oxidation complex	3.25
phbC3	*lpg2260*	Similar to poly(3-hydroxyalkanoate) synthetase	2.25
pta	*lpg2261*	Similar to phosphotransacetylase	2.12
pksJ	*lpg2186*	Similar to polyketide synthase of type I	2.17
lpg2837	*lpg2837*	phospholipase/lecithinase/hemolysin, lysophospholipase A	6.13

The data were collected from two independent biological replicates. See *Fig. 3.10* for qRT-PCR validation. $p < 0.01$ for all targets shown.

Table 3.2. Flagellar gene expression of PE phase *dksA* mutant *L. pneumophila*.

Gene Name	Gene.ID	Annotation	*dksA*/WT
Class I			
rpoN	*lpg0477*	RNA polymerase sigma factor (sigma-54)	-2.17
fleQ	*lpg0853*	Master regulator of flagellar gene transcription	---
Class IIa			
fliJ	*lpg1756*	Export and assembly	---
fliI	*lpg1757*	Flagellum-specific ATP synthase	---
fliH	*lpg1758*	Export and assembly	---
fliG	*lpg1759*	Motor switch protein	---
fliF	*lpg1760*	M-ring protein	-2.17
fliE	*lpg1761*	Hook-basal body complex protein	---
fleR	*lpg1762*	Two-component response regulator	---
fleS	*lpg1763*	Two-component sensor histidine kinase	---
flhA	*lpg1785*	Export and assembly	---
flhB	*lpg1786*	Export and assembly	---
fliR	*lpg1787*	Export and assembly	---
fliQ	*lpg1788*	Export and assembly	---
fliP	*lpg1789*	Export and assembly	---
fliO	*lpg1790*	Export and assembly	---
fliN	*lpg1791*	Motor switch protein	-2.85
fliM	*lpg1792*	Motor switch protein	---
Class IIb			
flgA	*lpg0908*	P-ring biosynthesis	---
flgB	*lpg1216*	Proximal rod protein	-2.38
flgC	*lpg1217*	Proximal rod protein	-4.55
flgD	*lpg1218*	Rod modification protein	-4.00
flgE	*lpg1219*	Flagellar hook protein	-11.11
flgF	*lpg1220*	Proximal rod protein	-4.34
flgG	*lpg1221*	Distal rod protein	---
flgH	*lpg1222*	L-ring protein precursor	---
flgI	*lpg1223*	P-ring protein precursor	---
flgJ	*lpg1224*	Peptidoglycan-hydrolyzing protein	---
flgK	*lpg1225*	Hook-associated protein	-2.70
*flgL**	*lpg1226*	Flagellar hook-associated protein	-12.50
fliK	*lpg1688*	Hook-length control protein	-3.57
fleN	*lpg1783*	Regulator of flagellar synthesis	-7.14
flhF	*lpg1784*	Biosynthesis regulator GTP-binding protein	-6.25
Class III			
*flgN**	*lpg0906*	Potential chaperone	-8.33
*flgM**	*lpg0907*	Anti-sigma-28 factor	-20.00
*motB**	*lpg1780*	Sodium-type motor protein	-16.67
*motA**	*lpg1781*	Sodium-type motor protein	-20.00
fliA	*lpg1782*	RNA polymerase sigma factor (sigma-28)	-20.00
motA2	*lpg2318*	Proton conductor component of motor	-10.00
motB2	*lpg2319*	Motor protein	-16.67
*flhB'**	*lpg2583*	Unknown	-10.00
Class IV			
*fliS**	*lpg1337*	Potential chaperone	-20.00
*fliD**	*lpg1338*	Filament cap	-25.00
*flaG**	*lpg1339*	Unknown	-33.33
*flaA**	*lpg1340*	Flagellin	-50.00
*motY**	*lpg2962*	Sodium-type motor protein	-7.14

The data were collected from two independent biological replicates. See *Fig. 3.11* for qRT-PCR validation. $p < 0.01$ for all targets shown. * indicates a gene regulated by *fliA* (σ^{28}) (Albert-Weissenberger et al.)

Table 3.3. Bacterial strains and plasmids

Strain	Relevant genotype/phenotype	Reference
E.coli		
DH5α	F-endA1 *hsdR17* (r- m+) *supE44 thi-1 recA1 gyrA* (Nal^r) *relA1* Δ(*lacZYA-argF*)$_{U169}$Φ80dLacZΔM15λ*pir*RK6	Laboratory collection
DY330	W3110 Δ*lacU169 gal490 λcl1857* Δ(*cro-bioA*)	(Yu et al., 2000)
L. pneumophila		
MB110	Lp02 wild-type, Str^R, Thy-, HsdR-	(Berger & Isberg, 1993)
MB699	Lp02 Δ*dksA*::FRT::*cat*::FRT mutant	This work
MB697	Lp02 *relA*::*gent spoT*::*kan* double mutant	(Dalebroux et al., 2009)
MB698	Lp02 p206-*gent*, vector control strain	This work
MB701	Lp02 Δ*dksA*::FRT::*cat*::FRT mutant p206-*gent*	This work
MB731	Lp02 Δ*dksA*::FRT::*cat*::FRT p*dksA*i-*gent*	This work
MB698	Lp02 p206-*cat*, vector control strain	(Dalebroux et al., 2009)
MB686	Lp02 *relA*::*gent spoT*::*kan* double mutant p206-*cat*	(Dalebroux et al., 2009)
MB687	Lp02 *relA*::*gent spoT*::*kan* double mutant p$relA^{L.p.}$	(Dalebroux et al., 2009)
MB700	Lp02 *relA*::*gent spoT*::*kan* double mutant p*dksA*i-*cat.*	This work
MB359	Lp02 p$relA^{E.c.}$	(Hammer & Swanson, 1999)
MB702	Lp02 Δ*dksA*::FRT::*cat*::FRT mutant p$relA^{E.c.}$	This work
MB355	Lp02 p*flaAgfp* td(Δ)I	(Hammer & Swanson, 1999)
MB732	Lp02 Δ*dksA*::FRT::*cat*::FRT mutant p*flaAgfp* td(Δ)I	This work
MB685	Lp02 *relA*::*gent spoT*::*kan* double mutant p*flaAgfp* td(Δ)I	(Dalebroux et al., 2009)
MB733	Lp02 p*fliAgfp* td(Δ)I	This work
MB735	Lp02 Δ*dksA*::FRT::*cat*::FRT mutant p*fliAgfp* td(Δ)I	This work
MB734	Lp02 *relA*::*gent spoT*::*kan* double mutant p*fliAgfp* td(Δ)I	This work
Plasmids		
pGEMT-Easy	MCS within coding region of B-lactamase α-fragment linearized with single-T overhangs, Amp^R	Promega
pGEM-Gent	pGEMT-Easy with 1.7 kb gentamycin cassette cloned into the MCS, source of 1.7 kb gentamycin resistance cassette, Amp^R, $Gent^R$	(Molofsky et al., 2005)
pKD3	Template plasmid for λ Red system, *bla* FRT::*cat*::FRT, *ori* R6K	(Datsenko & Wanner, 2000)
pGEM-*dksA*	pGEMT-Easy with 1.4 kb PCR amplified *dksA* chromosomal region ligated into T overhangs, Amp^R	This work
pGEM-Δ*dksA*::FRT::*cat*::FRT	pGEM-*dksA* with 1.1 kb FRT::*cat*::FRT cassette from pKD3 recombineered using strain DY330 to delete and replace the *dksA* coding sequence leaving ~500 bp homology on either side for Lp02 chromosomal recombination, Amp^R, Cam^R	This work
pGEM-*dksA*i	pGEMT-Easy with 540 bp PCR amplified *dksA* chromosomal region ligated into T overhangs, Amp^R	This work
p206-*cat*	pMMB66EH derivative, Δ*mob*, $lacI^q$,$P_{taclacUV5}$, Cam^R	(Morales et al., 1991)
p*dksA*i-*cat*	p206-*cat* with 540 bp *Sal*I/*Hin*dIII fragment from pGEM-*dksA*i ligated between the *Sal*I/*Hin*dIII sites in the MCS, collinear with the $P_{taclacUV5}$ promoter, $lacI^q$, Inducible *dksA* expression, Cam^R.	This work
p206-*gent*	p206-*cat* with the *cat* cassette replaced by the gentamycin resistance cassette from pGEM-Gent, $Gent^R$.	This work
p*dksA*i-*gent*	p206-*gent* with 540 bp *Sal*I/*Hin*dIII fragment from pGEM-*dksA*i ligated between the *Sal*I/*Hin*dIII sites in the MCS, collinear with the $P_{taclacUV5}$ promoter, $lacI^q$, Inducible *dksA* expression, $Gent^R$.	This work
p$relA^{E.c.}$	pMMB66EH derivative, $lacI^q$,$P_{taclacUV5}$, $Gent^R$ with a *relA* allele from *E. coli* encoding a truncated, metabolically active RelA, cloned into the MCS, collinear with the $P_{taclacUV5}$ promoter, $lacI^q$, Inducible $relA^{E.c.}$ expression, $Gent^R$.	(Hammer & Swanson, 1999)
p*flaAgfp*	150 bp *flaA* promoter fragment fused to GFPmut3 in pKB5 with P_{tac} and $lacI^q$ removed, td(Δ)i	(Hammer & Swanson, 1999)
p*fliAgfp*	304 bp *fliA* promoter fragment fused to GFPmut3 in pKB5 with P_{tac} and $lacI^q$ removed, td(Δ)i	This work

Table 3.4. Select list of transcripts down-regulated 90 min after relA$^{L.p.}$ induction.

Gene Name / Function	Gene.ID	Annotation	ppGpp^0 p*relA*$^{L.p.}$/p*empty*
ATP Synthesis			
atpC	*lpg2981*	Highly similar to H+transporting ATP synthase epsilon chain	-2.48
atpD	*lpg2983*	Highly similar to H+transporting ATP synthase beta chain	-2.54
atpG	*lpg2984*	Highly similar to H+transporting ATP synthase chain gamma	-3.00
atpA	*lpg2985*	Highly similar to H+transporting ATP synthase chain alpha	-2.67
atpH	*lpg2986*	Highly similar to H+transporting ATP synthase chain delta	-3.91
atpF	*lpg2987*	Highly similar to H+transporting ATP synthase chain b	-2.99
atpE	*lpg2988*	Highly similar to H+transporting ATP synthase chain c	-2.69
atpB	*lpg2989*	Highly similar to H+transporting ATP synthase chain a	-7.52
atpI	*lpg2990*	Highly similar to H+transporting ATP synthase subunit i	-6.54
Membrane Modification			
fabZ	*lpg0510*	(3R)-hydroxymyristoyl-[acyl carrier protein]dehydratase	-2.79
lpxA1	*lpg0511*	UDP-N-acetylglucosamine acyltransferase	-2.48
plsB	*lpg0551*	similar to 1-acyl-sn-glycerol-3-phosphate acyltransferase	-2.66
lag-1	*lpg0777*	O-acetyltransferase	-4.90
lpg1873	*lpg1873*	similar to membrane-bound lytic murein transglycosylase B precursor	-2.27
MFS Transporters			
phtE	*lpg0652*	Similar to major facilitator family transporter	-2.07
lpg2501	*lpg2501*	similar to multidrug resistance protein- MFS superfamily	-2.59
Protein Translocation			
secE	*lpg0316*	Preprotein translocase subunit	-2.27
secF	*lpg2000*	Preprotein translocase subunit	-2.17
secD	*lpg2001*	Preprotein translocase subunit	-2.55
Heat Shock Response			
hslV	*lpg0640*	Peptidase component of the HslUV protease	-2.96
hslU	*lpg0641*	ATP-dependent protease ATP-binding subunit	-2.96
dnaJ	*lpg2024*	Chaperone protein	-2.35
dnaK	*lpg2025*	Chaperone protein (HSP-7)	-2.37
grpE	*lpg2026*	Heat-shock protein (HSP-7 cofactor)	-3.04
Other Factors			
nusG	*lpg0317*	transcription antitermination protein	-2.11
mreB	*lpg0811*	Rod shape-determining protein	-3.29
comL	*lpg1186*	Similar to competence lipoprotein	-3.41
lpg1593	*lpg1593*	Similar to carbon storage regulator CsrA	-2.20
nusA	*lpg2773*	Transcription elongation protein	-2.18
yigC	*lpg2933*	oxidoreductase, 3-octaprenyl-4-hydroxybenzoate carboxy-lyase	-2.02
rho	*lpg2934*	transcription termination factor	-2.05
Translation			
rplk	*lpg0318*	50S ribosomal protein L11	-2.78
rplA	*lpg0319*	50S ribosomal protein L1	-2.43
rplL	*lpg0321*	50S ribosomal subunit protein L7/L12	-2.52
rpsL	*lpg0324*	30S ribosomal protein S12	-2.18
rpsJ	*lpg0328*	30S ribosomal subunit protein S1	-2.16
rplC	*lpg0329*	50S ribosomal subunit protein L3	-2.19
rplD	*lpg0330*	50S ribosomal subunit protein L4	-2.42
rplW	*lpg0331*	50S ribosomal subunit protein L23	-2.46
rpsS	*lpg0333*	30S ribosomal subunit protein S19	-2.18
rpsC	*lpg0335*	30S ribosomal protein S3	-1.97
rplP	*lpg0336*	50S ribosomal protein L16	-2.30
rpmC	*lpg0337*	50S ribosomal subunit protein L29	-2.23
rpsQ	*lpg0338*	30S ribosomal protein S17	-2.10
rplN	*lpg0339*	50S ribosomal protein L14	-2.67
rplX	*lpg0340*	50S ribosomal protein L24	-2.25
rplR	*lpg0341*	50S ribosomal subunit protein L18	-2.27
lpg0342	*lpg0342*	similar to methylated-DNA-protein-cysteine S-methyltransferase	-3.13
rplS	*lpg0343*	50S ribosomal protein L19	-3.02
trmD	*lpg0344*	Highly similar to tRNA (guanine-N1)-methyltransferase	-3.57
rimM	*lpg0397*	similar to 16S rRNA processing protein RimM	-3.27
rpsP	*lpg0398*	Highly similar to 30S ribosomal protein S16	-2.40
rpL33	*lpg0478*	50S ribosomal subunit protein L33	-3.91
rpmB	*lpg0479*	50S ribosomal protein L28	-4.44
lpg0607	*lpg0607*	similar to putative lysyl-tRNA synthetase	-2.27
ppt	*lpg1519*	putative pyrimidine phosphoribosyl transferase	-2.39
rpsR	*lpg1591*	30S ribosomal subunit protein S18	-2.93
rpsF	*lpg1592*	30S ribosomal protein S6	-3.88
pyrH	*lpg1712*	Uridylate kinase (UK) (Uridine monophosphate kinase)	-2.59

tsf	*lpg1713*	Elongation factor Ts (EF-Ts)	-2.88
rpsB	*lpg1714*	30S ribosomal protein S2	-3.21
rpsA	*lpg1740*	30S ribosomal protein S1	-1.98
rpsU	*lpg2358*	30S ribosomal protein S21	-2.28
lpg2593	*lpg2593*	similar to rRNA methylase (sun protein)	-2.26
rpsT	*lpg2636*	30S ribosomal subunit protein S2	-1.97
rplU	*lpg2651*	50S ribosomal protein L21	-5.71
pth	*lpg2653*	similar to peptidyl-tRNA hydrolase	-4.35
rpsI	*lpg2706*	30S ribosomal subunit protein S9	-2.69
ppiB	*lpg2726*	Peptidyl-prolyl cis-trans isomerase B	-3.18
tgt	*lpg2727*	Similar to queuine tRNA-ribosyltransferase	-3.25
rpsO	*lpg2769*	30S ribosomal protein S15	-5.29
truB	*lpg2770*	tRNA pseudouridine synthase B	-2.24
rbfA	*lpg2771*	Ribosome-binding factor A	-1.97
metG	*lpg2882*	methionyl-tRNA synthetase	-1.97
gidA	*lpg2889*	Highly similar to glucose-inhibited division protein A GidA,	-2.81
thdF	*lpg3001*	Similar to GTPase for tRNA modification trmE	-2.92
rpmH	*lpg3005*	50S ribosomal protein L34	-2.99

The data were collected from two independent biological replicates. See *Fig. 3.10* for qRT-PCR validation. $p < 0.01$ for all targets shown.

Table 3.5. List of primers

Primers for Cloning	Sequence	Amplicon Size
dksA1 fwd.	5'-AATCTCCCCCTAAAACAATACCAC -3'	
dksA2 rvs.	5'-CTCGCAGCAGGGACTAAATCT -3'	1.4 kb
dksAi1 fwd.	5'-GTCGACGAATGAAATATAGGGTCT-3'	
dksAi2 rvs.	5'-AAGCTTTGTTTAATGTTCAGAGGC-3'	540 bp
dksA-pKD3a fwd.	5'-TGTATAAAACTCAGTTTCAGATACAGAA TGAAATGTGTAGGCTGGAGCTGC-3'	
dksA-pKD3b rvs.	5'-GGAATTTATTGTTTAATGTTCAGAGGCA AGAAAGAACATATGAATATCCTCCTTAGTTCC-3'	1.1 kb
fliAP1 fwd.	5'-GGATCCATGTGCAGTTAGATTACCT-3'	
fliAP2 rvs.	5'-TCTAGACAGCAGATTGTAGTTATTAGT-3'	304 bp

Real-time PCR Primers	Sequence
letE1 fwd.	5'-AGCAGAACAATGGCTCGAAGGATG-3'
letE2 rvs.	5'-CGGCTATCGCACCACCAATTTCAA-3'
rpoS1 fwd.	5'-TCCTGCAGAGCTGCTAACCAATGA-3'
rpoS2 rvs.	5'-TACCAACGTCTTCCAGTGTCGCTT-3'
flgB1 fwd.	5'-GCCAAGGCGTTGATTGCAAGAGAT-3'
flgB2 rvs.	5'-TTACCGGCCATAGTTGCTGTCAGT-3'
ndk1 fwd.	5'-TTATGGGCGCGACAAATCCCAAAG-3'
ndk2 rvs.	5'-GCTGTCAGAACCATGCACAGCATT-3'
lag-1a fwd.	5'-TGGTCAATAGCAGTTGAGTGGTGG-3'
lag-1b rvs.	5'-ACCCAGAACCCAGAAACCATACCA-3'
lpg0260a fwd.	5'-TATAACGGGCTTACAGGCTTGGCA-3'
lpg0260b rvs.	5'-AGCAGTCAGCGCGTGAATAGAGAT-3'
lpg2096a fwd.	5'-AGCAATTGGGAGCGAGGTGATAGA-3'
lpg2096b rvs.	5'-AGGCTTGTTGATGATCGAGCAGTG-3'
fliA1 fwd.	5'-TCCTTAGCCTCACGCCCAAGTTTA-3'
fliA2 rvs.	5'-ATTGGGTGCCGCGTTCTGTTTATC-3'
flaA1 fwd.	5'-ATGACTGCACAAATTCGCGGGATG-3'
flaA2 rvs.	5'-TGT-TTCCTGCATTGCTCCTTCAGC-3'
fliD1 fwd.	5'-AGCGCCGACAACTCTGTATTGACA-3'
fliD2 rvs.	5'-TCGATGCGATAGTCACGGCAGAAA-3'

Figure 3.1.

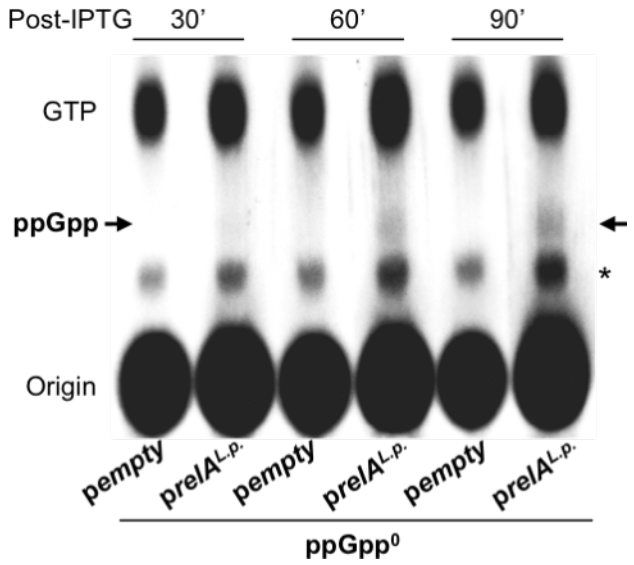

Figure 3.1. Kinetics of ppGpp accumulation after p*relA*$^{L.p.}$ induction.
To evaluate the timing of ppGpp accumulation after *relA*$^{L.p.}$ induction, early-E phase AYET broth cultures of ppGpp^0 p*empty* and ppGpp^0 p*relA*$^{L.p.}$ labeled with ^{32}P phosphoric acid were treated with 500 μM IPTG for the periods indicated, then nucleotides in cell extracts were separated by PEI-TLC. An arrow indicates the major ppGpp species, guanosine tetraphosphate; an asterisk denotes the position of guanosine pentaphosphate and an unidentified phosphorylated species. The autoradiogram shown represents one of two independent experiments.

Figure 3.2.

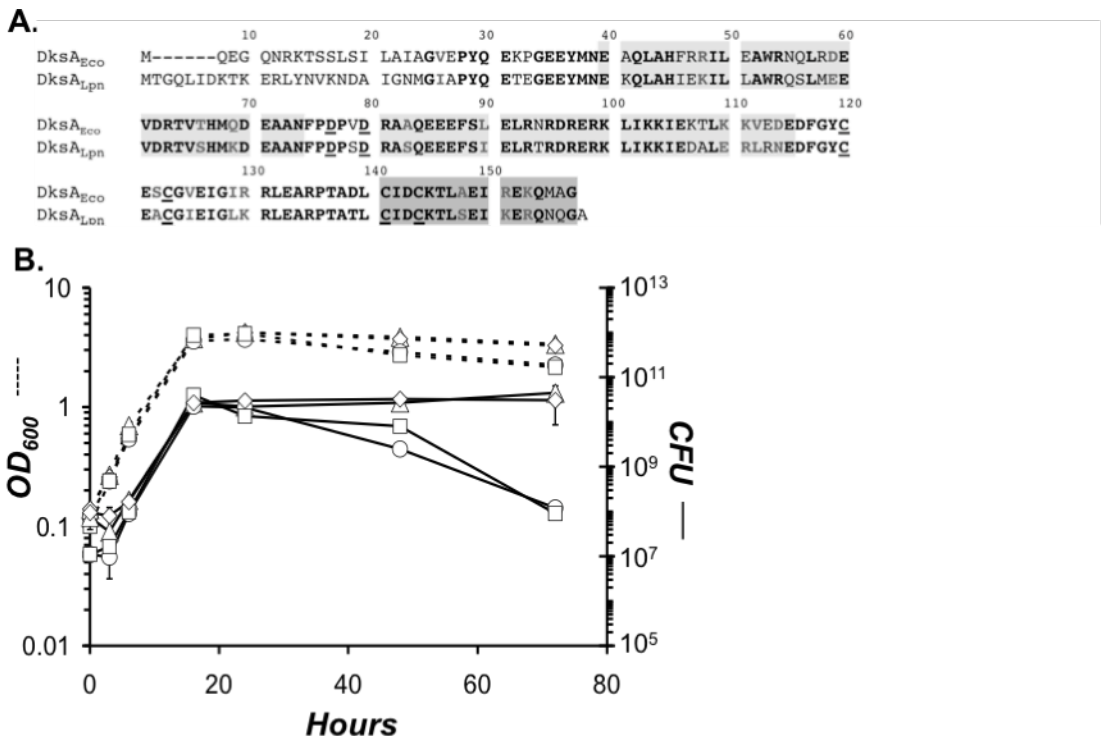

Figure 3.2. To survive stationary phase stress, *L. pneumophila* requires DksA and ppGpp.

A. *L. pneumophila* encodes a 158 amino acid protein that is similar to the 151 amino acid DksA protein of *E. coli* K-12. Residues in bold, black lettering are identical; those in bold and grey are conserved. The underlined aspartate residues comprise the acidic tip, conserved in DksA and other RNAP secondary channel interacting proteins, while the underlined cysteine residues comprise a zinc finger motif. Light grey shading denotes two α-helical domains sandwiching the acidic tip that adopt a coiled-coil structure in the *E. coli* protein (Perederina et al., 2004). Dark grey shading indicates a C-terminal α-helix. **B.** Early-E phase AYET broth cultures of WT (triangles) and ppGpp^0 (circles) carrying empty vector, and *dksA* mutants transformed with empty vector (squares) or p*dksA* (diamonds), were diluted to OD_{600} of 0.15, and bacteria were treated with 25 μM IPTG. At the times indicated, culture density and viability were quantified by reading OD_{600} (dashed lines) and enumerating colony forming units (CFU ± SE) ml^{-1} from duplicate samples on CYET (solid lines). The data represent one of three independent experiments.

Figure 3.3.

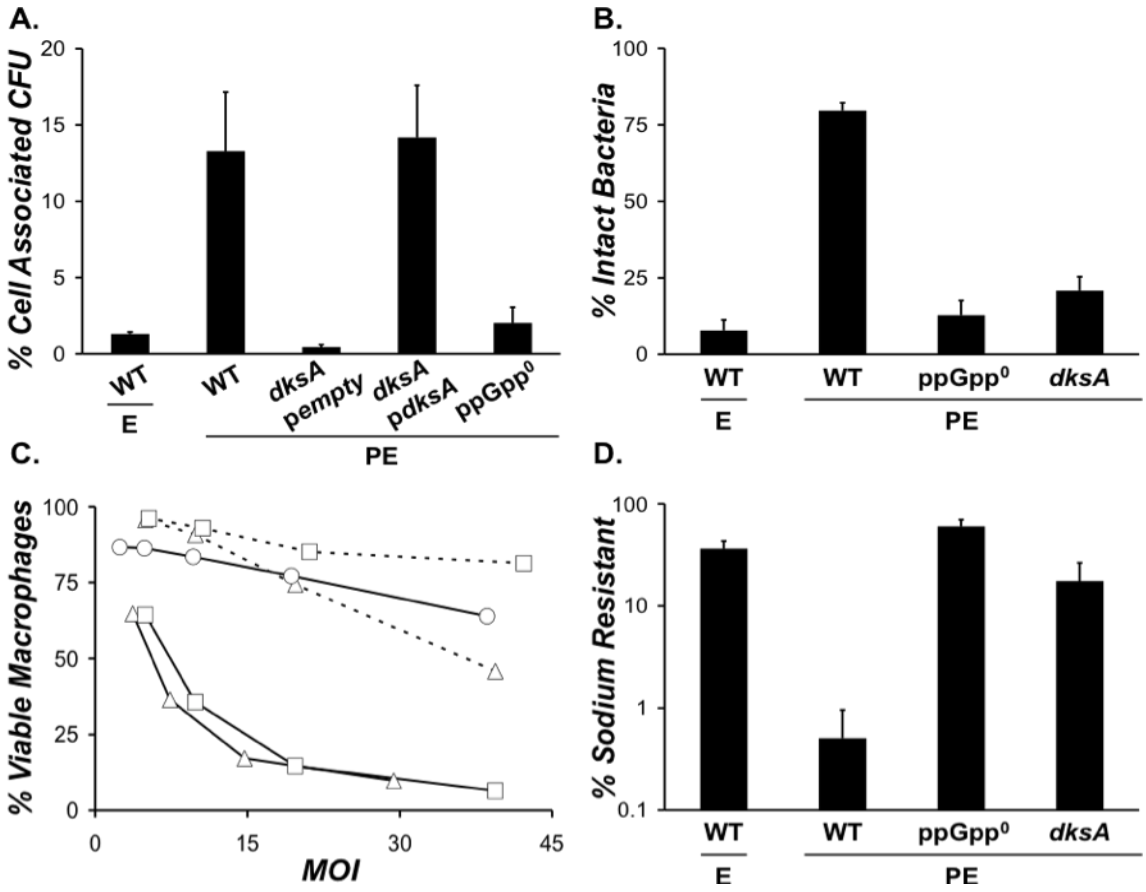

Figure 3.3. To activate PE phase transmission traits, *L. pneumophila* requires DksA and ppGpp.

A. To analyze whether DksA contributes to infectivity of PE phase *L. pneumophila*, macrophages were infected at an MOI of ~ 1 for 2 h with WT p*empty*, ppGpp^0 p*empty*, *dksA* p*empty*, or *dksA* p*dksA* cultured to the growth phase shown. Bacteria carrying plasmids were cultured from early-E phase to PE phase with 25 μM IPTG. Graphed are the mean percent of cell-associated CFU ± SE from duplicate wells in one of two independent experiments. **B.** The ability of bacteria to resist degradation in macrophage lysosomes was quantified using fluorescence microscopy by scoring the percent of intracellular bacteria that were intact at 2 h post-infection. Shown are the mean percentages from duplicate coverslips ± SE from three independent experiments. **C.** To determine the contribution of DksA to *L. pneumophila* cytotoxicity to macrophages, mid-E phase WT p*empty* (triangles, dashed lines), PE phase WT p*empty* (triangles, solid lines), PE phase ppGpp^0 p*empty* (circles, solid lines), PE phase *dksA* p*empty* (squares, dashed lines), or PE phase *dksA* p*dksA* (squares, solid lines) bacteria, cultured with IPTG as described in A, were added to triplicate wells of macrophages at the MOI shown. The values plotted represent the mean ± SE for triplicate samples determined in one of three similar experiments. **D.** To measure sodium resistance, E or PE phase bacteria of the strains depicted were plated onto medium with or without 100 mM NaCl. Shown are the mean percentages from duplicate samples ± SE from three independent experiments.

Figure 3.4.

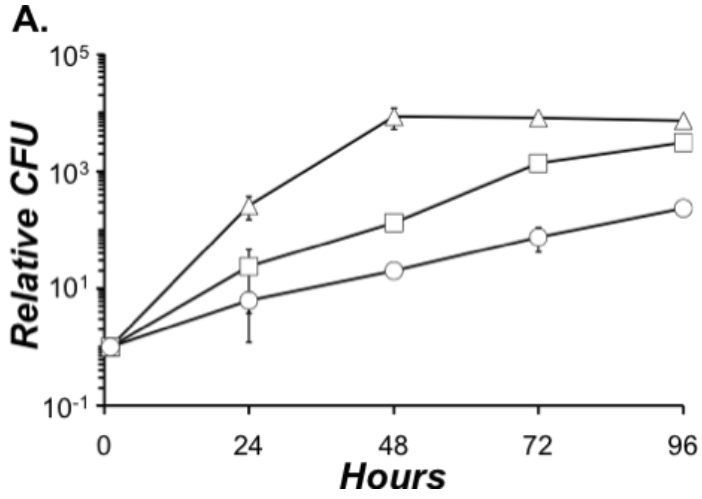

Figure 3.4. For optimal growth in *A. castellanii*, *L. pneumophila* requires both DksA and ppGpp.
Thy$^+$ derivatives of WT (triangles), *dksA* (squares) and ppGpp^0 (circles) bacteria were cultured to PE phase, then added to amoebae in infection buffer at an MOI of ~ 0.05. At the times shown aliquots were suspended and lysed, and lysates plated for CFU enumeration. To obtain relative CFU, viable counts for 24, 48, 72, and 96 were divided by the 1 h CFU value for each strain. Depicted are the relative CFU values from duplicate infections ± SE. The data represent one of two independent experiments.

Figure 3.5.

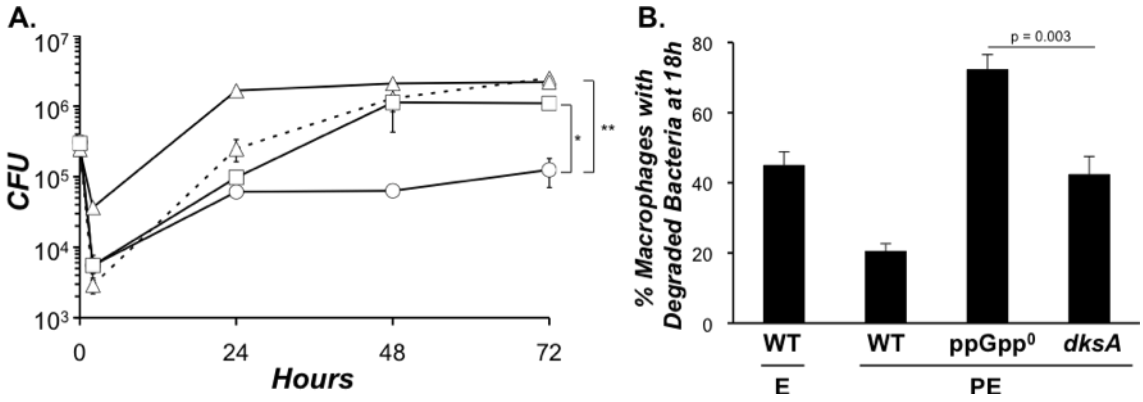

Figure 3.5. ***L. pneumophila*** **requires ppGpp for macrophage transmission; DksA is less critical.**
A. Macrophages were infected at an MOI of ~ 1 with E phase WT (triangles, dashed lines), PE phase WT (triangles, solid line), PE phase ppGpp^0 (circles), or PE phase *dksA* (squares) bacteria. The number of viable bacteria was determined at the time points shown. Depicted are the mean CFU ± SE from duplicate samples in one of four independent experiments. * Indicates that the difference in the mean CFU values calculated from four independent experiments for WT and ppGpp^0 mutant bacteria at 72 h was statistically significant by a paired, two-tailed Student *t* test (p = 0.047). ** Indicates that the difference between ppGpp^0 and *dksA* mutant bacteria at 72 h was also statistically significant (p = 0.047). **B.** Macrophages were infected at an MOI of ~ 1 with PE phase WT or an MOI ~ 3 of E phase WT, PE phase ppGpp^0 and PE phase *dksA* mutant bacteria, then at 18 h coverslips were fixed. A total of 100 infected macrophages from duplicate coverslips were scored as follows: macrophages with a single intact or a single degraded bacterium, and macrophages with multiple intact or multiple degraded bacteria. Shown is the mean percent ± SE of macrophages with degraded bacteria at 18 h from three independent experiments.

Figure 3.6.

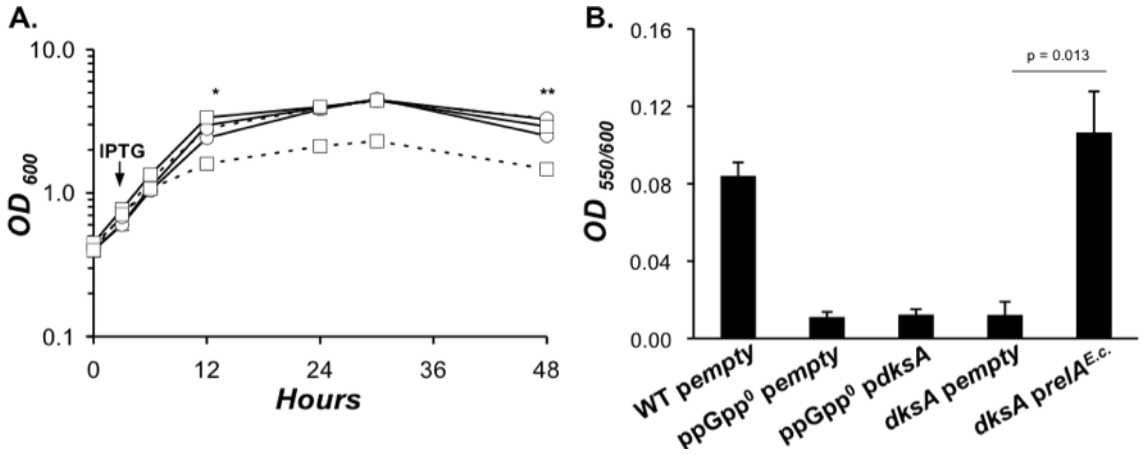

Figure 3.6. Constitutive ppGpp synthesis is sufficient to bypass the *dksA* requirement for cell cycle arrest and pigment production.
A. To test the effect of either *dksA* induction or constitutive ppGpp synthesis on the growth of *L. pneumophila* mutant bacteria, WT p*empty* (triangles), ppGpp^0 transformed with either p*empty* (circles, solid lines), or p*dksA* (circles, dashed lines), and *dksA* mutants carrying either p*empty* (squares, solid lines), or p*relA*$^{E.c.}$ (squares, dashed lines), a truncated and constitutively active form of *E. coli* RelA, were induced with IPTG in early-E phase AYET broth cultures, then culture density was monitored by reading OD_{600} over the time period shown. **B.** At 48 h post-IPTG (indicated in A by **), the OD_{550} of the supernatants was read to quantify extracellular pigment production. To account for differences in cell density, pigmentation values were normalized to culture density by dividing OD_{550} by OD_{600}. The graph depicts the mean OD_{550}/OD_{600} values ± SE from three independent experiments.

Figure 3.7.

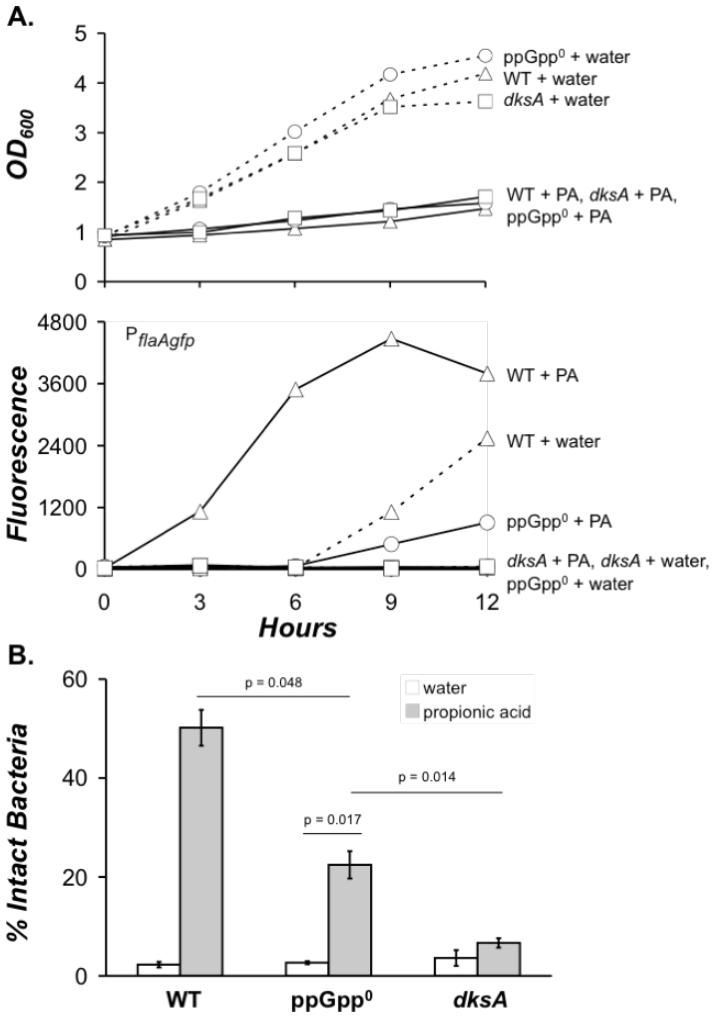

Figure 3.7. In response to fatty acid stress, DksA activates transmission traits with and independently of ppGpp.

A. To determine whether DksA is required for *flaA* promoter activation in response to excess propionic acid (PA), mid-E phase bacteria harboring the p*flaAgfp* reporter plasmid were normalized to an OD_{600} (0.75-0.85), then 10 mM (PA) (solid lines), or water (dashed lines) were added to WT (triangles), $ppGpp^0$ (circles), or *dksA* (squares) bacteria. At 3 h time intervals, cell density (top panel) and *flaA* promoter activity was quantified by measuring green fluorescent protein (gfp) accumulation using fluorometry. The data depicted represent one of three independent experiments. **B.** To determine if DksA contributes to increased resistance to degradation by macrophages after PA treatment, PA (light gray), or water (white) was added to cultures as described in A. After 3 h, bacteria were used to infect macrophages, and resistance to lysosomal degradation was scored by fluorescence microscopy. Shown are the mean percentages from triplicate coverslips ± SE from three independent experiments. In addition to the p values depicted, the mean percent resistance values for WT PA and *dksA* PA were statistically different by a two-tailed paired Student t-test (p = 0.005).

Figure 3.8.

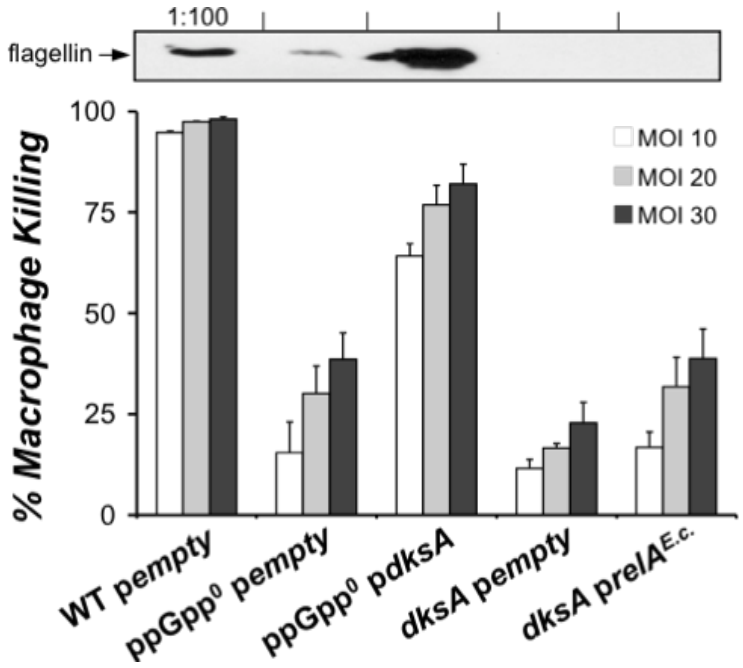

Figure 3.8. Induction of *dksA* is sufficient to bypass the requirement for ppGpp for flagellin-dependent phenotypes.
In the strains subjected to the induction described in Figure 3.6A, we assessed levels of flagellin and macrophage cytotoxicity. At 9 h post-IPTG (indicated in Fig. 3.6A by *), bacterial cell pellets were harvested, and levels of cell-associated flagellin were assessed by Western analysis (upper image). At 9 h post-IPTG, bacteria were also added to triplicate wells of macrophages at the MOIs shown (bottom panel), and cytotoxicity was measured. The values plotted represent the mean percent macrophage killing ± SE for triplicate samples determined in one of three similar experiments.

Figure 3.9.

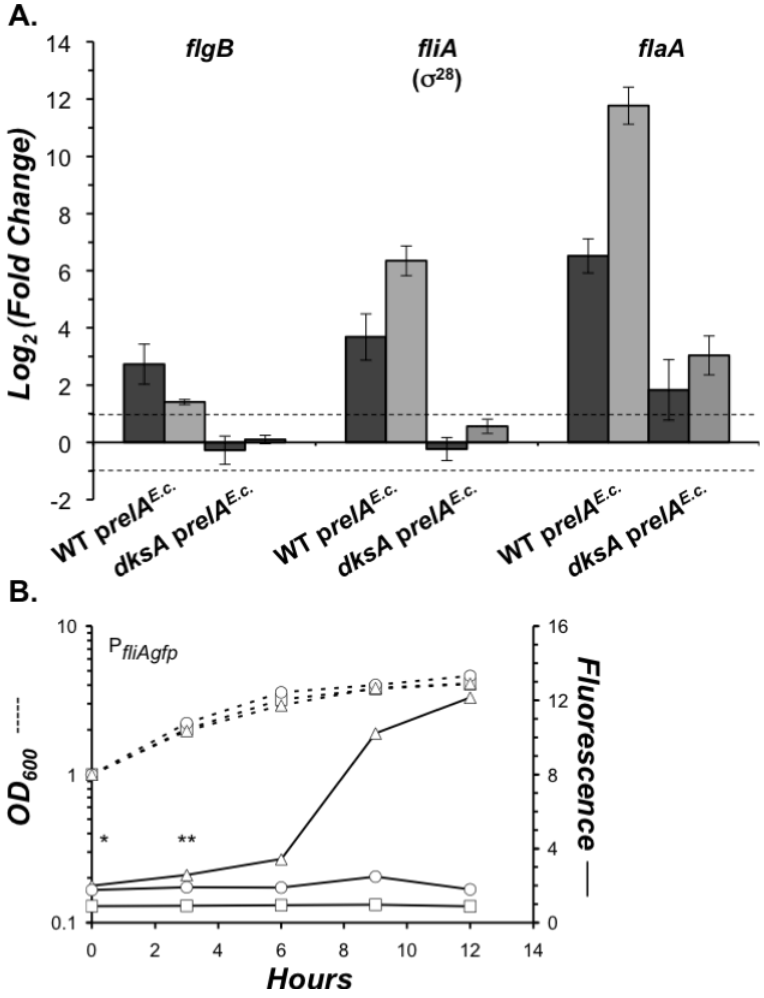

Figure 3.9. ***L. pneumophila*** **uses ppGpp to modulate DksA-dependent gene expression.**

A. To assess the contribution of DksA to gene expression by ppGpp, qRT-PCR was performed on transcripts isolated from mid-E phase AYET broth cultures of WT and *dksA* mutant bacteria induced to express p*relA*$^{E.c.}$ with 500 μM IPTG. RNA was harvested at 0, 90 and 300 minutes post-IPTG, and the relative transcript levels were assessed by dividing 90 min (dark grey) and 300 min (light grey) values by the 0 min value to give the fold increase for each target. Depicted are the mean log2 (fold increase) values from triplicate wells in three independent experiments ± SE. Dashed lines delineate the two-fold change cutoff. **B.** To monitor *fliA* promoter activity at the E to PE phase transition, mid-E phase WT (triangles), ppGpp^0 (circles), or *dksA* (squares) bacteria harboring the p*fliAgfp* reporter plasmid were diluted to an OD_{600} of 1.0. Cell density (dashed lines) and *fliA* promoter activity (solid lines) were quantified until bacteria entered stationary phase. Actual fluorescence values were 1000X greater than those shown. The data represent one of three independent experiments, and the differences between WT and *dksA*, and ppGpp^0 and *dksA* at 0* and 3 h** were statistically significant by a two-tailed paired Student t-test (p = 0.01*, 0.04** and 0.03*, 0.006**).

Figure 3.10.

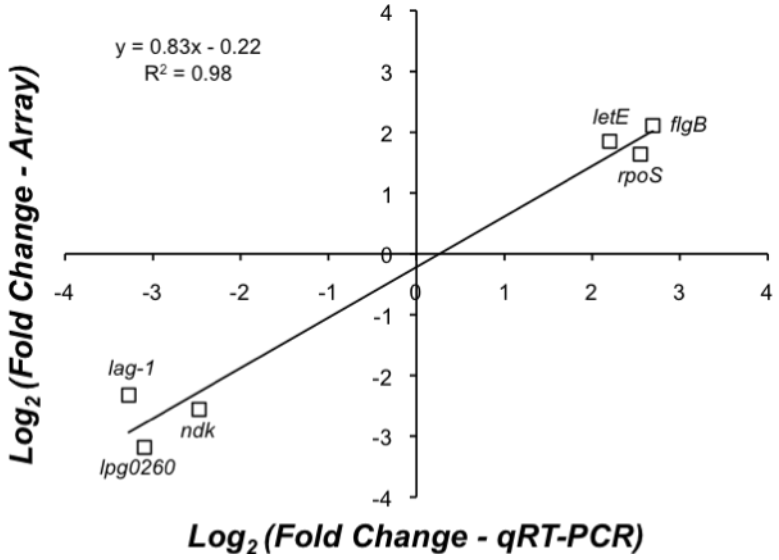

Figure 3.10. qRT-PCR validation of the *relA*$^{L.p.}$ induction microarray data set. *L. pneumophila* ppGpp^0 mutant bacteria carrying either p*empty* or p*relA*$^{L.p.}$ were grown in AYET to E phase (OD_{600} = 1.4) then treated with 500 μM IPTG. Cells were harvested for RNA isolation at 90 min post-IPTG and qRT-PCR was performed using primers listed in *Supplemental Table 3.2*. The experiment was performed in duplicate and the mean relative transcript level (p*relA*$^{L.p.}$/p*empty*) was calculated for six targets; *letE*, *rpoS*, *flgB*, *ndk*, *lag-1* and *lpg0260*. Mean values from both the qRT-PCR and microarray experiments were log2-transformed, reported as fold change and directly compared.

Figure 3.11.

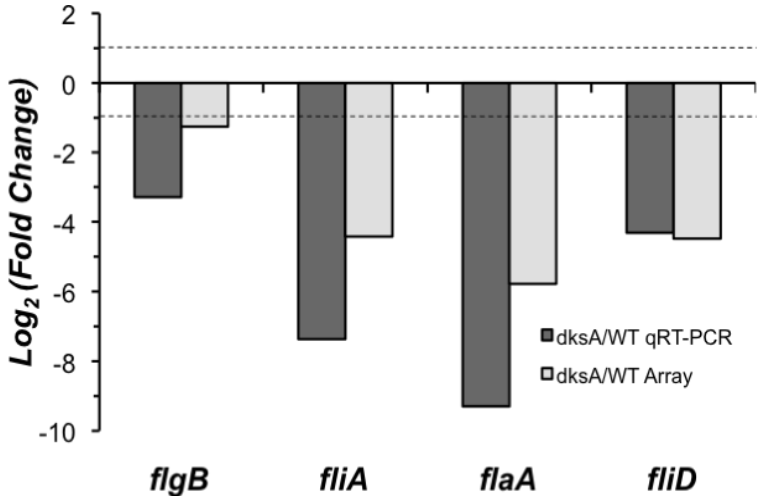

Figure 3.11. qRT-PCR validation of the PE phase *dksA*/WT microarray data set. Cultures of WT and *dksA* mutant *L. pneumophila* were grown synchronously in AYET to PE phase until 95-100% of the WT culture was motile. Cells were harvested for RNA isolation and qRT-PCR was performed using primers listed in *Supplemental Table 3.2*. The experiment was performed in duplicate and the mean relative transcript level (*dksA*/WT) was calculated for four flagellar transcripts; *flgB*, *fliA*, *flaA*, *and fliD*. Mean values from both the qRT-PCR and microarray experiments were log2-transformed, reported as fold change and plotted alongside one another. Dashed lines delineate the two-fold change cutoff.

Figure 3.12.

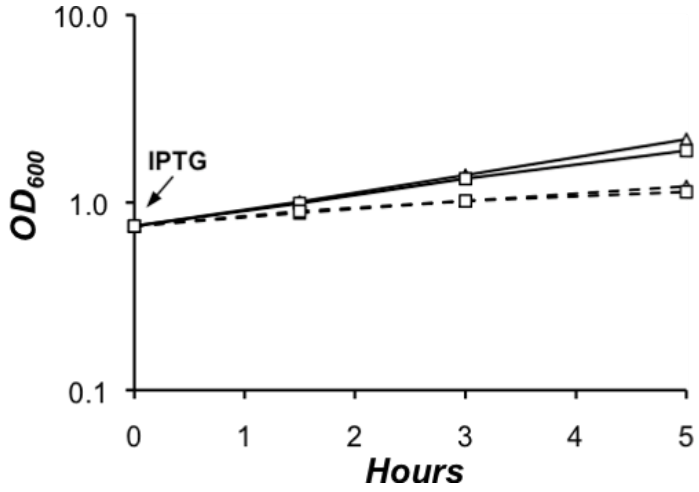

Figure 3.12. Growth of WT and *dksA* mutant *L. pneumophila* induced to express p*relA*$^{E.c.}$.

As an indirect measure of the amount of ppGpp produced after induction of the constitutive ppGpp synthetase, *relA*$^{E.c.}$, we monitored the growth response of WT and *dksA* mutant bacteria. Culturing conditions were similar to those described in *Figure 3.9A*. OD_{600} readings of WT p*empty* (solid lines, triangles), WT p*relA*$^{E.c.}$ (dashed lines, triangles), *dksA* p*empty* (solid lines, squares), and *dksA* p*relA*$^{E.c.}$ (dashed lines, squares) were taken at 0, 1.5, 3 and 5 h post-IPTG. The slopes of the curves were calculated between 0 and 5 h post-IPTG: WT p*empty* = 0.28 h^{-1}, *dksA* p*empty* = 0.23 h^{-1}, WT p*relA*$^{E.c}$ = 0.09 h^{-1}, and *dksA* p*relA*$^{E.c.}$ = 0.08 h^{-1}.

CHAPTER IV

ppGpp activates two regulatory RNAs to relieve CsrA-mediated repression of *Legionella pneumophila* flagellar biogenesis.

Summary

In host cells, *Legionella pneumophila* cycles between at least two virulence programs, the replicative and the transmissive. To control bacterial differentiation, *L. pneumophila* employs the ppGpp nucleotide and the RNA-binding protein CsrA. Flagellar mediated motility and resistance to lysosomal degradation, two hallmark virulence traits of transmissive *L. pneumophila*, are activated by ppGpp and repressed by CsrA. Here, we demonstrate that by activating two CsrA-binding regulatory RNAs, RsmY and RsmZ, ppGpp promotes derepression by CsrA. Activation of *rsmZ* occurs at the level of transcription initiation and involves the LetA response regulator, the RpoS (σ^{38}) sigma factor and DksA. The regulatory RNAs control *L. pneumophila* flagellar biosynthesis, since *rsmY rsmZ* double mutant bacteria are amotile and fail to activate the gene encoding the flagellar sigma factor, FliA (σ^{28}). RsmY and RsmZ likely control the ability of CsrA to regulate a *fliA* gene activator, since *csrA* induction reduces the total amount of *fliA* mRNA, but does not decrease *fliA* mRNA stability. Activation of RsmY/Z by ppGpp alleviates CsrA repression of *L. pneumophila* flagellar biosynthesis.

Introduction

To maximize their fitness in host environments, pathogenic bacteria exert tight control over virulence mechanisms. Pathogens process internal and external cues using complex signal transduction networks to fine-tune transcription, translation and activity of specialized virulence factors. For several pathogens, these specialized networks have been integrated into general stress response signaling pathways. Hence, the physiological status of the cell can dictate virulence expression.

The Gram-negative intravacuolar pathogen *Legionella pneumophila* monitors local cues to balance the pool of guanosine tetra-phosphate (ppGpp). Synthesis of ppGpp initiates differentiation of *L. pneumophila* to the transmissive state, a form equipped to transit between macrophages (Molofsky & Swanson, 2004, Dalebroux et al., 2009, Dalebroux *et al.*, 2010). Consistent with behavior of intracellular bacteria, synthesis of ppGpp by exponential (E), replicative phase broth-grown *L. pneumophila* triggers differentiation to the post-exponential (PE), transmissive form (Hammer & Swanson, 1999, Dalebroux et al., 2009, Dalebroux et al., 2010). In host cells, alarmone accumulation promotes increased resilience of bacteria during periods of uptake and egress (Dalebroux et al., 2009, Dalebroux et al., 2010). Hydrolysis of ppGpp is also critical for intracellular growth. Low levels of ppGpp permit activation factors needed for replication and repression of those critical for host-to-host transmission (Dalebroux et al., 2009). Therefore, ppGpp influences both replicative and transmissive *L. pneumophila* virulence programs in macrophages.

In most γ-proteobacteria, ppGpp modulates RNA polymerase (RNAP) to control gene transcription. Specifically, ppGpp interacts with RNAP and the RNAP secondary channel interacting protein DksA to control transcription initiation (Potrykus & Cashel,

2008, Haugen et al., 2008). Gene targets of ppGpp and DksA can be activated or deactivated depending on the promoter, and their control can be either cooperative, or independent (Lyzen et al., 2009, Merrikh et al., 2009b). How ppGpp and DksA act independently and even oppositely at particular promoters is poorly understood.

Independent regulation may be mediated by physical interactions between ppGpp and factors other than RNAP (Buglino et al., 2002, Wang et al., 2007, Persky et al., 2009). Recently, the Gram-negative intestinal pathogen *S. enterica* servovar Typhimurium was found to employ ppGpp for dimerization and DNA-binding of SlyA, a transcriptional activator of this pathogen's intracellular virulence program (Zhao et al., 2008). *Francisella tularensis* uses ppGpp to promote physical interaction between the RNAP-associated MglA-SspA complex and the putative DNA-binding factor PigR to control activation of the Francisella Pathogenicity Island (Charity *et al.*, 2009). Thus, pathogenic bacteria use ppGpp to control the activity of critical virulence regulators.

In contrast to ppGpp, the RNA binding protein CsrA negatively regulates *L. pneumophila* transmission. Conditional *csrA* mutant bacteria are locked in the transmissive state, exhibiting motility and resistance to lysosomal degradation independent of starvation. Consequently, *csrA* mutant bacteria are unable to replicate in macrophages (Molofsky & Swanson, 2003).

In *E. coli*, *Pseudomonas fluorescens* and *Erwina carotovora* CsrA influences the stability of specific mRNAs (Babitzke & Romeo, 2007). Interaction between CsrA and GGA motifs in the untranslated leader sequence and/or ribosome-binding site (Shine-Dalgarno) of mRNAs prevents their translation and promotes their degradation (Babitzke & Romeo, 2007). In contrast, some *E. coli* mRNAs such as *flhDC* are stabilized by their

interaction with CsrA, leading to increased activity of the proteins (Wei *et al.*, 2001). The mechanism by which CsrA confers stability to an mRNA by direct physical interaction is unknown.

In many Gram-negative bacteria, particular non-coding regulatory RNAs interact with CsrA to antagonize its activity. *E. coli* encodes two cognate regulatory RNAs (CsrB and CsrC) peppered with GGA motifs; *P. fluorescens* encodes three (RsmZ, RsmY and RsmX) (Babitzke & Romeo, 2007, Lapouge *et al.*, 2008). The abundance of CsrA contact sites enables these non-coding RNAs to sequester multiple CsrA dimers, preventing contact with target mRNAs (Babitzke & Romeo, 2007). In this manner, the regulatory RNAs derepress transcripts targeted by CsrA.

Just as the CsrA system is broadly conserved, so too is activation of the regulatory RNAs. In many γ-proteobacteria, the GacS/A two component regulatory system (BarA/UvrY in *E. coli*, and LetA/S in *L. pneumophila*) controls activation of the RNAs (Lapouge et al., 2008, Hovel-Miner et al., 2009, Sahr et al., 2009, Rasis & Segal, 2009, Brencic *et al.*, 2009). By undefined mechanisms, the inner membrane associated sensor kinase (GacS, BarA, or LetS) becomes activated, often at the exponential to stationary phase transition in broth. This leads to phosphorylation of the cognate response regulator (GacA, UvrY, or LetA). The response regulator then binds DNA upstream of the regulatory RNAs and initiates their transcription (Lapouge et al., 2008). This conserved regulatory mechanism enables a variety of bacteria to control CsrA activity in response to environmental cues.

In *L. pneumophila*, CsrA represses factors critical for specialized virulence programs. Overexpression of *csrA* leads to reduced levels of the mRNA for the flagellar

gene activator *fliA* (σ^{28}), while conditional *csrA* mutant bacteria express increased levels of *fliA* relative to WT bacteria during nutrient abundance (Fettes et al., 2001, Forsbach-Birk et al., 2004), suggesting CsrA represses *fliA*. In addition, CsrA prevents translation of secreted substrates of the *L. pneumophila* Type IV secretion system (Rasis & Segal, 2009). Type IV secretion is essential for *L. pneumophila* to inhibit phagosome-lysosome fusion and promotes formation of an ER-derived vacuolar replication niche (Isberg et al., 2009). Therefore, CsrA controls key elements of *L. pneumophila* pathogenesis.

Recently, two non-coding CsrB-like regulatory RNAs were identified in *L. pneumophila*, RsmY and RsmZ. As in other bacteria their expression is induced at the exponential to stationary phase transition, and both interact with CsrA directly (Sahr et al., 2009, Rasis & Segal, 2009). Their activation requires the LetA/S two component system and the stationary phase sigma factor RpoS (σ^{38}) (Hovel-Miner et al., 2009, Sahr et al., 2009, Rasis & Segal, 2009). LetA binds to a consensus DNA sequence upstream of RsmY and RsmZ to activate their transcription; however, the mechanism by which RpoS activates *rsmY* and *rsmZ* is unknown (Sahr et al., 2009, Rasis & Segal, 2009). By sequestering CsrA, RsmY and RsmZ derepress factors critical for *L. pneumophila* virulence.

Previously, we showed that ppGpp synthesis leads to rapid accumulation of RsmZ in *L. pneumophila* (Dalebroux et al., 2010). Additionally, ppGpp and DksA play distinct roles in activating *fliA*, a proposed target of CsrA. Therefore, we tested whether ppGpp is a physiological stimulus for RsmY/Z transcription in *L. pneumophila*, and asked whether the regulatory RNAs control robust *fliA* and *flaA* gene activation in stationary phase by modulating CsrA activity.

Experimental Procedures

Bacterial strains and culture. *L. pneumophila* strain Lp02 (*thyA hsdR rpsL*; MB110), a virulent thymine auxotroph derived from Philadelphia 1 (Berger & Isberg, 1993), was the parental strain for all the strains analyzed. *L. pneumophila* was cultured at 37°C with agitation in 5 ml of *N*-(2-acetamido)-2-aminoethanesulfonic acid (ACES; Sigma)-buffered yeast extract (AYE) broth or on ACES-buffered charcoal yeast extract (CYE), supplemented with 100 μg/ml thymidine (AYET, CYET) when necessary. Bacteria from colonies <5 days old were cultured in broth overnight, then subcultured into fresh AYET prior to experiments. Exponential (E) cultures had an optical density at 600 nm (OD_{600}) of 0.3 to 2.0; post-exponential (PE) cultures had an OD_{600} of 3.0 to 4.5. Where indicated, ampicillin (amp; Fisher) was added to a final concentration of 100 μg ml^{-1}; gentamycin (gent; Fisher) to 10 μg ml^{-1}; rifampin to 100 μg ml^{-1}; kanamycin (kan; Roche) to 25 μg ml^{-1} chloramphenicol (cam; Roche) to 5 μg ml^{-1} for *L. pneumophila* and 10 ug ml^{-1} for *E. coli*; and, isopropyl-beta-D-thiogalactopyranoside (IPTG) to the concentrations specified. To determine colony forming units (CFU), serial dilutions of *L. pneumophila* were plated on CYET and incubated at 37°C for 4-5 days.

rsmY, *rsmZ* and *rsmY rsmZ mutant strain construction.* The Δ*rsmY*::FRT::*cat*::FRT mutant allele was constructed by first generating a deletion insertion allele using recombineering (Datsenko & Wanner, 2000, Yu et al., 2000). DNA encoding *rsmY* plus 5' and 3' flanking sequence (for RecA dependent natural competence in *L. pneumophila*) was amplified from the *L. pneumophila* chromosome by PCR using primers rsmY1, rsmY2. The fragments were ligated into pGEM generating pGEM-*rsmY*. Next, the

FRT::*cat*::FRT cassette from pKD3 was amplified using primers rsmY-pKD3a and rsmY-pKD3b. The resulting fragment with overhangs homologous to the 5' and 3' flanking regions of *rsmY* was used for recombineering (Datsenko & Wanner, 2000, Yu et al., 2000). Recombinant plasmids were isolated from DY330 and used to transform DH5α. Plasmid DNA from PCR positive, antibiotic resistant transformants was isolated. The pGEM-Δ*rsmY*::FRT::*cat*::FRT plasmid was used to transform *L. pneumophila* by natural competence, generating Δ*rsmY*::FRT::*cat*::FRT single mutant *L. pneumophila* (ZD286; Table 4.1).

The Δ*rsmZ*::*kan* deletion insertion allele was cloned differently. Briefly, the Δ*rsmZ*::*kan* deletion insertion allele was amplified from the chromosome of Δ*rsmZ*::*kan* mutant *L. pneumophila* strain Paris using the primers rsmZ1 and rsmZ2 (Sahr et al., 2009). Primers were designed to amplify a fragment containing the *rsmZ* deletion-insertion allele and ~ 500 bp of overhanging sequence to facilitate recombination onto the chromosome of *L. pneumophila* strain Philadelphia. This fragment was used to generate pGEM-Δ*rsmZ*::*kan*, and the plasmid used to transform DH5α. Resistant transformants were isolated and screened by colony PCR. The pGEM-Δ*rsmZ*::*kan* was used to transform both WT and Δ*rsmY*::FRT::*cat*::FRT mutant *L. pneumophila* by natural competence, generating Δ*rsmZ*::*kan* single and Δ*rsmY*::FRT::*cat*::FRT Δ*rsmZ*::*kan* double mutant *L. pneumophila* (ZD274 and ZD288, respectively; Table 4.1).

rsmZ-gfp promoter fusions. A 340 bp segment upstream of *rsmZ* containing the LetA binding site (Rasis & Segal, 2009) was amplified using primers rsmZ P1 and P2. The

fragment was ligated into pKB5-*gfp* to generate the *rsmZ-gfp* transcriptional reporter plasmid (Hammer & Swanson, 1999).

Fluorometry. To quantify promoter activity, E phase cultures were diluted to OD_{600} = 1.0 (t = 0) and cultured to stationary phase. Cultures were normalized to OD_{600} = 3.0 and fluorescence was detected as described (Edwards et al., 2009).

Quantitative Real-time PCR. To assess whether ppGpp and DksA induce *rsmY* and *rsmZ* transcript accumulation (Fig. 4.1), WT and *dksA* mutant bacteria carrying p*relA*$^{E.c.}$ were cultured to E phase OD_{600} (0.7-0.8) and treated with 500 μM IPTG. Bacteria were harvested at 0, 90 and 300 min post-IPTG. By 300 min, WT p*relA*$^{E.c.}$ bacteria were in the transmissive state and were fully motile. RNA isolation, cDNA synthesis, and Real-time PCR (RT-PCR) were carried out as described previously (Dalebroux et al., 2010). Primers for qRT-PCR are listed (Table 4.2).

To determine the affect of *csrA* overexpression on mRNA levels and stability (Fig. 4.5), WT bacteria carrying either p*empty* or p*csrA* were cultured to mid-E phase (OD_{600} ~ 1.2) and induced with 300 μM IPTG. Bacteria were cultured in the presence of IPTG for ~ six hours (OD_{600} ~ 3.5), at which time WT p*empty* bacteria exhibited ~ 25 % motility. Then, rifampin (100 μg ml^{-1}) was added to each culture to inhibit transcription. At 0, 5, 10 and 15 min post-rifampin addition, 1 ml of culture was added to 500 μl of ice cold AYET, as described (Charpentier *et al.*, 2008). Cells were spun for 30 sec at 4°C and pellets were resuspended in 1.5 ml of TRIzol (Invitrogen). RNA isolation, cDNA

synthesis, and Real-time PCR (RT-PCR) were carried out as described previously (Dalebroux et al., 2010). Primers for qRT-PCR are listed (Table 4.2).

Macrophage cultures. Macrophages were isolated from femurs of female A/J mice (Jackson Laboratory) and cultured in RPMI-1640 containing 10% heat-inactivated fetal bovine serum (RPMI/FBS; Gibco BRL) as described (Swanson & Isberg, 1995). Following a 7-day incubation in L-cell supernatant-conditioned media, macrophages were plated at 5×10^4 per well for cytotoxicity assays or 2.5×10^5 per well for lysosomal degradation assays.

Degradation in macrophages. The percentage of intracellular *L. pneumophila* that remain intact after a 2 h infection was quantified by fluorescence microscopy (Bachman & Swanson, 2001).

Cytotoxicity. To measure contact-dependent cytotoxicity, *L. pneumophila* were added to macrophages at the indicated MOI, and cytotoxicity was measured spectrophoretically as described (Molofsky et al., 2005).

Results

ppGpp activates RsmY and RsmZ expression.

To determine whether ppGpp and DksA activate expression of RsmY and RsmZ, we induced synchronous ppGpp synthesis in WT and *dksA* mutant *L. pneumophila* and then analyzed expression of each regulatory RNA at 0, 90 and 300 min post-induction. The fold-change relative to the t = 0 min time point was calculated at 90 and 300 min for

both RsmY and RsmZ. Alarmone accumulation was synchronized using a truncated, constitutively active RelA enzyme from *E. coli* (Schreiber et al., 1991, Hammer & Swanson, 1999, Dalebroux et al., 2010). *L. pneumophila* transformed with p*relA*$^{E.c.}$ accumulate increasing levels of ppGpp as a function of time with inducer, isopropyl-beta-D-thiogalactopyranoside (IPTG). In WT bacteria, p*relA*$^{E.c.}$ induction leads to rapid growth inhibition and differentiation to the transmissive form (Hammer & Swanson, 1999).

At 90 min, the levels of RsmY and RsmZ had increased in WT bacteria, suggesting ppGpp rapidly activates expression of both RNAs (Fig. 4.1). By 300 min RsmY and RsmZ levels continued to increase, suggesting ppGpp induces prolonged and continuous activation of both RNAs.

Immediate activation of RsmY and RsmZ by ppGpp required DksA, as transcripts failed to accumulate in *dksA* mutant *L. pneumophila* by 90 min (Fig. 4.1). By 300 min, the DksA requirement was partially bypassed, as *dksA* mutant bacteria exhibited an ~ two-fold increase in both RsmY and RsmZ. Thus, ppGpp activates RsmY and RsmZ in *L. pneumophila*; DksA enhances this effect, but its role can be partially bypassed by increasing alarmone levels.

Activation of the rsmZ promoter requires ppGpp, DksA, LetA and RpoS.

As bacteria enter stationary phase, *L. pneumophila* employ the response regulator LetA and the alternative sigma factor RpoS (σ^{38}) to activate the *rsmZ* promoter (Rasis & Segal, 2009). Activation by LetA is likely direct, as the protein binds to a consensus

DNA sequence upstream of *rsmZ* (Sahr et al., 2009, Rasis & Segal, 2009); however, it is not known whether RpoS acts directly.

To investigate which factors cooperate to activate transcription of either *rsmY* or *rsmZ*, we generated transcriptional reporter plasmids. Briefly, promoter fragments containing the LetA-binding site and putative -35 and -10 elements of both promoters were cloned in front of the ribosome-binding site (Shine-Dalgarno) of the green fluorescent protein gene (*gfp*) (Hammer & Swanson, 1999, Sahr et al., 2009, Rasis & Segal, 2009). While the *rsmZ* reporter showed activity, the *rsmY* reporter was virtually inactive. Therefore, a different reporter was designed using published *rsmY* promoter coordinates (Rasis & Segal, 2009). Again, fluorescence readings failed to exceed background levels in WT bacteria throughout exponential and stationary phase (Yagi, data not shown). This suggests either that *rsmY* promoter activity is below the limit of our detection, or post-transcriptional mechanisms contribute to RsmY activation in the Philadelphia Lp02 strain of *L. pneumophila*.

Promoter activity of *rsmZ* was monitored at the E to PE phase transition in WT, ppGpp^0, *dksA*, *letA* and *rpoS* mutant *L. pneumophila* using the *rsmZ-gfp* reporter. The reporter was functional, since WT bacteria exhibited increasing fluorescence upon entering PE phase (Fig. 4.2A). Consistent with previous findings, PE phase activation of the *rsmZ* promoter required both LetA and RpoS, as mutant bacteria failed to achieve WT fluorescence values (Fig. 4.2A). In line with our qRT-PCR results (Fig. 4.1), ppGpp and DksA also contributed to activation (Fig. 4.2A).

Consistent with the finding that ppGpp is sufficient to partially bypass the DksA requirement for RsmZ activation (Fig. 4.1), *dksA* mutant *L. pneumophila* moderately

activated the *rsmZ* promoter in PE phase to a level that was statistically different from that of both ppGpp^0 and *letA* mutant bacteria (Fig. 4.2B). We also consistently observed a modest increase in *rsmZ-gfp* fluorescence in *rpoS* mutant bacteria, although final PE phase values were not statistically different from those of ppGpp^0 and *letA* mutant *L. pneumophila* (Fig. 4.2B). Therefore, DksA, LetA, RpoS and ppGpp contribute to activation RsmZ transcription by mechanisms that remain to be defined.

rsmZ induction leads to ppGpp-independent transmission trait activation.

During exponential growth, *L. pneumophila* represses several virulence phenotypes important for host-to-host transmission using CsrA (Molofsky & Swanson, 2003). At the exponential to stationary phase transition many of these same traits are activated by ppGpp (Hammer & Swanson, 1999, Dalebroux et al., 2009, Dalebroux et al., 2010). Since RsmY and RsmZ are known to relieve CsrA repression, we investigated whether activation of *rsmZ* by ppGpp contributes to differentiation. In particular, we induced expression of *rsmZ* in ppGpp^0 mutant bacteria and assessed transmissive phenotypes.

Indeed, independently of alarmone, induction of *rsmZ* increased cell rounding, motility (~10% of any given culture aliquot), resistance to lysosomal degradation, and cytotoxicity of ppGpp^0 mutant *L. pneumophila* (Fig. 4.3A and B; data not shown). In contrast, the macrophage infectivity, pigment production, and sodium sensitivity defects of ppGpp^0 bacteria could not be bypassed by *rsmZ* induction (data not shown). Therefore, when induced from a plasmid, *rsmZ* can partially rescue ppGpp^0 mutant defects for some transmissive phase traits, but not others.

rsmY and rsmZ contribute to PE phase flagellar gene expression.

Several lines of evidence suggest CsrA represses a regulator of flagellar gene expression in *L. pneumophila* (Fettes et al., 2001, Molofsky & Swanson, 2003, Forsbach-Birk et al., 2004). To determine if the RsmY and RsmZ regulatory RNAs are required to relieve CsrA repression, *rsmY*, *rsmZ* and *rsmY rsmZ* double mutant *L. pneumophila* were constructed. Whereas *rsmY* and *rsmZ* single mutant bacteria exhibited 95-100% motility in PE phase, *rsmY rsmZ* double mutant *L. pneumophila* were filamentous and amotile (data not shown). Therefore, RsmY and RsmZ appear to cooperate to control PE phase flagellar biogenesis of *L. pneumophila*.

One flagellar gene regulator CsrA might target is FliA. This sigma factor activates expression of *flaA*, which encodes the major subunit of the flagellar filament. To determine whether RsmY and RsmZ affect activation or activity of FliA, we transformed *rsmY rsmZ* double mutant *L. pneumophila* with *fliA-gfp* and *flaA-gfp* reporter plasmids, and monitored fluorescence at the E to PE phase transition (Hammer & Swanson, 1999, Dalebroux et al., 2010). Fluorescence of ppGpp^0 and *dksA* mutant *L. pneumophila* strains carrying each plasmid was monitored as negative controls. Consistent with previous observations (Dalebroux et al., 2010), WT and ppGpp^0 mutant bacteria exhibited significant *fliA* promoter activity in E phase but *dksA* mutant strains did not (Fig. 4.4A). Therefore, DksA contributes to basal activation of the promoter. The regulatory RNAs were dispensable for this activity, since *rsmY rsmZ* double mutant bacteria exhibited fluorescence values equivalent to those of WT in E phase. However, the RNAs were required for robust activation of the *fliA* promoter at the transition to

stationary phase, since fluorescence values increased only slightly in *rsmY rsmZ* mutants relative to WT bacteria (Fig. 4.4A). The RsmY/Z requirement for *fliA* activation was partially relieved after prolonged incubation in stationary phase, as by 12 h into stationary phase (corresponding to the 18 h time point of Fig. 4.4), double mutant bacteria routinely achieved *fliA-gfp* fluorescence values > 5000 units (data not shown).

Surprisingly, the basal level of *fliA* promoter activity observed in E phase bacteria was not sufficient to activate the *flaA* promoter, as WT, $ppGpp^0$ and *rsmY rsmZ* double mutant bacteria exhibited essentially background levels of *flaA-gfp* fluorescence in E phase (Fig. 4.4B). Consistent with minimal *fliA* promoter activity in PE phase *rsmY rsmZ* bacteria, *flaA* promoter activity was dramatically reduced in double mutants relative to WT bacteria (Fig. 4.4B). However, as with the *fliA* promoter, late in stationary phase corresponding to the 18 h time point in Fig. 4.4, *rsmY rsmZ* double mutants often achieved fluorescence levels of > 3000 units from *flaA-gfp* (data not shown). Therefore, in PE phase, RsmY/Z contribute to optimal *fliA* and *flaA* promoter activation. Furthermore, *L. pneumophila* appear to regulate FliA activity during replicative growth, since WT bacteria exhibit significant *fliA* promoter activity in E phase (Fig. 4A), yet the *flaA* promoter remains inactive under these conditions (Fig. 4B).

In an effort to verify that RsmY and RsmZ regulate the expression of certain transmission traits, genetic complementation experiments were performed for *rsmY rsmZ* double mutant *L. pneumophila*. In these studies, motility, pigment production and cell rounding were fully restored in *rsmY rsmZ* mutant *L. pneumophila* when *rsmY* expression was induced from an IPTG-inducible promoter (data not shown). However, several attempts to complement the double mutant's defects with *rsmZ in trans* were not

successful. In particular, when two different *rsmZ* constructs and a range of different IPTG concentrations were analyzed, no effect of induction was observed. Therefore, the phenotypes of the *rsmY rsmZ* double mutants described above may not be solely attributed to deletion of the regulatory RNAs. Currently, a new *rsmZ* deletion insertion allele is being constructed by recombineering for our ongoing studies.

Overexpression of csrA affects fliA mRNA levels and stability.

It has been suggested that CsrA regulates *fliA* directly in *L. pneumophila* (Fettes et al., 2001, Forsbach-Birk et al., 2004). Therefore, we tested whether overexpression of *csrA* impacts the stability of *fliA* mRNA. Wild-type *L. pneumophila* express CsrA during the replication period in broth and macrophages (Molofsky & Swanson, 2003). Therefore, we induced expression of *csrA* from mid-E phase to early-PE phase in WT *L. pneumophila* and compared these bacteria to mock-treated WT p*empty* bacteria (Molofsky & Swanson, 2003). To test mRNA stability, *L. pneumophila* transcription was inhibited with 100 μg/ml of rifampin during the period when ~25% of WT p*empty* bacteria exhibit motility (OD_{600} = 3.5) and < 1% of WT p*csrA* bacteria do so (data not shown). Culture aliquots were collected at 0, 5, 10, and 15 min post-rifampin addition, and RNA was isolated. Since new transcripts do not form after rifampin addition, we evaluated the impact of excess CsrA protein on turnover of particular mRNAs by monitoring their fold decrease over time relative to the level at the time of rifampin addition to WT p*csrA* and WT p*empty* bacterial cultures.

To begin, the relative amount of *fliA* mRNA in WT p*csrA* bacteria compared to WT p*empty* at the time of rifampin addition (~ 6 h post-IPTG; OD_{600} ~ 3.5) was

calculated using qRT-PCR. Consistent with Northern blot analysis (Fettes et al., 2001), *csrA* overexpression resulted in an approximately twelvefold reduction in *fliA* mRNA levels (Fig. 4.5A), confirming that CsrA reduces *fliA* mRNA levels. To determine if this reduction was a result of reduced stability of the *fliA* transcript, we monitored its degradation. Surprisingly, we found that induction of *csrA* led to increased stability of the *fliA* transcript, as degradation was less pronounced in WT p*csrA* bacteria relative to WT p*empty* (Fig. 4.5B, left panel). Thus, reduced *fliA* expression in bacteria overexpressing *csrA* is not a result of decreased transcript stability, but rather likely reflects decreased *fliA* promoter activation.

Accordingly, we next asked whether *csrA* over-expression impacts either the amount or stability of the mRNA encoding RpoS and FleQ, two *fliA* gene activators (Bachman & Swanson, 2004a, Hovel-Miner et al., 2009, Albert-Weissenberger et al.). Unlike the *fliA* transcript, the levels and stability of *rpoS* and *fleQ* RNA were only modestly impacted by *csrA* over-expression (Fig. 4.5 and data not shown). Thus, *csrA* induction only modestly affects the *fliA* transcriptional regulators, *rpoS* and *fleQ*. Together, these preliminary data indicate that over-expression of *csrA* reduces *fliA* mRNA levels by repressing *fliA* promoter activation, directly or indirectly.

Discussion

When replicating *L. pneumophila* experience metabolic stress, the ppGpp alarmone accumulates and orchestrates bacterial differentiation. One hallmark of this process is the rapid assembly of a monopolar flagellum. *L. pneumophila* flagellar biogenesis requires a transcriptional cascade of at least four different gene classes. Several factors contribute, including the master regulator FleQ (Class II and III), the

response regulator FleR, and the alternative sigma factors RpoN (σ^{54}) (Class II) and FliA (σ^{28}) (Class III and IV) (Bruggemann et al., 2006, Heuner, 2007, Albert-Weissenberger et al.). Unlike other *L. pneumophila* flagellar gene regulators, *fliA* is unique in that its expression is highly induced in stationary phase by ppGpp (Dalebroux et al., 2010).

Regulation of FliA must be tightly controlled. The sigma factor controls the costly production of the final components needed for flagellar assembly, as well as several factors unrelated to the flagellar regulon; it is also required for growth in particular amoebic hosts (Heuner et al., 2002, Bruggemann et al., 2006, Albert-Weissenberger et al.). During starvation, elevated ppGpp induces robust *fliA* promoter activity and continuous accumulation of *fliA* mRNA (Dalebroux et al., 2010). Given the recent finding that CsrA activity is regulated by RsmY and RsmZ (Rasis & Segal, 2009), and that CsrA represses *fliA* in *L. pneumophila* (Fettes et al., 2001, Forsbach-Birk et al., 2004), we sought to determine if ppGpp controls production of the non-coding regulatory RNAs to regulate *fliA* expression.

In *E. coli*, *P. aeruginosa* and *L. pneumophila*, expression of the non-coding regulatory RNAs that sequester CsrA occurs as bacteria enter stationary phase (Gudapaty *et al.*, 2001, Weilbacher *et al.*, 2003, Hovel-Miner et al., 2009, Sahr et al., 2009, Rasis & Segal, 2009, Brencic et al., 2009). In *E. coli*, this induction is independent of ppGpp (Jonas *et al.*, 2006), whereas in *L. pneumophila* the alarmone is a physiological stimulus for rapid and prolonged accumulation of both *rsmY* and *rsmZ* (Fig. 4.1).

Stationary phase activation of *rsmZ* transcription is complex, requiring an arsenal of *L. pneumophila* regulators including: ppGpp, LetA, RpoS and DksA (Fig. 4.2). Activation is unlikely to occur by conventional mechanisms involving ppGpp, DksA and

RNAP, since ppGpp^0 and *dksA* mutant *L. pneumophila* exhibit different patterns of *rsmZ* promoter activation (Fig. 4.2). While ppGpp is absolutely required, DksA is partially dispensable. Therefore, two distinct ppGpp-dependent pathways may contribute, one requiring DksA, the other independent of DksA (Fig. 4.6). The partial DksA requirement for *rsmZ* activation is of particular interest in the pathogenesis field, since *L. pneumophila* exhibits differential requirements for ppGpp and DksA during macrophage transmission (Dalebroux et al., 2010). One possibility is that ppGpp and/or DksA act indirectly by inducing expression of other activators of the *rsmZ* promoter, such as LetA and RpoS.

In many bacteria, RpoS is regulated by post-transcriptional mechanisms (Hengge, 2009). For example, *E. coli* employs ppGpp and DksA to control transcription of anti-adaptor proteins that stabilize the RpoS protein (Bougdour & Gottesman, 2007, Merrikh *et al.*, 2009a). *L. pneumophila* may also regulate RpoS by post-transcriptional mechanisms. Indeed, replicating *L. pneumophila* have high levels of RpoS mRNA, while the transcript is virtually undetectable in stationary phase cells (Bachman & Swanson, 2004a), suggesting *L. pneumophila* mediates a stress-induced decrease in RpoS mRNA. Given that RpoS protein activates *rsmY* and *rsmZ* in stationary phase (Hovel-Miner et al., 2009, Rasis & Segal, 2009), this decrease in *rpoS* mRNA correlates with an increase in the sigma factor's activity. Therefore, ppGpp and DksA may equip *L. pneumophila* to promote RpoS translation in stationary phase.

Concomitantly, ppGpp likely influences expression or activity of the LetA/S system. *L. pneumophila* that lack ppGpp and *letA* exhibit different *rsmZ* promoter activity then *dksA* mutant bacteria in stationary phase (Fig. 4.2), indicating they

contribute to activation of *rsmZ* independently of DksA. *S. enterica* serovar Typhimurium and *F. tularensis* are known to employ ppGpp to control the activity of specialized virulence regulators by direct physical interactions with the alarmone (Zhao et al., 2008, Charity et al., 2009). Perhaps *L. pneumophila* also enlists ppGpp in non-canonical mechanisms that control activation of LetA/S. For example, the alarmone might influence the activation state of LetS, perhaps even by acting as a phosphodonor for LetS. Alternatively, ppGpp could facilitate LetA DNA-binding to activate *rsmZ* transcription. Our genetic data will inform future biochemical experiments designed to elucidate these regulatory interactions.

Two lines of evidence suggest activation of RsmY and RsmZ is critical for *L. pneumophila* to express transmission traits. First, overexpression of *rsmZ* partially bypasses the requirement for ppGpp for motility, lysosomal degradation resistance and macrophage cytotoxicity of *L. pneumophila* (Fig. 4.3; data not shown). Therefore, ppGpp activates *rsmZ* to control these phenotypes. Second, *rsmY rsmZ* double mutant *L. pneumophila* are amotile and exhibit minimal activation of the *fliA* and *flaA* promoters in broth, suggesting the RNAs contribute to flagellar gene expression in *L. pneumophila* (Fig. 4.4). However, the *rsmY rsmZ* double mutant data set must be validated, since *rsmZ* induction did not restore transmission phenotypes to this strain (data not shown). Additionally, our results contradict those reported for the related *L. pneumophila* strain Paris, in which *rsmY rsmZ* double mutants are flagellated and fully motile (Sahr et al., 2009). The mutant phenotypes in strain Paris are in contrast to bacteria that lack the upstream activator LetA, which are amotile and lack flagella in both *L. pneumophila* Paris and Philadelphia. Therefore, our colleagues proposed that flagellar biogenesis

requires additional LetA-dependent factors, perhaps even additional regulatory RNAs. Until complementation of our *rsmY rsmZ* double mutant in the Philadelphia Lp02 strain is demonstrated, whether other LetA-dependent factors impact flagellar expression is an open question.

In *E. coli* and *P. aeruginosa,* CsrA controls stability of particular mRNAs, but the mechanism by which CsrA represses *fliA* in *L. pneumophila* is unknown. Consistent with previous studies, over-expression of *csrA* dramatically reduces *fliA* mRNA (Fig. 4.5A) (Fettes et al., 2001, Forsbach-Birk et al., 2004). However, decreased mRNA is not a result of increased mRNA turnover, since *csrA* over-expression stabilizes the *fliA* transcript (Fig. 4.5B). Therefore, we postulated that robust activation of the *fliA* promoter requires that *L. pneumophila* derepress an activator of *fliA* (Fig. 4.6). Two candidates were tested, RpoS and FleQ (Bachman & Swanson, 2004a, Hovel-Miner et al., 2009, Albert-Weissenberger et al.). While *csrA* induction modestly reduces the level of *rpoS* and *fleQ* mRNAs (Fig. 4.5A), it does not affect their stability (Fig. 4.5B; data not shown). Therefore, *rpoS* and *fleQ* mRNAs do not appear to be direct targets of CsrA.

One limitation to our experimental design is that, as *csrA* is induced from mid-E phase to early-PE phase, a portion of the total CsrA protein in the cell is likely sequestered by RsmY/Z. To avoid counteracting effects of these regulatory RNAs, an alternative approach would be to deplete CsrA and then assess mRNA levels and stability. Fortunately, a conditional *csrA* null mutant strain of *L. pneumophila* has been constructed and analyzed. When *csrA* is removed, *L. pneumophila* activate transmission traits prematurely, including flagellar synthesis and motility (Molofsky & Swanson,

2003). Transcript decay studies employing conditional *csrA* mutant *L. pneumophila* are currently underway.

To our surprise, over-expression of *csrA* increased the stability of the *fliA* mRNA (Fig. 4.5B). Because a longer half-life cannot account for the decrease in *fliA* levels observed, our results suggest that CsrA regulates gene expression by more than one mechanism. By decreasing *fliA* promoter activity, CsrA could prevent excessive accumulation of *fliA* mRNA during the replication period (Dalebroux et al., 2010). Simultaneously, CsrA may stabilize the basal level of *fliA* transcripts by a direct interaction to maintain a low level of FliA protein in exponential phase (Fig. 4.6). In fact, *E. coli* utilizes a similar mechanism to control its master regulators of flagellar gene transcription. By interacting directly with the *flhDC* transcripts, *E. coli* CsrA stabilizes the mRNA and facilitates their translation (Wei et al., 2001). Consistent with the idea that a similar regulatory circuit maintains a steady state level of FliA in exponential phase *L. pneumophila*, DksA promotes basal *fliA* promoter activity and mRNA accumulation during the replication period (Fig. 4.4A) (Dalebroux et al., 2010). However, the FliA protein is presumably inactive or present at levels insufficient to compete with σ^{70} in exponential phase, since the *flaA* promoter remains quiescent until WT bacteria enter stationary phase (Fig. 4.4B). Although protein levels have not been measured, it is possible that FliA is also post-translationally regulated in *L. pneumophila* (Fig. 4.6), a mechanism common to other bacteria (Chevance & Hughes, 2008).

In summary, *L. pneumophila* employs ppGpp to activate transcription of two CsrA-binding regulatory RNAs, RsmY and RsmZ (Fig. 4.6). Since these RNAs sequester CsrA, their production enables *L. pneumophila* to relieve CsrA repression when

protein or fatty acid biosynthesis is impaired (Dalebroux et al., 2009). In particular, our genetic data suggest that ppGpp controls *fliA* transcription by promoting CsrA derepression of a *fliA* gene activator. By this model, when ppGpp levels are high, expression of this unidentified activator results in robust *fliA* mRNA accumulation. Alarmone control of CsrA activity is critical for *L. pneumophila* differentiation, since it governs final assembly of the flagellum. This regulatory mechanism can be expanded to include effectors of Dot/Icm Type IV secretion system, as well as elements critical for *L. pneumophila* resistance to lysosomal degradation, since in addition to flagellar biogenesis, CsrA has been shown to repress these systems in *L. pneumophila* (Molofsky & Swanson, 2003, Rasis & Segal, 2009). Knowledge of the mechanism(s) by which ppGpp initiates transcription of the RsmY and RsmZ regulatory RNAs of *L. pneumophila* will broaden our understanding of the contribution of ppGpp to bacterial developmental biology and pathogenesis.

Table 4.1. Bacterial strains and plasmids

Strain	Relevant genotype/phenotype	Reference
E.coli		
DH5α	F-endA1 *hsdR17* (r- m+) *supE44 thi-1 recA1 gyrA* (Nal^r) *relA1* Δ(*lacZYA-argF*)$_{U169}$Φ80dLacZΔM15*λpir*RK6	Laboratory collection
DY330	W3110 Δ*lacU169 gal490 λcl1857* Δ(*cro-bioA*)	(Yu et al., 2000)
L. pneumophila		
MB110	Lp02 wild-type, Str^R, Thy-, HsdR-	(Berger & Isberg, 1993)
MB699	Lp02 Δ*dksA*::FRT::*cat*::FRT mutant	(Dalebroux et al., 2010)
MB697	Lp02 *relA*::*gent spoT*::*kan* double mutant	(Dalebroux et al., 2009)
MB379	Lp02 *rpoS*::*kan* mutant	(Bachman & Swanson, 2001)
MB413	Lp02 *letA 22-3*::*kan* mutant	(Hammer et al., 2002)
ZD287	Lp02 Δ*rsmY*::FRT::*cat*::FRT mutant	This work
ZD274	Lp02 Δ*rsmZ*::*kan*	This work
ZD288	Lp02 Δ*rsmY*::FRT::*cat*::FRT Δ*rsmZ*:: *kan*, double mutant	This work
MB359	Lp02 wild-type, p*relA*$^{E.c.}$	(Hammer & Swanson, 1999)
MB702	Lp02 Δ*dksA*::FRT::*cat*::FRT, p*relA*$^{E.c.}$	(Dalebroux et al., 2010)
ZD216	Lp02 *relA*::*gent spoT*::*kan*, p*rsmZ*	This work
MB355	Lp02 wild-type, p*flaAgfp* td(Δ)I	(Hammer & Swanson, 1999)
MB732	Lp02 Δ*dksA*::FRT::*cat*::FRT, p*flaAgfp* td(Δ)I	(Dalebroux et al., 2010)
MB685	Lp02 *relA*::*gent spoT*::*kan*, p*flaAgfp* td(Δ)I	(Dalebroux et al., 2009)
ZD310	Lp02 Δ*rsmY*::FRT::*cat*::FRT Δ*rsmZ*::*kan*, p*flaAgfp* td(Δ)I	This work
MB733	Lp02 wild-type, p*fliAgfp* td(Δ)I	(Dalebroux et al., 2010)
MB735	Lp02 Δ*dksA*::FRT::*cat*::FRT, p*fliAgfp* td(Δ)I	(Dalebroux et al., 2010)
MB734	Lp02 *relA*::*gent spoT*::*kan*, p*fliAgfp* td(Δ)I	(Dalebroux et al., 2010)
ZD311	Lp02 Δ*rsmY*::FRT::*cat*::FRT Δ*rsmZ*::*kan*, p*fliAgfp* td(Δ)I	This work
BY60	Lp02 wild-type, p*rsmZgfp* td(Δ)I	This work
BY65	Lp02 Δ*dksA*::FRT::*cat*::FRT, p*rsmZgfp* td(Δ)I	This work
BY63	Lp02 *relA*::*gent spoT*::*kan*, p*rsmZgfp* td(Δ)I	This work
BY107	Lp02 *rpoS*::*kan*, p*rsmZgfp* td(Δ)I	This work
BY98	Lp02 *letA 22-3*::*kan*, p*rsmZgfp* td(Δ)I	This work
Plasmids		
pGEMT-Easy	MCS within coding region of B-lactamase α-fragment linearized with single-T overhangs, Amp^R	Promega
pKD3	Template plasmid for λ Red system, *bla* FRT::*cat*::FRT, *ori* R6K	(Datsenko & Wanner, 2000)
pGEM-*rsmY*	pGEMT-Easy with 1.0 kb PCR amplified *rsmY* chromosomal region ligated into T overhangs, Amp^R	This work
pGEM-Δ*rsmY*::FRT::*cat*::FRT	pGEM-*rsmY* with 1.1 kb FRT::*cat*::FRT cassette from pKD3 recombineered using strain DY330 to delete and replace *rsmY* leaving ~500 bp homology on either side for Lp02 chromosomal recombination, Amp^R, Cam^R	This work
pGEM-Δ*rsmZ*::*kan*	pGEM with the ~ 2.6 kb Δ*rsmZ*::*kan* deletion insertion allele amplified from the chromosome of Δ*rsmZ*::*kan* mutant *L. pneumophila* strain Paris with ~500 bp homology on either side for Lp02 chromosomal recombination, Amp^R, Kan^R	This work
pMMB207C	pMMB207, Δ*mobA*	(Chen et al., 2004)
prsmZ	pMMB207C, with promoterless *rsmZ* cloned into the MCS, collinear with the $P_{taclacUV5}$ promoter, *lacI*^{q}, Inducible *rsmZ* expression, Cm^R.	(Sahr et al., 2009)
p*relA*$^{E.c.}$	pMMB66EH derivative, *lacI*^{q},$P_{taclacUV5}$, $Gent^R$ with a *relA* allele from *E. coli* encoding a truncated, metabolically active RelA, cloned into the MCS, colinear with the $P_{taclacUV5}$ promoter, *lacI*^{q}, Inducible *relA*$^{E.c.}$ expression, $Gent^R$.	(Hammer & Swanson, 1999)
p*flaAgfp*	150 bp *flaA* promoter fragment fused to GFPmut3 in pKB5 with P_{tac} and *lacI*^{q} removed, td(Δ)i	(Hammer & Swanson, 1999)
p*fliAgfp*	304 bp *fliA* promoter fragment fused to GFPmut3 in pKB5 with P_{tac} and *lacI*^{q} removed, td(Δ)i	(Dalebroux et al., 2010)
p*rsmZgfp*	340 bp *rsmZ* promoter fragment fused to GFPmut3 in pKB5 with P_{tac} and *lacI*^{q} removed, td(Δ)	This work

Table 4.2. List of primers

Primers for Cloning	Sequence	Amplicon Size
rsmY1 fwd.	5’-AGGAAGATCCTAACTACCATCGCTT-3’	1024bp
rsmY2 rvs.	5’-GGCGCCTATATTCAACGTGGA-3’	
rsmZ1 fwd.	5’-GCGGAGTATCCAGTCGCTC-3’	1163bp
rsmZ2 rvs.	5’-TTAGCCACAAGGCCCCTC-3’	
rsmY-pKD3a fwd.	5’-CTCGATGACTTATACTGCCAATTGGTTA TACTAAAAACATACAGTGTAGGCTGGAGCTGC-3’	
rsmY-pKD3b rvs.	5’-ATAATGCCTTTTGATTGCTTAAATGAAT AAATTGAGCATATGAATATCCTCCTTAGTTCC-3’	1093bp
rsmZ P1	5' -GGATCCTTTCTTGGTTGCATTTAGTT-3’	
rsmZ P2	5' -TCTAGAATATCCATATAAGTCTTGATTAAAATTTA-3’	340 bp

Real-time PCR Primers	Sequence
lpg2096a fwd.	5’-AGCAATTGGGAGCGAGGTGATAGA-3’
lpg2096b rvs.	5’-AGGCTTGTTGATGATCGAGCAGTG-3’
rsmY1 fwd.	5’-CATACAGGGATGACATGGATATG-3’
rsmY2 rvs.	5’-CGTCCTTGCAGCGAAGTACA-3’
rsmZ1 fwd.	5’-TGGATATGAGTCGTGCAAATGG-3’
rsmZ2 rvs.	5’-GACTCAGCCCTGGCTTTTC-3’
fliA1 fwd.	5’-TCCTTAGCCTCACGCCCAAGTTTA-3’
fliA2 rvs.	5’-ATTGGGTGCCGCGTTCTGTTTATC-3’
fleQ1 fwd.	5’-AGCAAGTAGCCGATACTGAAGCCA-3’
fleQ2 rvs.	5’-CCCGGGATTGCTCCACAATTGATA-3’
rpoS1 fwd.	5’-TCCTGCAGAGCTGCTAACCAATGA-3’
rpoS2 rvs.	5’-TACCAACGTCTTCCAGTGTCGCTT-3’

Figure 4.1.

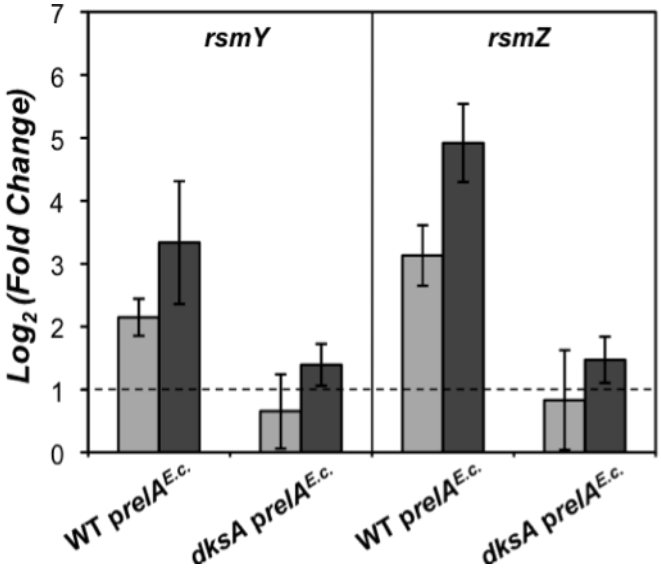

Figure. 4.1. ppGpp synthesis triggers accumulation of the RsmY and RsmZ non-coding regulatory RNAs.
To test whether ppGpp accumulation activates *rsmY/Z* expression in *L. pneumophila*, qRT-PCR was performed on transcripts isolated from mid-E phase AYET broth cultures of WT and *dksA* mutant bacteria induced to express p*relA*$^{E.c.}$ with 500 μM IPTG. RNA was harvested at 0, 90 and 300 minutes post-IPTG, and the relative transcript levels were assessed by dividing 90 min (light grey) and 300 min (dark grey) values by the 0 min value to give the fold increase for each target. Depicted are the mean log2 (fold increase) values from triplicate wells in three independent experiments ± SE. The dashed line delineates the two-fold change cutoff.

Figure 4.2.

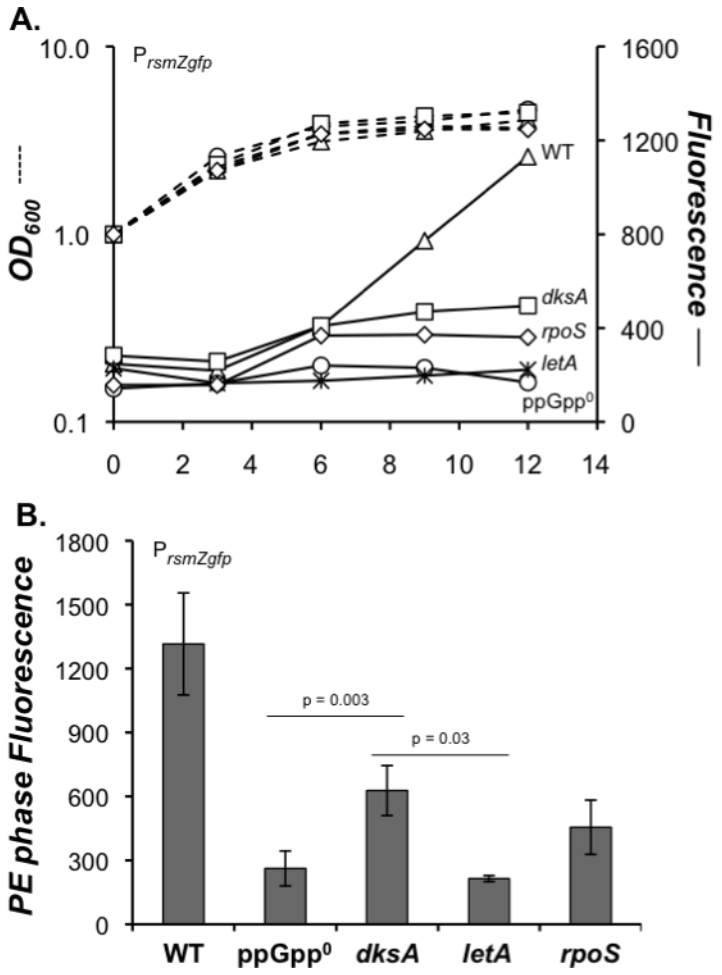

Figure 4.2. *L. pneumophila* requires ppGpp, DksA, RpoS and LetA for PE phase activation of the *rsmZ* promoter.
A. To monitor *rsmZ* promoter activity at the E to PE phase transition, mid-E phase WT (triangles), ppGpp^0 (circles), *dksA* (squares), *rpoS* (diamonds), and *letA* mutant bacteria (asterisks) harboring the p*rsmZgfp* reporter plasmid were diluted to an OD_{600} of 1.0. Cell density (dashed lines) and *rsmZ* promoter activity (solid lines) were quantified until bacteria entered stationary phase. The data represent one of three independent experiments. **B.** The 12 h fluorescence values (depicted in A) were averaged from three independent experiments ± SE. In addition to the P values shown, each mutant was statistically different than WT by a two-tailed paired Student t-test ($p < 0.01$).

Figure 4.3.

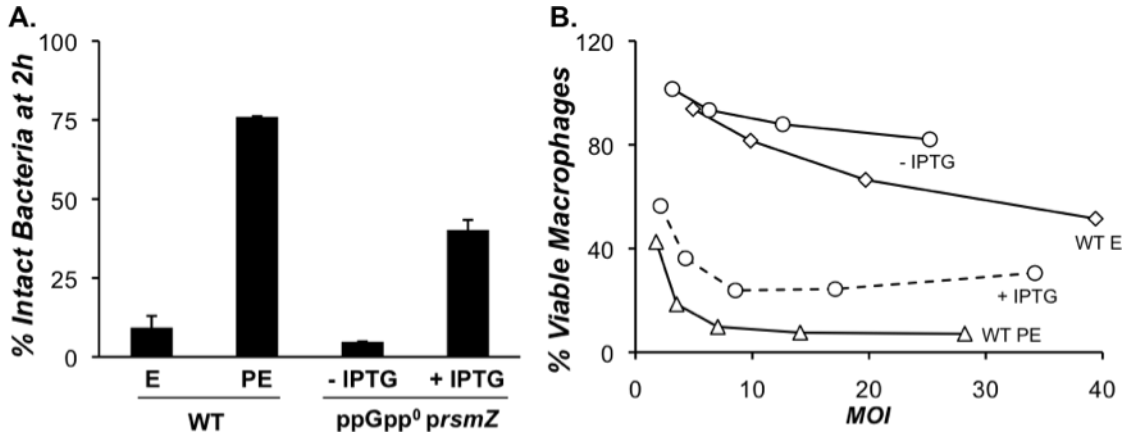

Figure 4.3. Induction of *rsmZ* is sufficient to bypass ppGpp for macrophage degradation resistance and cytotoxicity.
To test the effect of *rsmZ* induction on the ability of ppGpp^0 mutant *L. pneumophila* to resist lysosomal degradation or exhibit toxicity toward macrophages, E phase ppGpp^0 mutant bacteria harboring p*rsmZ* were cultured PE phase with or without 200 μM IPTG before infecting macrophages; E and PE phase WT bacteria served as negative and positive controls, respectively. **A.** Lysosomal degradation was scored by fluorescence microscopy. Shown are the mean percentages from duplicate coverslips ± SE from one experiment. **B.** To measure cytotoxicity, triplicate macrophages were infected with either E phase WT (triangle, dashed lines), or PE phase WT (triangles, solid lines), and ppGpp^0 p*rsmZ* mutant bacteria with IPTG (circles, solid lines), or without IPTG (circles, dashed lines) at the multiplicities of infection (moi) shown. The values plotted represent the mean percent viable macrophages in one experiment.

Figure 4.4.

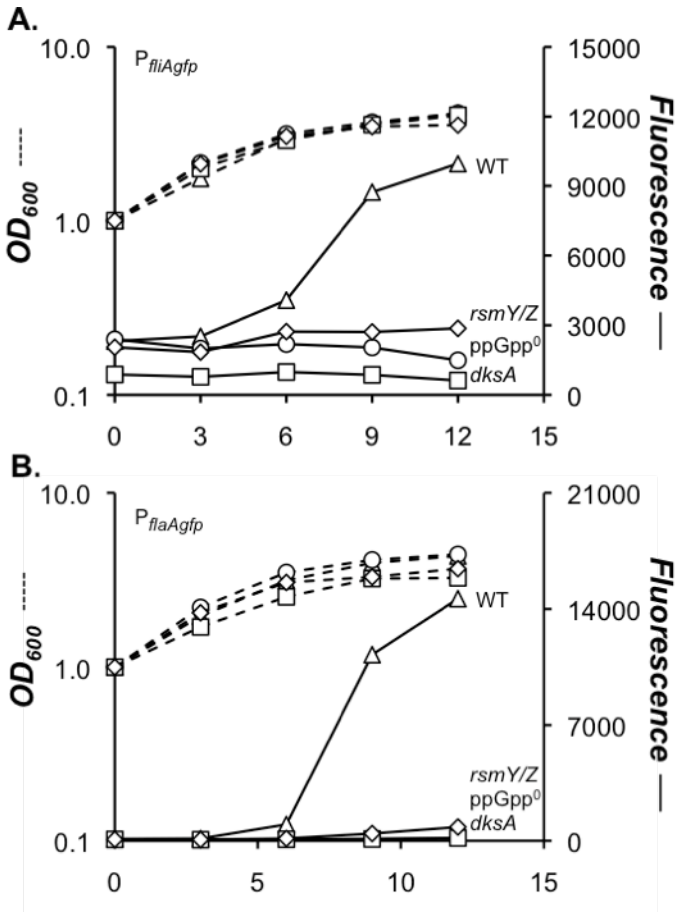

Figure 4.4. RsmY/Z contribute to PE phase activation of *fliA* and *flaA*.
To monitor *fliA* and *flaA* promoter activity at the E to PE phase transition, mid-E phase WT (triangles), ppGpp^0 (circles), *dksA* (squares), and *rsmY rsmZ* double mutant bacteria (diamonds), harboring either the p*fliAgfp* reporter plasmid (**A**), or the p*flaAgfp* reporter plasmid (**B**) were diluted to an OD_{600} of 1.0. Cell density (dashed lines) and promoter activity (solid lines) were quantified until bacteria entered stationary phase. The data represent one of two independent experiments.

Figure 4.5.

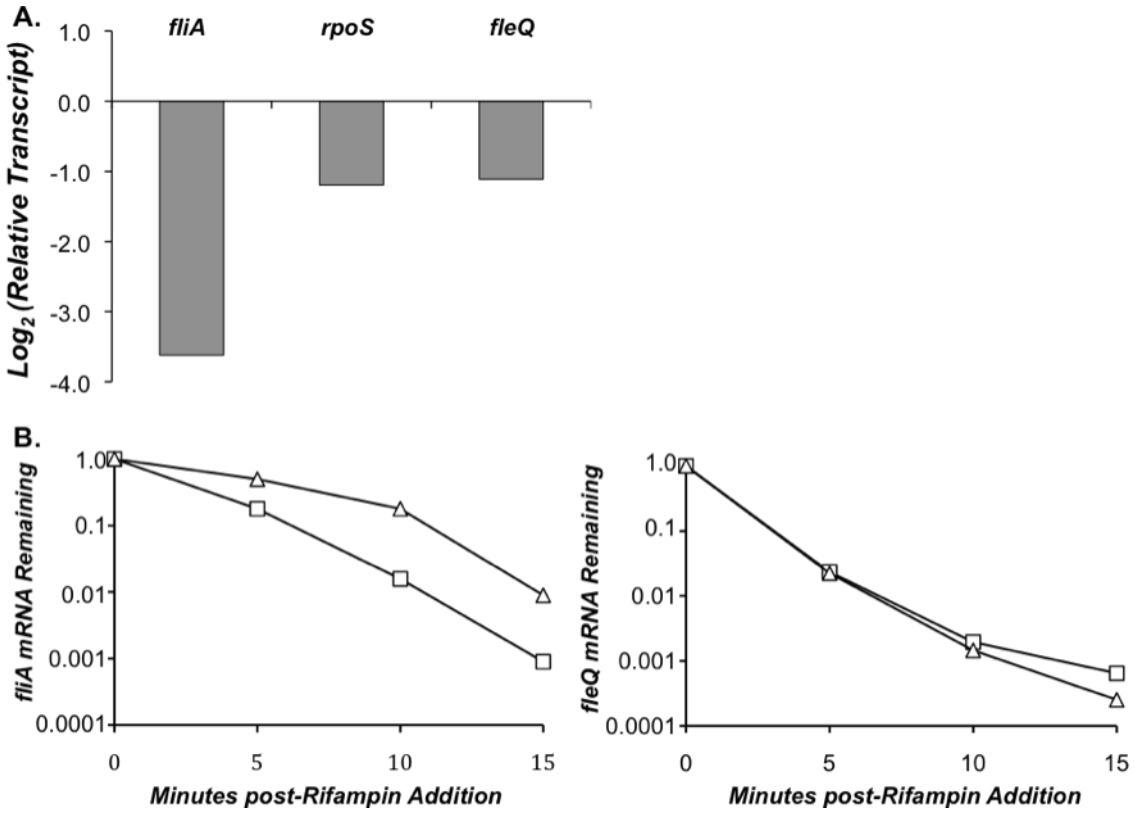

Figure 4.5. Induction of *csrA* leads to reduced *fliA* mRNA, but increased stability. To determine whether *csrA* overexpression impacts the level of *fliA*, *rpoS*, or *fleQ* transcripts, and/or their stability, E phase WT bacteria harboring either p*empty* or p*csrA* were treated with IPTG and cultured into early-PE phase before rifampin addition. **A.** To test whether *csrA* induction impacts the amount of *fliA*, *rpoS*, or *fleQ* mRNA at the E to PE phase transition, relative transcript levels were obtained by comparing WT p*csrA* and WT p*empty* at the time of rifampin addition (t = 0). **B**. To determine if *csrA* induction impacts transcript stability, *fliA* (left panel) and *fleQ* (right panel) mRNAs were quantified by qRT-PCR at 0, 5, 10 and 15 post-rifampin addition. Data are expressed relative to the initial amount of mRNA at the time of transcription inhibition for each strain; WT p*csrA* (triangles), WT p*empty* (squares). The data are from one experiment.

Figure 4.6.

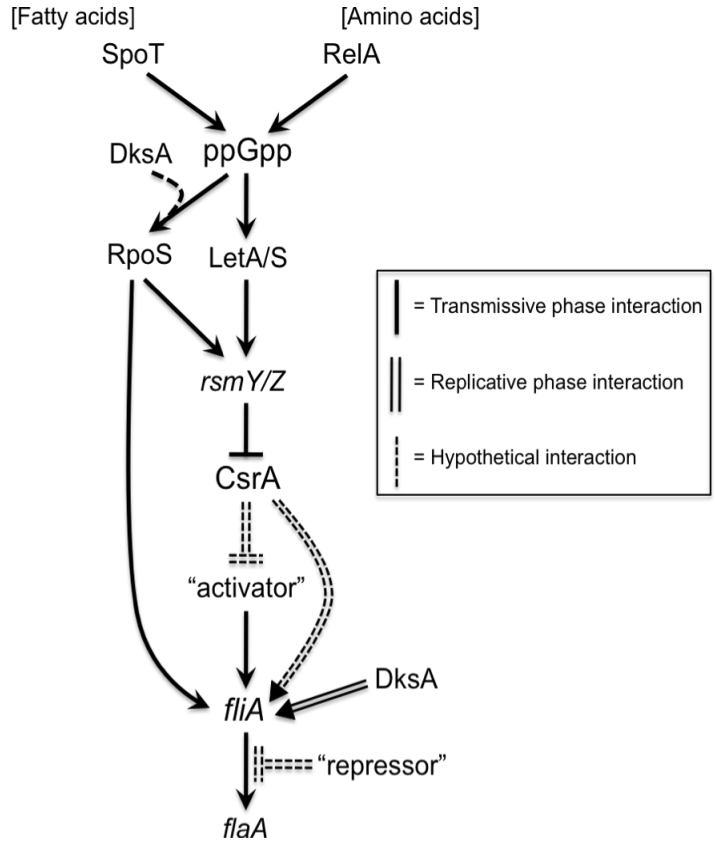

Figure 4.6. ppGpp synthesis leads to activation of RsmY/Z and derepression of a *fliA* gene activator.
In response to fatty acid and amino acid stress, SpoT and RelA synthesize ppGpp, leading to activation of the alternative sigma factor RpoS and the LetA/LetS two-component regulatory system. LetA and RpoS activate transcription of *rsmY* and *rsmZ*, two regulatory RNAs. RsmY/Z bind to CsrA to derepress an unidentified activator of *fliA* transcription. The FliA sigma factor activates the promoter of the *flaA* gene, encoding the filament subunit, facilitating final assembly of the flagellum. By an independent mechanism, RpoS also activates transcription of *fliA* in PE phase (Bachman & Swanson, 2004a)(Dalebroux, unpublished). In E phase, DksA activates the *fliA* promoter. Translation of *fliA* in E phase may be facilitated by a direct mechanism involving CsrA, since CsrA stabilizes *fliA* message (Fig. 4.5). However, the activity or amount of FliA protein is repressed in E phase. Solid, single lines indicate interactions occurring in the transmissive bacteria, double lines indicate interactions occurring in replicative bacteria, and dashed lines indicate speculative interactions.

CHAPTER V

CONCLUSION

Introduction

L. pneumophila can be isolated from aquatic environments around the world. In these ecosystems, bacteria reside in specialized vacuoles of freshwater protozoa and in complex surface associated communities like biofilms. As a consequence of their association with protozoa, the bacteria have evolved survival strategies that enable opportunistic infection of human alveolar macrophages. No case of human-to-human transmission of *L. pneumophila* has been reported. Therefore, man is indeed an accidental host and represents an evolutionary dead-end for the pathogen. Accordingly, selective pressure on *L. pneumophila* has come from association with amoebae.

Like all bacteria, the goal of *L. pneumophila* is to survive and replicate. As such, *L. pneumophila* has evolved to differentiate during its life cycle. Immediately prior to egress from an exhausted host, bacteria transform into the transmissive state. The motile, highly infectious, resistant form of the bacterium is equipped to disperse in the environment and primed to withstand defenses deployed upon its ingestion by a new host. Traits critical during transitory periods are superfluous once the new host has been breached. Thus, *L. pneumophila* has adapted to repress factors critical for transmission and activate those needed for replication. This highly controlled survival strategy makes *L. pneumophila* a model intracellular pathogen.

Metabolic cues and the bifunctional nature of SpoT

I began my thesis work studying novel metabolites that triggered *L. pneumophila* differentiation (Appendix A). My goal was to understand how metabolism impacted the life cycle of *L. pneumophila*. Previous work suggested amino acid availability dictates bacterial differentiation (Hammer & Swanson, 1999, Sauer et al., 2005); however, we demonstrated that *L. pneumophila* also monitors fatty acid metabolism. While studying the genetic requirements for the response to short chain fatty acids (SCFA; Appendix A), I confronted a discrepancy in the literature.

For some time, ppGpp accumulation was known to trigger rapid differentiation of bacteria. Seminal work by a former graduate student in our lab revealed that artificial induction of ppGpp synthesis led to immediate activation of *L. pneumophila* transmission traits (Hammer & Swanson, 1999). Subsequent studies questioned the validity of these findings. An independent group had demonstrated that the primary source of ppGpp in *L. pneumophila*, the RelA enzyme, was dispensable for intracellular growth in both amoebae and macrophages (Zusman et al., 2002). Given its dependence on amino acids for carbon (Tesh et al., 1983), and the long established role of RelA in responding to amino acid starvation in other γ-proteobacteria (Potrykus & Cashel, 2008), the finding that RelA was dispensable for *L. pneumophila* pathogenesis was surprising. More importantly, it directly challenged our model, which held that ppGpp initiates differentiation in broth and host cells.

At the time, much attention was being given to the bifunctional ppGpp synthetase/hydrolase SpoT and its exciting new role in monitoring fatty acid metabolism in *E.coli* and other Gram-negative bacteria (Battesti & Bouveret, 2006, Battesti &

Bouveret, 2009). Therefore, I predicted that SpoT might not only be critical for the response to SCFA but also could provide the enzymatic activity necessary for intracellular bacteria to differentiate. Indeed, this was the case (Appendix A, Chapter II). By studying SpoT, I embarked on a journey into the magical world of ppGpp.

Investigating the role of SpoT in *L. pneumophila* pathogenesis proved to be even more rewarding than expected. An under appreciated aspect of the differentiation model is that *L. pneumophila* undergoes two distinct transformations in host cells. The most commonly studied is the transition from the replicative to the transmissive state. However, when transmissive bacteria have successfully evaded host defenses, this virulence program becomes superfluous and bacteria must activate the replicative virulence program. To do so, *L. pneumophila* employs the RNA-binding protein CsrA to repress transmission traits and activate those needed for replication (Molofsky & Swanson, 2003). Previous studies suggested that *L. pneumophila* monitors nutrient availability to control replication. Specifically, mutants lacking the phagosomal transporter protein PhtA are locked in the transmissive state and fail to replicate in macrophages and amoebae (Sauer et al., 2005). Since PhtA functions as a transporter of the amino acid threonine, we deduced that *L. pneumophila* monitors metabolic cues to determine when conditions are suitable for replication. Consistent with this model, I determined that *L. pneumophila* mutants lacking SpoT hydrolase activity also fail to initiate replication in host cells (Chapter II). Therefore, ppGpp levels dictate the initiation of *L. pneumophila* replication in macrophages. Together, these findings allowed me to build the following working model.

Shortly after uptake, when bacteria have successfully evaded degradation in host lysosomes, *L. pneumophila* monitors vacuolar amino acid levels to control the ppGpp pool. If amino acids are abundant, SpoT degrades the alarmone. Data presented in Chapter IV suggests ppGpp transcription initiation of regulatory RNAs that repress CsrA activity, by decreasing the alarmone pool, *L. pneumophila* permits repression of transmission factors by CsrA. Thus, bacterial replication ensues.

L. pneumophila encodes 11 Pht paralogues, several of which are essential for intracellular replication (Chen et al., 2008)(Sauer, unpublished). In addition to amino acids, some Phts balance cellular nucleotide pools (Fonseca, unpublished). With the PhtA paradigm as a model, it will be important to determine the mechanism by which *L. pneumophila* controls degradation of ppGpp when nutrients are sufficient to sustain replication. Work outlined in this thesis suggests that in addition to its synthesis, ppGpp hydrolysis is also regulated (Chapter II). As such, genetic analyses of the ability of *relA* and *spoT* mutations to suppress the differentiation defect of intracellular *pht* mutant bacteria should prove fruitful.

Consistent with the longstanding model that ppGpp controls differentiation in host cells, the genetic analyses described in Chapter II demonstrate that SpoT is also critical for *L. pneumophila* to transit between macrophages. Although predicted, the result was telling for a number of reasons. For many years our lab has studied regulators of the replicative to transmissive phase transition. Factors like the LetA/LetS two-component system, the FliA sigma factor and the LetE transmission trait enhancer are critical for differentiation in broth, but dispensable for bacteria to transit between macrophages (Hammer et al., 2002), suggesting differentiation in broth does not fully model infection.

The intracellular survival defects of *relA spoT* double mutant *L. pneumophila* reveal that, unlike other regulators, ppGpp is essential during infection. Therefore, the *relA spoT* double mutant will be a valuable tool to discern the genetic requirements for *L. pneumophila* to spread in macrophage culture.

Another intriguing aspect of the macrophage infection biology is that SpoT activity is sufficient. Relative to the RelA enzyme, SpoT is a weak ppGpp synthetase that accounts for a modest amount of the total pool of ppGpp in broth grown bacteria (Chapter II). However, SpoT-dependent ppGpp is sufficient to activate nearly all transmission traits in broth and to facilitate host-to-host transmission in macrophages (Chapter II). The specific requirement for SpoT during infection is not limited to *L. pneumophila*. In other pathogenic γ-proteobacteria, like *Yersinia pestis*, and *S. enterica* serovar Typhimurium, SpoT is sufficient to cause disease in mammals (Sun *et al.*, 2009, Pizarro-Cerda & Tedin, 2004). These patterns raise the possibility that a classic, RelA-dependent stringent response to amino acid starvation is rarely demanded within mammalian hosts. Perhaps during each of these bacterial infections, stimuli that trigger ppGpp accumulation *in vivo* are sensed by SpoT specifically. It is also possible that a modest amount of alarmone is sufficient to induce the essential subset of virulence factors, since SpoT is a weaker synthetase than RelA in some bacteria (Potrykus & Cashel, 2008). Alternatively, for bacteria to survive in hostile host environments, the ability of SpoT to balance alarmone levels through hydrolysis may outweigh the need for robust ppGpp synthesis. In particular, an interaction between SpoT and a protein involved in fatty acid biosynthesis, acyl-carrier protein (ACP), may be critical for

pathogens like *E. coli*, *P. aeruginosa* and *L. pneumophila* to regulate the dual activities of the SpoT enzyme (Battesti & Bouveret, 2009)(Chapter II).

L. pneumophila's differentiation in response to fatty acid supplementation (Appendix A) led to the hypothesis that SpoT monitors fatty acid biosynthesis through specific interactions with key components of this pathway to influence bacterial differentiation. Indeed, bacteria expressing a *spoT* allele predicted to encode a protein incapable of interacting with ACP failed to respond to fatty acid perturbation and were locked in the transmissive state (Chapter II). These findings suggested that *L. pneumophila* has evolved a SpoT-dependent stringent response to fatty acid stress, likely through an interaction between SpoT and ACP that controls enzyme activity. Currently, the specific stimuli that trigger intracellular *L. pneumophila* to differentiate are unknown. However, taken together, the potential ACP-SpoT interaction, the phenotypic response to SCFA, and the dual role of SpoT during macrophage infection motivate further study of how fatty acid metabolism impacts the behavior of intracellular *L. pneumophila*.

DksA and flagellar regulation by ppGpp

The first part of my thesis focused on understanding how metabolic cues influence ppGpp levels in *L. pneumophila*, and then the second half was devoted to understanding downstream regulation by the alarmone. These investigations led to the characterization of a second factor important for the *L. pneumophila* stringent response, namely DksA, and also exposed ppGpp as a critical regulator of mRNA stability in *L. pneumophila*.

The rationale to study DksA was strong given its role in mediating the cellular response to ppGpp in other γ-proteobacteria (Chapter I). Independent regulation by ppGpp and DksA is an exciting new avenue of research in the RNAP field (Magnusson et al., 2007, Aberg et al., 2009, Lyzen et al., 2009, Merrikh et al., 2009b). *L. pneumophila* proved an important example of a pathogen that has evolved DksA to work in concert with, and independently, of ppGpp.

Studies using broth grown bacteria suggest *L. pneumophila* requires cooperative regulation by ppGpp and DksA for activation of all transmission traits; however, to move from macrophage-to-macrophage, bacteria require ppGpp but not DksA (Chapter III). Thus, DksA-independent factors contribute to host-to-host transmission of *L. pneumophila*. Since other *L. pneumophila* regulators exhibit a similar phenotypic pattern (required in broth, but dispensable in macrophages), the impact of DksA was not entirely surprising. However, my phenotypic analysis was the first indication that the regulatory relationship between ppGpp and DksA was complex. Upon closer inspection, independent regulation was also observed during flagellar biogenesis, a hallmark of *L. pneumophila* differentiation.

Gene expression studies show that *L. pneumophila* responds to elevated ppGpp by deactivating ribosomal gene expression and inducing flagellar gene expression (Chapter III). Although both protein synthesis and flagellar biosynthesis likely confer significant energetic costs on the bacterial cell, the need for *L. pneumophila* to find its next meal demands a mode of transportation. Therefore, *L. pneumophila* has evolved signal transduction pathways that use ppGpp and DksA to initiate flagellar assembly.

The typical bacterial flagellum is comprised of ~ 20,000 filament subunits (Chevance & Hughes, 2008). In *L. pneumophila*, the FliA sigma factor controls transcriptional activation of the gene encoding the major flagellar filament subunit, *flaA*. My work demonstrates that to control final assembly of the flagellum, *L. pneumophila* regulates FliA on numerous levels. During replication, DksA maintains a basal level of *fliA* transcription, independently of ppGpp (Chapter III). Studies described in Chapter IV suggest CsrA may facilitate translation of this basal message when nutrients are abundant, since over-expression of *csrA* stabilizes the *fliA* transcript. Any FliA protein produced during replication is not sufficient to activate the *flaA* promoter, since it remains quiescent under these conditions. These findings are the first to suggest that even with nutrients present *L. pneumophila* regulates *fliA* at the level of transcription, and perhaps even post-transcriptionally.

More specifically, *L. pneumophila* likely maintains a basal level of FliA protein during replication, but holds the transcription factor inactive until conditions deteriorate. Electromobility shift assays with purified CsrA and *fliA* mRNA to test direct interaction, along with application of *fliA* translational fusions and FliA westerns to monitor protein production and levels should prove fruitful in understanding this regulation.

Deteriorating vacuolar conditions necessitate rapid robust activation of the flagellar sigma factor. As ppGpp accumulates, both the activity of the *fliA* promoter and the level of *fliA* mRNA increase (Chapter III). Concomitant with *fliA* mRNA accumulation, the activity of the *flaA* promoter increases, as does *flaA* message, indicating that the FliA protein is now active. In Chapter IV, I demonstrate that ppGpp governs the activation of *fliA* and *flaA* in part by controlling transcriptional activation of

the non-coding regulatory RNAs, RsmY and RsmZ. The exact mechanism remains to be determined, but the alarmone likely influences the activity of RpoS and/or LetA/S, which we and others have shown also contribute (Chapter IV) (Hovel-Miner et al., 2009, Sahr et al., 2009, Rasis & Segal, 2009). Studies of how ppGpp controls the activity of the LetA/S system in particular should prove to be rewarding, as ppGpp might regulate the activity of the proteins themselves.

By controlling expression of RsmY/Z, ppGpp influences the ability of CsrA to control an undefined activator of *fliA* transcription (Fig. 5.1). Although, two candidates were tested, RpoS and FleQ, neither proved promising. Thus, it is possible that an additional activator of the *fliA* promoter exists in *L. pneumophila* and that the mRNA encoding this factor is destabilized by CsrA. In addition to flagellar biogenesis, CsrA has been shown to repress expression of effectors of the Dot/Icm Type IV secretion system, as well as elements critical for *L. pneumophila* resistance to lysosomal degradation (Molofsky & Swanson, 2003, Rasis & Segal, 2009). Thus, *L. pneumophila* has evolved multiple mechanisms to exploit ppGpp to control mRNA stability of key virulence pathways.

Concluding remarks

To monitor and adapt to their environment, bacteria rely on sensory systems to regulate complex physiological processes. A wide variety of pathogens couple their specific virulence pathways with more general adaptations, like stress resistance, by integrating dedicated regulators with global signaling networks. Many pathogenic bacteria rely on nucleotide alarmones to cue metabolic disturbances and coordinate

survival and virulence programs. Work described here has merely broken the surface of the vast regulatory roles ppGpp plays for *L. pneumophila*. I have demonstrated that alarmone levels must be controlled when vacuolar conditions demand bacterial replication, as well as when they necessitate escape. Thus, *L. pneumophila* monitors intracellular cues to control both the replicative and the transmissive virulence programs. As with many pathogens, the signals sensed by *L. pneumophila* in the host environment remain elusive. By understanding why *L. pneumophila* differentiates, and in response to what, our knowledge of its host-pathogen relationship will continue to expand. Its strict reliance on ppGpp during its intracellular lifecycle makes *L. pneumophila* an attractive experimental model to understand how metabolic cues are transmitted by second messenger signaling pathways to govern bacterial virulence.

Figure 5.1.

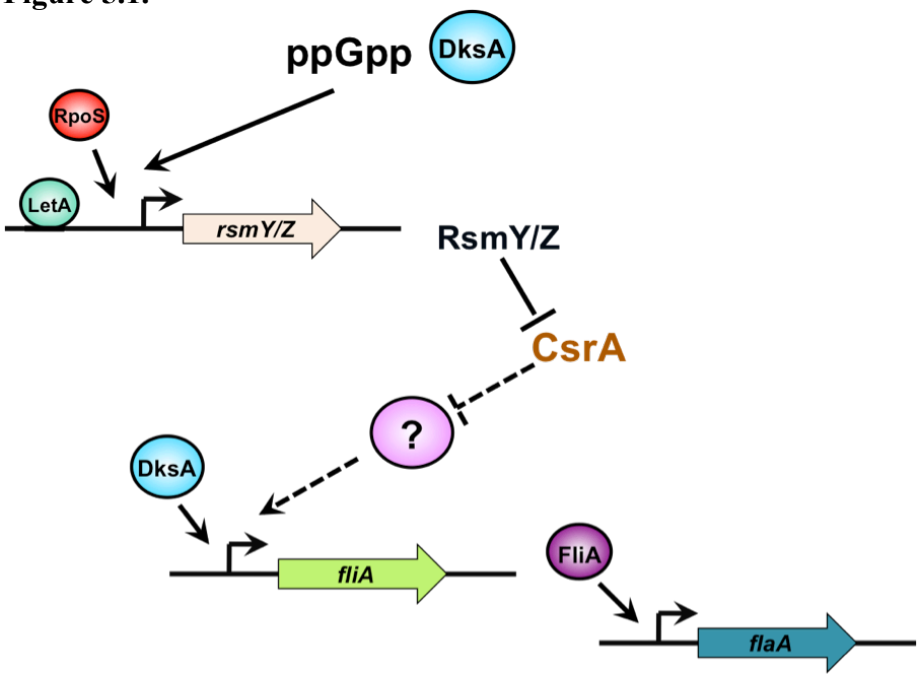

Figure 5.1. ppGpp and DksA control transcriptional activation of RsmY/Z to derepress *fliA*, a transcriptional activator of *flaA*.
During stress, ppGpp and DksA activate transcription of two regulatory RNAs *rsmY* and *rsmZ* likely by an indirect mechanism involving LetA and RpoS. RsmY/Z bind to CsrA to derepress an unidentified activator of *fliA* transcription. The FliA sigma factor activates the promoter of the *flaA* gene, encoding the filament subunit, facilitating final assembly of the flagellum. When nutrients are abundant, DksA activates the *fliA* promoter, a mechanism that may serve to maintain a basal level of *fliA* mRNA, and or protein. Solid lines indicate known regulatory interactions and dashed lines indicate putative interactions.

APPENDIX

Legionella pneumophila couples fatty acid flux to microbial differentiation and virulence

Summary

During its life cycle, *Legionella pneumophila* alternates between at least two phenotypes: a resilient, infectious form equipped for transmission and a replicative cell type that grows in amoebae and macrophages. Considering its versatility, we postulated that multiple cues regulate *L. pneumophila* differentiation. Beginning with a Biolog Phenotype MicroArray screen, we demonstrate that excess short chain fatty acids (SCFAs) trigger replicative cells to cease growth and activate their panel of transmissive traits. To coordinate their response to SCFAs, *L. pneumophila* utilizes the LetA/LetS two-component system, but not phosphotransacetylase or acetyl kinase, two enzymes that generate high-energy phosphate intermediates. Instead, the stringent response enzyme SpoT appears to monitor fatty acid biosynthesis to govern transmission trait expression, as an altered distribution of acylated acyl carrier proteins correlated with the SpoT-dependent differentiation of cells treated with either excess SCFAs or the fatty acid biosynthesis inhibitors cerulenin and TOFA. We postulate that, by exploiting the stringent response pathway to couple cellular differentiation to its metabolic state, *L. pneumophila* swiftly acclimates to stresses encountered in its host or the environment, thereby enhancing its overall fitness.

Introduction

Legionella pneumophila is a promiscuous, gram-negative pathogen commonly found in freshwater systems. In these environments, *L. pneumophila* efficiently parasitizes many different species of amoebae and protozoa (Fields *et al.*, 2002). Moreover, *Legionella* can establish biofilms in both natural and potable water systems, which serve as reservoirs of contamination (Fields et al., 2002). If susceptible individuals aspirate bacteria-laden aerosols, the pathogen can colonize the alveolar macrophages to cause the severe pneumonia Legionnaires' disease. Due to the disparate conditions under which *L. pneumophila* can survive, the bacteria must utilize mechanisms to monitor their milieu and swiftly acclimate to their surroundings.

To tolerate environmental fluctuations, many bacteria alter their cellular physiology and morphology, a process known as differentiation. The sexually transmitted bacterium *Chlamydia trachomatis* alternates between an extracellular, metabolically inert elementary body required for transmission and an intracellular, metabolically active reticulate body that undergoes repeated cycles of cell division (Abdelrahman & Belland, 2005). Likewise, the etiologic agent of human Q fever, *Coxiella burnetii*, differentiates between a replicative large cell variant and a resilient small cell variant (Heinzen *et al.*, 1999). Within biofilm communities, the opportunistic pathogen *Pseudomonas aeruginosa* alternates between distinct motile and non-motile cell types (Purevdorj-Gage *et al.*, 2005). By employing cellular differentiation, bacterial pathogens can evade host defense mechanisms and promote self-preservation. Ground-breaking work by Rowbotham revealed that, within amoebae, *L. pneumophila* also exhibits two distinct phenotypes: a non-motile, thin-walled replicative form and a motile, thick-walled infectious form that contains stores of an energy-rich polymer

(Rowbotham, 1986). Corroborating these early findings, subsequent genetic and molecular studies determined that the replicative and transmissive phases of the *L. pneumophila* life cycle are reciprocal (Byrne & Swanson, 1998, Hammer & Swanson, 1999, Alli et al., 2000, Watarai *et al.*, 2001, Wieland *et al.*, 2002, Molofsky & Swanson, 2003). The current model suggests that when phagocytes cells engulf transmissive *L. pneumophila*, the bacteria avoid lysosomal degradation by establishing vacuoles isolated from the endosomal network, a process mediated by the Dot/Icm type IV secretion system and its substrates, as well as by vesicles shed from the outer membrane (Berger & Isberg, 1993, Berger *et al.*, 1994, Joshi *et al.*, 2001, Fernandez-Moreira et al., 2006). If conditions in the vacuole are favorable, the RNA-binding protein CsrA and the sRNA chaperone Hfq repress transmissive traits, enabling *L. pneumophila* to replicate profusely (Fettes et al., 2001, Molofsky & Swanson, 2003, McNealy *et al.*, 2005). Once nutrient supplies are exhausted, replication halts, and the progeny initiate a global change in their physiology known as the stringent response (Hammer & Swanson, 1999, Zusman et al., 2002). This pathway generates the alarmone ppGpp, which coordinates bacterial differentiation. In particular, a major shift in the *L. pneumophila* transcriptional profile is mediated by alternative sigma factors, while the LetA/LetS two-component system relieves CsrA repression on transmissive traits (Hammer & Swanson, 1999, Hammer et al., 2002, Molofsky & Swanson, 2003, Bruggemann et al., 2006). As a consequence, *L. pneumophila* expresses a panel of traits that are vital for dissemination, including cytotoxicity, motility and lysosome evasion (Bachman & Swanson, 2001, Hammer et al., 2002, Lynch *et al.*, 2003, Bachman & Swanson, 2004a, Bachman & Swanson, 2004b, Jacobi et al., 2004). In addition, *L. pneumophila* may further develop into the highly

resilient and infectious mature intracellular form (MIF) under defined conditions (Faulkner & Garduno, 2002). Eventually, the exhausted host cell lyses, releasing transmissive *L. pneumophila* into the environment, which can then initiate subsequent rounds of infection.

Amino acid concentrations appear to be a critical metabolic cue, as fluctuations in their availability alter the developmental state of the microbe. For example, intracellular *L. pneumophila* rely on PhtA, a transporter of the Major Facilitator Superfamily, to gauge whether the threonine supply is sufficient to sustain growth (Sauer et al., 2005). Furthermore, macrophages require the amino acid transporter SLC1A5 to support replication of intracellular *L. pneumophila* (Wieland *et al.*, 2005). Studies of broth cultures predict that when amino acid supplies are depleted, uncharged tRNAs accumulate, and the stringent response enzyme RelA produces the ppGpp signaling molecule which triggers *L. pneumophila* differentiation (Hammer & Swanson, 1999, Zusman et al., 2002).

Since *Legionella* persist in diverse environments, we postulated that signals other than amino acids also induce their differentiation. Indeed, for transmission between macrophages, *L. pneumophila* requires SpoT (Dalebroux et al., 2009), a second ppGpp synthetase known to equip *Escherichia coli* to generate the alarmone in response to a variety of stresses, such as phosphate starvation or inhibition of fatty acid biosynthesis (Seyfzadeh et al., 1993, Zusman et al., 2002, Gong *et al.*, 2002, Magnusson et al., 2005, Battesti & Bouveret, 2006). By screening hundreds of metabolites via Biolog Phenotype MicroArrays, and then applying a series of pharmacological, biochemical and genetic tests, we determined that, in response to perturbations in fatty acid biosynthesis,

replicative *L. pneumophila* rely on SpoT to activate the stringent response pathway and coordinately express transmissive traits, thereby coupling phase differentiation to their metabolic state.

Experimental procedures

Bacterial strains and cultures. *L. pneumophila* strain Lp02 (*thyA hsdR rpsL*; MB110), a virulent thymine auxotroph, was the parent for all strains constructed (Berger & Isberg, 1993). To obtain *letA* and *letS* mutants lacking pflaG (MB413 and MB416, respectively), the mutant alleles from MB414 and MB417 were transferred onto the Lp02 chromosome by natural competence (Stone & Kwaik, 1999). *relA* (MB696) and *relA spoT* (MB697) mutants were generated using standard techniques as described elsewhere (Dalebroux et al., 2009). To monitor the induction of the *flaA* promoter, the *relA* and *relA spoT* mutants were transformed with the pflaG plasmid to generate MB684 and MB685, respectively (Table A.1).

To construct a *pta ackA2* (*lpg2261* and *lpg2262*) deletion mutant, the 3.3 kb *pta ackA2* locus was amplified from Lp02 genomic DNA using forward primer 5'-GCAACTCGTATGCCATAC and reverse primer 5'-GTAAATCCATCGCTTTGGG. The PCR fragment was purified and ligated to pGEM-T (Promega), transformed into *E. coli* DH5α, and the resulting plasmid designated as pGEM-T-PtaAckA2 (MB619). A 1.8 kb region of the *pta ackA2* open reading frame was removed by digestion with XmaI and NheI, and the remaining pGEM-T-PtaAckA2 fragment was blunted with Klenow and treated with Antarctic phosphatase (New England Biolabs). The 1.3 kb kanamycin resistance cassette from pUC4K was removed via EcoRI digestion, blunted with Klenow and ligated into the digested pGEM-T-PtaAckA2 plasmid to create pGEM-T-

PtaAckA2::Kan (MB681). After verification by PCR, the deletion/insertion alleles were transformed into Lp02 via natural competence and selected for by antibiotic resistance (Stone & Kwaik, 1999). The desired chromosomal mutation was confirmed by PCR and the resulting strain designated as MB641 (Table A.1). To monitor the induction of the *flaA* promoter by fluorometry, MB641 was transformed with pflaG. Two independent isolates were tested in fluorometry assays and found to be similar; MB682 data are displayed.

Bacteria were cultured at 37°C in 5 ml aliquots of *N*-(2-acetamido)-2-aminoethanesulfonic acid (ACES; Sigma)-buffered yeast extract (AYE) broth and supplemented with 100 µg/ml thymidine when necessary. Cultures having an optical density at 600 nm (OD_{600}) of 0.5 to 0.85 were defined as exponential (E), and those of OD_{600} 3.4 to 4.5 as post-exponential (PE). To obtain colony-forming units (CFU), *L. pneumophila* were plated on ACES-buffered charcoal-yeast extract agar supplemented with 100 µg/ml thymidine (CYET) and incubated at 37°C for 4-5 days.

Biolog Phenotype MicroArray™ Analysis. Phenotype MicroArray™ (PM) plates were purchased from Biolog (Hayward, CA; Table A.2). 100 µl of E phase MB355 cultured in AYE media was added to each well of the Biolog plates, and the plates were incubated at 37°C while shaking. After 3 or 6 h, 200 µL of each culture was transferred to black, clear-bottom tissue culture plates (Costar), and the relative fluorescence intensity was quantified using a Synergy™ HT microplate reader (Bio-Tek) using 485 nm excitation, 530 nm emission and sensitivity of 50. Inducers were defined as having a 1.4-7.3 mean

fold increase in fluorescence at 6 h when compared to the negative control well of the Biolog plates in at least 3 independent experiments.

Fluorometry. To monitor expression of the flagellin promoter, *L. pneumophila* strains containing the *flaAgfp* reporter plasmid pflaG were cultured in AYE media. At OD_{600} = 0.50-0.85 (T = 0), the cultures were supplemented with 10 mM acid, 0.5 μg/ml cerulenin (Sigma) or 5 μg/ml 5-(tetradecyloxy)-2-furoic acid (TOFA; Cayman Chemical). Cultures supplemented with water or DMSO served as negative and vehicle controls, respectively. At the times indicated, culture cell density was measured as OD_{600}. To analyze similar bacterial numbers, aliquots were collected by centrifugation, and the cell densities were normalized to OD_{600} = 0.01 in PBS. The fluorescence intensity of a 200 μL aliquot was measured as described above.

Motility. To qualitatively assess motility, 10 μl wet mounts of broth-grown *L. pneumophila* were prepared and immediately examined by phase-contrast microscopy. Relative motility levels were based on at least 3 independent observations of fields that contained several hundred microbes. A scored of (-) was assigned to cultures that were <10% motile; (+) indicates cultures that were 10-25% motile; (++) indicates cultures that were 25-75% motile; and (+++) indicates that > 75% of the microbes within the fields displayed high levels of directed motility.

Macrophages. Macrophages isolated from femurs of female A/J mice (Jackson Laboratory) were cultured in RPMI-1640 containing 10% heat-inactivated fetal bovine

serum (RPMI/FBS; Gibco BRL) as described previously (Swanson & Isberg, 1995). Following a 7-day incubation in medium supplemented with L-cell supernatant, macrophages were plated at either 5×10^4 or 2.5×10^5 per well for cytotoxicity and degradation assays, respectively.

Cytotoxicity. To measure contact-dependent cytotoxicity of *L. pneumophila* for macrophages, PE bacteria or E phase cultures supplemented with water or 10 mM fatty acids for 3 h were added to monolayers at the indicated multiplicities of infection (MOI). After centrifugation at 400 × g for 10 min at 4°C (Molofsky et al., 2005), the cells were incubated for 1 h at 37°C. To quantify macrophage viability, RPMI/FBS containing 10% alamarBlue™ (Trek Diagnostic Systems) was added to the monolayers for 6-12 h, and the reduction of the colorimetric dye was measured spectrophotometrically as described (Molofsky et al., 2005).

Degradation. The percentage of intracellular *L. pneumophila* that remained intact after a 2 h macrophage infection was quantified by fluorescence microscopy. Briefly, macrophages were plated at 2.5×10^5 onto coverslips in 24 well plates. Then, PE bacteria or E phase microbes exposed to either water or 10 mM fatty acids for 3 h were added to macrophage monolayers at an MOI ~ 1. The cells were centrifuged at 400 × g for 10 min at 4°C and then incubated for 2 h at 37°C. Uninternalized bacteria were removed by washing the monolayers with 3 × 0.5 ml RPMI/FBS. Control experiments indicate that < 1% of the cell-associated bacteria are extracellular following the three RPMI/FBS washes (Bachman & Swanson, 2004a)(data not shown). Macrophages were

then fixed, permeabilized and stained for *L. pneumophila* as described (Molofsky et al., 2005).

Sodium sensitivity. To calculate the percentage of *L. pneumophila* that are sensitive to sodium, PE bacteria or E cultures supplemented with either water or 10 mM fatty acids for 3 h were plated onto CYET with or without100 mM NaCl. After 6 days at 37°C, CFUs were enumerated and the percentage of sodium sensitive microbes calculated as described (Byrne & Swanson, 1998).

Analysis of acyl-ACPs. For purification of acyl-ACPs, WT *L. pneumophila* were cultured to the E phase at 37°C on an orbital shaker in 250 ml AYE containing 100 μg/ml thymidine. Upon reaching an OD_{600} between 0.5-0.85, the cultures were supplemented with water, 10 mM fatty acid or 0.5 μg/ml cerulenin and then cultured for an additional 3 h. After collection by centrifugation at 4,000 × g for 20 min at 4°C, the cell pellets were stored at -80°C. Once thawed on ice, the pellets were resuspended in 12.5 ml ACP buffer (200 mM NaCl, 20 mM Tris-HCl, pH 6, 1 mM EDTA). To reduce protein degradation, one tablet of a protease inhibitor cocktail (Roche) was added to each 12.5 ml suspension. Cells were lysed by sonication and the lysates cleared by centrifugation at 7,000 × g for 1 h at 4°C. Since *L. pneumophila* is predicted to contain three ACPs ranging from 8.6-15.3 kDa, large molecular weight proteins were removed from the lysates via 50K and 30K centrifugal filter devices (Amicon Ultra, Millipore # UFC905024 and UFC903024, respectively). The remaining ACP fractions were concentrated with 5K centrifugal filter devices (# UFC800524), which also removed small molecular weight proteins and salts.

The protein concentration of each sample was determined using the Bio-Rad Protein Assay, and samples were stored at -20°C. To visualize the profile of intracellular acyl-ACPs, 13% nondenaturing gels were prepared, and urea was added to either 0.5 M or 2.5 M for short chain fatty acid (SCFA) or long chain fatty acid (LCFA) gels, respectively (Rock & Cronan, 1981, Jackowski & Rock, 1983, Post-Beittenmiller *et al.*, 1991). After electrophoresis in 192 mM glycine, 25 mM Tris buffer, samples were transferred to polyvinylidene difluoride (PVDF) membrane (Bio-Rad), and the membranes blocked in TBS-T (20 mM Tris-HCl, pH 7.5, 500 mM NaCl, 0.05% Tween 20) containing 5% nonfat milk. To detect the *L. pneumophila* ACP proteins, the membranes were probed with a primary antibody generated by *E. coli* ACP (gift from C. O. Rock, Memphis, TN) diluted 1:500 and a secondary goat anti-rabbit antibody conjugated to horseradish peroxidase (Pierce) diluted 1:8000 (Jackowski & Rock, 1983) and then developed with SuperSignal® West Pico Chemiluminescent Substrate (Pierce).

Detection of ppGpp. Accumulation of the ppGpp signaling molecule in response to flux in fatty acid metabolism was detected by thin-layer chromatography (TLC) as described (Cashel & Gallant, 1969, Cashel, 1994, Hammer & Swanson, 1999). Briefly, E phase *relA* (MB696) and *relA spoT* (MB697) cultures were diluted to an $OD_{600} = 0.25$ and cultured at 37°C on a roller drum with approximately 100 µCi/ml carrier-free [^{32}P]-phosphoric acid (ICN Pharmaceuticals) for 6 h, or two generation times. Next, cultures were supplemented with water, 10 mM acetic acid or 10 mM propionic acid and incubated for an additional 1.5 h at 37°C. To extract the nucleotides, 50 µl aliquots were removed from each culture and added to 13 M formic acid and then incubated on ice for

15 min. Samples were subjected to two freeze-thaw cycles and stored at -80°C until used for chromatography. Formic acid extracts (25 µl) were applied to a PEI-cellulose TLC plate (20 × 20) and developed with 1.5 M KH_2PO_4, pH 3.4 as described (Cashel & Gallant, 1969, Cashel, 1994, Hammer & Swanson, 1999). TLC plates were exposed to autoradiography film for 72 h and developed in a phosphoimager. To monitor growth following water or fatty acid supplementation, optical densities were determined for non-radioactive cultures grown under identical conditions.

Results

Biolog Phenotype MicroArrays identify novel cues of L. pneumophila differentiation

To identify signals that trigger *L. pneumophila* differentiation, we employed Biolog Phenotype MicroArrays to screen various sources of carbon, nitrogen, sulfur and phosphorous. Exponential (E) *L. pneumophila* carrying a *gfp* reporter for flagellin, a marker of transmissive bacteria (Table A.1), were cultured in the plates, and their relative fluorescence was monitored. Of the 387 compounds screened, only 22 (6%) induced *flaAgfp* expression prematurely (Table A.2). Among these were deoxyadenosine, deoxyribose, 2-deoxy-D-glucose-6-phosphate, dihydroxyacetone, nitrite, hydroxylamine, parabanic acid and methionine-alanine dipeptide. However, the predominant class of compounds (12 of 22) was carboxylic acids. In particular, the four SCFAs formic, acetic, propionic and butyric acid and the medium chain fatty acid caproic acid all triggered *flaAgfp* expression. Also eliciting a positive response were two detergents, Tween 20 and Tween 80; however, both detergents also contain the carboxylic acid groups lauric and oleic acid, respectively. Indeed, when exposed to 5 mM lauric acid, a 12 carbon carboxylic acid, *L. pneumophila* also stopped replicating and expressed *flaAgfp* (data not

shown). However, the response to lauric acid was slower and less robust than to the SCFAs, likely due to the requirement for receptor-mediated transport across the *L. pneumophila* membrane and perhaps the time needed for the fatty acid to be degraded via b-oxidation. Interestingly, high concentrations of SCFAs inhibit the growth of many microorganisms (Bohnhoff *et al.*, 1964), including *L. pneumophila* (Warren & Miller, 1979). Moreover, acetate, propionate and butyrate regulate *Salmonella enterica* serovar Typhimurium invasion gene expression *in vitro* at concentrations that correlate with their abundance in the intestinal tract (Lawhon *et al.*, 2002). Therefore, we postulated that *L. pneumophila* monitors SCFAs to coordinate its life cycle.

Excess short chain fatty acids inhibit L. pneumophila growth and induce motility

Acetic and propionic acid, which were identified in the Phenotype MicroArrays screen, were selected for further analysis as previous data indicate that both regulate virulence genes in *S. enterica* serovar Typhimurium, albeit with opposite effects (Lawhon et al., 2002). As predicted, when E cultures were treated with either 10 mM acetic or propionic acid, *L. pneumophila* immediately stopped replicating (Fig. A.1A and C) and activated the *flaA* promoter (Fig. A.1B and D). The growth inhibition of *L. pneumophila* treated with the SCFAs was not attributed to a loss in viability, as judged by enumerating CFU (data not shown). In contrast, control cultures supplemented with water did not induce the *flaA* promoter or halt replication until the transition to the post-exponential (PE) phase, which occurred at 9 h (corresponds to $OD_{600} \approx 3.4$; Fig. A.1, circles). Titration experiments analyzing a range from 2.5 to 20 mM demonstrated that 10 mM

SCFAs was optimal, as lower concentrations failed to significantly activate the *flaA* promoter or inhibit growth (data not shown).

To determine whether the response by *L. pneumophila* to SCFAs was a consequence of alterations in pH, E bacteria were instead treated with two inorganic acids. When added to concentrations of 1.25 to 20 mM, neither hydrochloric nor perchloric acid triggered growth inhibition or induction of the *flaAgfp* promoter (Fig. A.1E and F; data not shown). Moreover, the pH of *L. pneumophila* cultures supplemented with acetic or propionic acid did not differ significantly from those supplemented with water, nor did the pH of the treated cultures vary detectably over the course of the experiment (data not shown). Finally, when E cultures were supplemented with non-acidic forms of acetate, *L. pneumophila* stopped replicating and activated the flagellin promoter. For example, when supplemented with 10 mM calcium acetate for 6 h, E cultures showed a 28 fold induction of *flaAgfp* when compared to water control (data not shown). Therefore, *L. pneumophila* respond to a signal generated by SCFAs that is distinct from pH.

Fatty acid supplementation stimulates L. pneumophila differentiation

We next investigated whether SCFAs trigger other *L. pneumophila* transmissive phase phenotypes, including motility, cytotoxicity to phagocytic cells, avoidance of lysosomal degradation and sodium sensitivity (Byrne & Swanson, 1998). As expected from the *flaAgfp* data (Fig. A.1), microscopic examination revealed that 10 mM SCFAs induced motility (Table A.3). Also, after supplementation with either acetic or propionic acid, E phase *L. pneumophila* became as cytotoxic to macrophages as PE control cultures

(Fig. A.2A). Importantly, the addition of SCFAs alone was not cytotoxic (Table A.3, *letA* and *letS* mutants; data not shown). Further, although only 15% of E phase control *L. pneumophila* avoided degradation, > 50% of those exposed to acetic or propionic acid remained intact (Fig. A.2B). Finally, 10 mM acetic or propionic acid also triggered sodium sensitivity in E phase microbes (Fig. A.2C). Thus, our Biolog screen accurately predicted that exposure to 10 mM SCFAs induces the *L. pneumophila* transmissive phenotype. Moreover, our studies support previous data that indicate that the *flaAgfp* reporter is a valid marker of *L. pneumophila* differentiation (Hammer & Swanson, 1999, Sauer et al., 2005, Bruggemann et al., 2006). Accordingly, we next investigated two potential modes of action: Excess SCFA may either generate high-energy intermediates that activate two-component phosphorelay systems or instead alter fatty acid metabolism.

To respond to fatty acids, L. pneumophila requires the LetA/LetS two-component system, but not generation of acetyl-phosphate and propionyl-phosphate

The *L. pneumophila* two-component system LetA/LetS regulates all known transmissive phase phenotypes (Lynch et al., 2003). To discern whether the response of *L. pneumophila* to SCFAs depends on this signal transduction system, we exploited *letA* and *letS* mutants. When confronted with SCFAs, the *letA* and *letS* mutants resembled wild-type (WT) *L. pneumophila* by restricting their growth (Table A.3). However, *L. pneumophila* required the LetA/LetS system to induce flagellin expression in response to 10 mM acetic acid (Fig. A.3A) and also the stronger inducer propionic acid, albeit to a lesser degree (Fig. A.3B). The two-component system was also largely required for expression of four other transmissive traits: motility, cytotoxicity, lysosome avoidance

and sodium sensitivity (Table A.3). Therefore, when *L. pneumophila* encounters a sudden increase in SCFAs, a pathway that includes LetA/LetS coordinates bacterial differentiation.

The response regulators of many two-component systems can use the high-energy intermediates acetyl-phosphate and propionyl-phosphate to catalyze their own phosphorylation (Wolfe, 2005). Therefore, we tested whether exogenous acetic or propionic acid are first converted to acetyl- and propionyl-phosphate before activating the LetA/LetS signal transduction system by analyzing a *L. pneumophila* mutant that lacks the two enzymes that synthesize the phosphate intermediates, phosphotransacetylase and acetyl kinase, encoded by the *pta* and *ackA2* genes, respectively (McCleary *et al.*, 1993, Wolfe, 2005). By monitoring the activity of the flagellin promoter, it was evident that neither the phosphotransacetylase nor the acetyl kinase enzyme was needed for *L. pneumophila* to differentiate when confronted by excess acetic or propionic acid (Fig. A.4). Thus, unlike *Salmonella* and *Bordetella* (Boucher *et al.*, 1994, Lawhon et al., 2002), SCFAs trigger *L. pneumophila* differentiation by a mechanism other than generating acetyl- and propionyl -phosphate intermediates to activate LetA/LetS.

Perturbations in fatty acid biosynthesis trigger L. pneumophila differentiation

To begin to test the hypothesis that SCFA supplements impinge upon either fatty acid degradation or biosynthesis, we tested whether acetic and propionic acid trigger *L. pneumophila* differentiation when the irreversible conversion of acetyl-CoA to malonyl-CoA is blocked. In mammalian cells, the competitive inhibitor TOFA blocks the acetyl-CoA carboxylase (ACC) complex (*accA*, *lpg0785*; *accB*, *lpg0463*; *accC*, *lpg0462* and

accD, *lpg1341*) and prevents acetate from being incorporated into fatty acids (Fig. A.5A; (McCune & Harris, 1979, Magnuson *et al.*, 1993, Panek *et al.*, 1977). Accordingly, malonyl-CoA levels in the cell are significantly reduced and fatty acid biosynthesis is halted. When cultures were simultaneously supplemented with SCFAs and TOFA, the majority of bacteria failed to differentiate (Fig. A.5B and C). When treated with TOFA alone, *L. pneumophila* did not differentiate, although their growth was restricted and viability was maintained, as the number of CFU was similar between 0 and 24 h after TOFA treatment (data not shown). Since activity of the ACC complex appeared to be required for SCFAs to initiate *L. pneumophila* differentiation, we deduced that addition of 10 mM acetic or propionic acid likely affects the fatty acid biosynthetic pathway.

As an independent test of this model, we exploited the antibiotic cerulenin, which has been well documented in *E. coli* to irreversibly blocks two key fatty acid enzymes, FabB (*lpg0102*, *lpg0361* and *lpg0362*) and FabF (Vance *et al.*, 1972, Buttke & Ingram, 1978, Ulrich *et al.*, 1983). Rather than depleting malonyl-CoA, cerulenin causes this precursor to accumulate in the cell (Heath et al., 1994). When E phase WT *L. pneumophila* were treated with 0.5 μg/ml cerulenin, bacterial replication stopped (data not shown) and the *flaA* promoter was activated (Fig. A.5D). This response was largely dependent on the LetA/LetS two-component system since WT cultures treated with cerulenin exhibited a 54 ± 15 fold change in fluorescence when compared to the DMSO control sample at 6 h, whereas the signal from *letA* and *letS* cultures increased only 12 ± 2.7 and 16 ± 3.9 fold, respectively (data reported for each strain are the means ± SEM in three independent experiments). In eukaryotic cells, the simultaneous addition of cerulenin and TOFA decreases malonyl-CoA levels and blocks fatty acid biosynthesis

(Pizer *et al.*, 2000). Whereas cerulenin activates the *L. pneumophila flaA* promoter, cultures treated with both cerulenin and TOFA did not differentiate (Fig. A.5E), implicating malonyl-CoA accumulation as a prerequisite for the stress response. Taken together, the effects of both SCFA supplementation and the pharmacological inhibitors of particular biosynthetic enzymes indicate that, when fatty acid biosynthesis is disrupted, *L. pneumophila* differentiates to the transmissive phase. Moreover, although it is well documented that in mammalian cells TOFA inhibits the ACC complex (McCune & Harris, 1979, Panek et al., 1977), this is the first indication that TOFA might elicit a similar response in microbes.

Short chain fatty acid supplements alter the profile of acylated acyl carrier proteins

A critical component of fatty acid and lipid biosynthesis is acyl carrier protein (ACP). In *E. coli*, once ACP is modified by a 4'-phosphopantetheine group, the small, acidic protein carries the growing fatty acid chain through successive rounds of elongation (Magnuson et al., 1993). To ascertain by an independent, biochemical approach whether SCFA supplementation alters the *L. pneumophila* fatty acid biosynthetic pathway, we analyzed their acyl-ACP pools.

When E cultures were supplemented with 10 mM acetic or propionic acid for 3 h, the profiles of acyl-ACPs were significantly different from those supplemented with water (Fig. A.6). In particular, cultures treated with the SCFAs resembled the PE control, as similar ACP bands were depleted. A similar pattern was observed after treatment with cerulenin (Fig. A.6). Therefore, these biochemical data are consistent with the model that flux in fatty acid biosynthesis triggers *L. pneumophila* differentiation.

Alterations in the fatty acid biosynthetic pathway stimulates the stringent response

Many microbes produce ppGpp from GTP to adapt to nutritional and metabolic stresses such as deprivation of amino acids, carbon, iron, phosphorous and fatty acids (Srivatsan & Wang, 2008). Moreover, a regulatory role for ACP in the stringent response has recently been described in *E. coli*: SpoT directly interacts with the functional form of ACP, and single amino acid substitutions that disrupt this interaction abrogates SpoT-dependent ppGpp accumulation when fatty acid biosynthesis is inhibited (DiRusso & Nystrom, 1998, Battesti & Bouveret, 2006). Likewise, when fatty acid biosynthesis is inhibited pharmacologically in *L. pneumophila*, ppGpp accumulates by a mechanism that requires SpoT, but not RelA (Dalebroux et al., 2009). Therefore, we investigated whether *L. pneumophila* also accumulates ppGpp in response to excess SCFAs. When supplemented with either 10 mM acetic or propionic acid, *relA* mutant cultures exhibited a trace level of ppGpp when compared to either the water or the *relA spoT* control (Fig. A.7A; Table A.1). The weak ppGpp signal detected in *relA* cells is consistent with that observed when WT and *relA* mutant *L. pneumophila* are treated with cerulenin, an inhibitor of fatty acid biosynthesis (Dalebroux et al., 2009)(data not shown). Because the slight ppGpp accumulation by *L. pneumophila* exposed to SCFAs was not conclusive, we analyzed genetically whether either the RelA or SpoT enzyme was required to coordinate this differentiation.

When E cultures were supplemented with either 10 mM acetic or propionic acid, *relA* mutant *L. pneumophila* differentiated, similar to WT (Fig. A.7B and C). In contrast, *relA spoT* mutants were unable to trigger the phenotypic switch (Fig. A.7B and C).

Together, our phenotypic, biochemical, and genetic data presented both here and elsewhere (Dalebroux et al., 2009) demonstrate that, when fatty acid biosynthesis is perturbed, SpoT equips *L. pneumophila* to invoke the stringent response pathway to initiate a swift differentiation program and rapidly adapt to metabolic stress.

Discussion

Since *L. pneumophila* persist within many diverse environments, we predicted that various metabolites cue its differentiation. By screening several hundred compounds via Phenotype MicroArrays, we identified 22 inducers of *L. pneumophila* differentiation and focused on carboxylic acids, which trigger a premature transition from the replicative to the transmissive phase (Fig. A.2, Tables A.2, A.3 and data not shown). Previous studies postulated that when amino acid concentrations are limiting, uncharged tRNAs accumulate and the RelA enzyme synthesizes ppGpp, an alarmone that activates the regulatory cascade that governs *L. pneumophila* differentiation (Hammer & Swanson, 1999, Zusman et al., 2002). We have expanded this model by showing that SpoT coordinates transmission trait expression either when fatty acids are excessive or their biosynthesis is perturbed, likely mediated by a regulatory interaction between SpoT and ACP (Battesti & Bouveret, 2006). Genetic data suggest that when SpoT can no longer bind ACP, *L. pneumophila* fails to differentiate in response to alterations in fatty acid biosynthesis (Dalebroux et al., 2009). Thus, we extend the paradigm of microbial differentiation by reporting that the stringent response machinery equips *L. pneumophila* to monitor both protein and fatty acid biosynthesis to regulate its virulence expression and govern transmission.

The mechanism by which bacteria detect fluctuations in fatty acid biosynthesis remains to be elucidated. In *E. coli*, SpoT might sense either an accumulation or a depletion of an intermediate in this biosynthetic pathway (DiRusso & Nystrom, 1998, Battesti & Bouveret, 2006). For *Bacillus subtilis*, a key regulator of lipid metabolism is malonyl-CoA, a molecule that may act as a signal during stress and starvation (Schujman *et al.*, 2008). Similarly, E phase *L. pneumophila* immediately induce the *flaAgfp* reporter when treated with cerulenin (Fig. A.5D), an inhibitor of fatty acid biosynthesis that causes malonyl-CoA to accumulate (Heath et al., 1994). On the other hand, TOFA, which is predicted to deplete the levels of malonyl-CoA present in the cell (McCune & Harris, 1979, Cook *et al.*, 1978), fails to stimulate E phase *L. pneumophila* to activate the *flaA* promoter (Fig. A.5B, C and E). Therefore, *L. pneumophila* may monitor the levels of malonyl-CoA in the cell to regulate its phenotypic switch.

Alternatively, *L. pneumophila* may gauge the acyl chains attached to ACP. Perhaps the bacteria recognize either an accumulation or a depletion of one or more of the acyl-ACP species (e.g., Fig. A.6) or an altered ratio of acyl-ACP to apo-ACP. In *E. coli*, there are numerous intermediates in the fatty acid biosynthetic pathway; accordingly, more detailed studies are needed to determine which, if any, intermediate(s) triggers *L. pneumophila* differentiation. *L. pneumophila* also encodes three putative ACPs (*lpg0359*, *lpg1396* and *lpg2233*) that are each predicted to be modified by 4'-phosphopantetheine (Magnuson et al., 1993). Our data do not address which ACP(s) is involved, as the specificity of the ACP antibody has not been determined (Fig. A.6). Therefore, whether each ACP plays a unique or redundant role in the *L. pneumophila* life cycle remains to be determined.

By analogy to *E. coli*, we favor a model by which ppGpp-dependent sigma factor competition enables *L. pneumophila* to fine-tune its gene expression profile (Magnusson et al., 2005, Bachman & Swanson, 2004a). The quantity of ppGpp observed in response to SCFAs and cerulenin is considerably less than that of PE bacteria (Fig. A.7A)(Dalebroux et al., 2009). This is consistent with the previous report that *E. coli* produces low levels of ppGpp in response to fatty acid starvation (Seyfzadeh et al., 1993). The difficulty in detecting ppGpp may reflect our labeling conditions: Due to its fastidious nature, a phosphate-limited media to label nucleotides efficiently is not a viable option for *L. pneumophila* studies. Nevertheless, since every PE trait is induced when E phase *L. pneumophila* are treated with excess SCFAs or cerulenin, even a modest level of ppGpp may be sufficient to trigger differentiation (Fig. A.2, Table A.3). Presumably, when fatty acid biosynthesis is altered, *L. pneumophila* produces a quantity of ppGpp sufficient to recruit to RNA polymerase the appropriate cohort of its six alternative sigma factors (Chien *et al.*, 2004, Cazalet *et al.*, 2004) to induce the PE traits that promote transmission to a new host and survival in the environment.

Several circumstances could alter the quantity of fatty acids in *L. pneumophila's* intracellular niche. When the TCA cycle does not operate completely, or when bacterial cells are flooded with excess carbon, microbes excrete acetate into their extracellular milieu (Wolfe, 2005). *L. pneumophila* also possesses lipolytic enzymes that may generate free fatty acids by degrading membranes of their own or their host (Hood *et al.*, 1986, Archuleta *et al.*, 2005). Alternatively, *L. pneumophila* may monitor external sources of fatty acids that are derived from the host plasma or phagosomal membranes. Consistent with this idea, within A/J mouse macrophages *L. pneumophila* replicate within

a lysosomal compartment (Sturgill-Koszycki & Swanson, 2000), the site for membrane degradation. Interestingly, the alveolar macrophages of rats can ingest pulmonary surfactant, which is rich in phosphatidylcholine and phosphatidylglycerol (Grabner & Meerbach, 1991), two substrates for the phospholipase A secreted by *L. pneumophila* (Flieger *et al.*, 2000). By this scenario, the accidental human host may exacerbate pathogenesis by stimulating synthesis of the transmission factor flagellin, which provokes a macrophage inflammatory cell death pathway (Molofsky et al., 2005, Molofsky et al., 2006, Ren *et al.*, 2006).

Additional metabolites that are present on the Biolog arrays may also cue intracellular differentiation of *L. pneumophila*. First, the permeability of *L. pneumophila* for each of the compounds on the Phenotype MicroArrays is unknown. Also, the plates include one concentration of each compound, yet titration curves indicate that several inducers only trigger differentiation within a narrow concentration range (data not shown). Indeed, nicotinic acid, which is present on Biolog plate PM5 at 10 μM, did not cause growth restriction or induction of the *flaAgfp* reporter, whereas 5 mM nicotinic acid does trigger *L. pneumophila* differentiation, as judged by both microarray and phenotypic data (Edwards, *et al.*, unpublished).

L. pneumophila can monitor perturbations in fatty acid biosynthesis to regulate its differentiation *in vitro*, but whether SCFAs also induce transmission traits within vacuoles of phagocytic cells is not known, as their composition has yet to be elucidated. Although *relA* is dispensable for intracellular growth in both human macrophages and amoebae (Hammer & Swanson, 1999, Zusman et al., 2002), *L. pneumophila* does require SpoT not only for transmission between mouse macrophages, but also to differentiate

from the replicative to the transmissive phase (Dalebroux et al., 2009). Therefore, *L. pneumophila* employs SpoT to monitor fatty acids or some other metabolite in macrophage vacuoles to govern its life cycle. By linking central metabolism to differentiation and virulence, *L. pneumophila* augments its fitness by adapting to fluctuating environments.

Table A.1. Bacterial strains and plasmids

Strain or Plasmid	Relevant genotype/phenotype	Reference or Source
Strains		
E. coli		
DH5a	F$^-$ *endA1 hsdR17* (r$^-$ m$^+$) *supE44 thi-l recA1 gryA* (Nal^1) *relA1* Δ (l*acZYA-argF*$^-$)$_{U169}$ ϕ80d*lacZ*Δ*M15* λ*pir*RK6	Laboratory collection
MB619	DH5a pGEM-T-PtaAckA2	This work
MB681	DH5a pGEM-T-PtaAckA2::Kan	This work
L. pneumophila		
MB110	wild type; *thyA hsdR rpsL*	(Berger & Isberg, 1993)
MB355	pflaG	(Hammer & Swanson, 1999)
MB413	*letA-22*::*kan*	(Hammer et al., 2002)
MB414	*letA-22*::*kan* pflaG	(Hammer et al., 2002)
MB416	*letS-36*::*kan*	(Hammer et al., 2002)
MB417	*letS-36*::*kan* pflaG	(Hammer et al., 2002)
MB641	*pta ackA2*::*kan*	This work
MB682	*pta ackA2*::*kan* pflaG	This work
MB684	*relA*::*kan* pflaG	(Dalebroux et al., 2009)
MB685	*relA*::*gent spoT*::*kan* pflaG	(Dalebroux et al., 2009)
Plasmids		
pGEM-T	Multiple cloning site within coding region of b-lactamase a fragment linearized with single-T overhangs; 3 kb; Amp^R	Promega
pflaG	150 bp *flaA* promoter fragment fused to GFP, encodes thymidylate synthetase; 10.5 kb; Amp^R	(Hammer & Swanson, 1999)
pGEM-T-PtaAckA2	pGEM-T containing 3.3 kb *pta ackA2 locus* PCR amplified from Lp02 chromosome and ligated into T overhangs; 6.3 kb; Amp^R	This work
pUC4K	pUC4 containing 1.3 kb kanamycin cassette	Pharmacia
pGEM-T-PtaAckA2::Kan	pGEM-T-PtaAckA2 with 1.3 kb kanamycin cassette inserted between *Xma*I and *Nhe*I sites in the *pta ackA2* ORF resulting in a 1.8 kb deletion	This work

Table A.2. Compounds that trigger premature differentiation in *L. pneumophila*

Compound*	Biolog Plate	Mean FC# at 6 h
Carboxylic Acids		
Formic acid	PM1	7.3 ± 0.6
Acetic acid	PM1	1.8 ± 0.2
Propionic acid	PM1	4.8 ± 1.9
Butyric acid	PM2A	3.3 ± 1.9
α-Ketovaleric acid	PM2A	1.9 ± 0.7
Caproic acid	PM2A	7.2 ± 1.4
Itaconic acid	PM2A	2.4 ± 0.8
Sorbic acid	PM2A	2.6 ± 0.4
4-hydroxybenzoic acid	PM2A	1.8 ± 0.4
m-hydroxy phenyl acetic acid	PM1	3.8 ± 2.1
p-hydroxy phenyl acetic acid	PM1	2.9 ± 1.4
Monomethyl succinate	PM1	4.7 ± 0.6
Detergents		
Polyoxyethylene sorbitan monolaurate (Tween 20)	PM5	3.2 ± 1.2
Polyoxyethylene sorbitan monooleate (Tween 80)	PM1	1.4 ± 0.1
Other		
2-Deoxy D glucose-6-phosphate	PM4A	2.0 ± 1.3
Deoxyadenosine	PM1	2.0 ± 0.2
Deoxyribose	PM2A	2.3 ± 0.4
Dihydroxyacetone	PM2A	2.7 ± 0.4
Hydroxylamine	PM3B	1.7 ± 0.2
Met-Ala dipeptide	PM3B	1.6 ± 0.4
Nitrite	PM3B	5.1 ± 1.8
Parabanic acid	PM3B	1.5 ± 0.3

*Approximate concentrations: 5-20 mM carbon sources, 2-5 mM nitrogen sources, and 0.1 to 2 mM phosphorus and sulfur sources.
#FC indicates the fold change ± SD in fluorescence between the compound and the negative control well on the Phenotype MicroArray plate.

Table A.3. Phenotypic response of *letA* and *letS* mutants 3 hours after fatty acid supplementation

Strain	Culture conditions	Growth inhibition[a]	Motility[b]	Cytotoxicity[c]	Degradation[d]	Na sensitivity[e]
WT	PE Control	+	+++	+ (9% ± 2%)	+ (82% ± 2%)	+ (0.3% ± 0.2%)
	E + H_2O	–	–	– (68% ± 15%)	– (16% ± 2%)	– (85% ± 13%)
	E + Acetic Acid	+	++	+ (16% ± 3%)	+ (52% ± 5%)	+ (8% ± 2%)
	E + Propionic Acid	+	++	+ (23% ± 8%)	+ (62% ± 5%)	+ (9% ± 3%)
letA	PE Control	+	–	– (74% ± 13%)	– (38% ± 5%)	– (48% ± 21%)
	E + H_2O	–	–	– (78% ± 12%)	– (18% ± 3%)	– (118% ± 16%)
	E + Acetic Acid	+	–	– (65% ± 2%)	– (27% ± 3%)	– (14% ± 7%)
	E + Propionic Acid	+	–	– (76% ± 9%)	– (35% ± 7%)	– (18% ± 7%)
letS	PE Control	+	–	– (82% ± 10%)	– (47% ± 5%)	– (64% ± 6%)
	E + H_2O	–	–	– (81% ± 8%)	– (18% ± 3%)	– (105% ± 4%)
	E + Acetic Acid	+	–	– (71% ± 15%)	– (29% ± 2%)	– (22% ± 10%)
	E + Propionic Acid	+	–	– (69% ± 16%)	– (41% ± 3%)	– (26% ± 20%)

a. Growth of *L. pneumophila* was monitored by measuring the OD_{600} of the cultures three hours after supplementation. Although *letA* and *letS* cultures supplemented with fatty acids do not display PE phenotypes, bacterial growth is completely inhibited. (+) indicates growth inhibition, while (-) indicates normal growth kinetics. Data represent at least three independent experiments.

b. Motility was assessed by phase-contrast microscopy and is based on numerous independent observations. (-) indicates cultures that were <10% motile, (+) indicates 10-25% motility, (++) indicates 25-75% motility, and (+++) indicates high levels of directed motility (>75%).

c. Cytotoxicity of *L. pneumophila* for macrophages was assessed by measuring the reduction of alamarBlue™ following a 1 h incubation. Data points that fell within an MOI range of 5-10 were pooled. (+) indicates that less than 50% of the macrophages were viable, whereas (-) represents greater than 50% macrophage viability. In parentheses, the mean percent of viable macrophages ± SEM is shown from at least three independent experiments performed in triplicate.

d. The percent of bacteria that remain intact following a 2 h incubation within macrophages was determined by fluorescence microscopy. (+) indicates that >50% of the bacteria were able to avoid degradation and (-) indicates that <50% of the bacteria avoided degradation. Data represent the mean ± SEM from at least three independent experiments performed in duplicate.

e. The percent of sodium sensitive bacteria was calculated by comparing CFU of cultures plated onto media with and without 100 mM NaCl. (+) indicates < 10% inhibition and (-) indicates > 10% inhibition. The values represent the mean ± SEM for at least three independent experiments performed in duplicate.

Figure A.1.

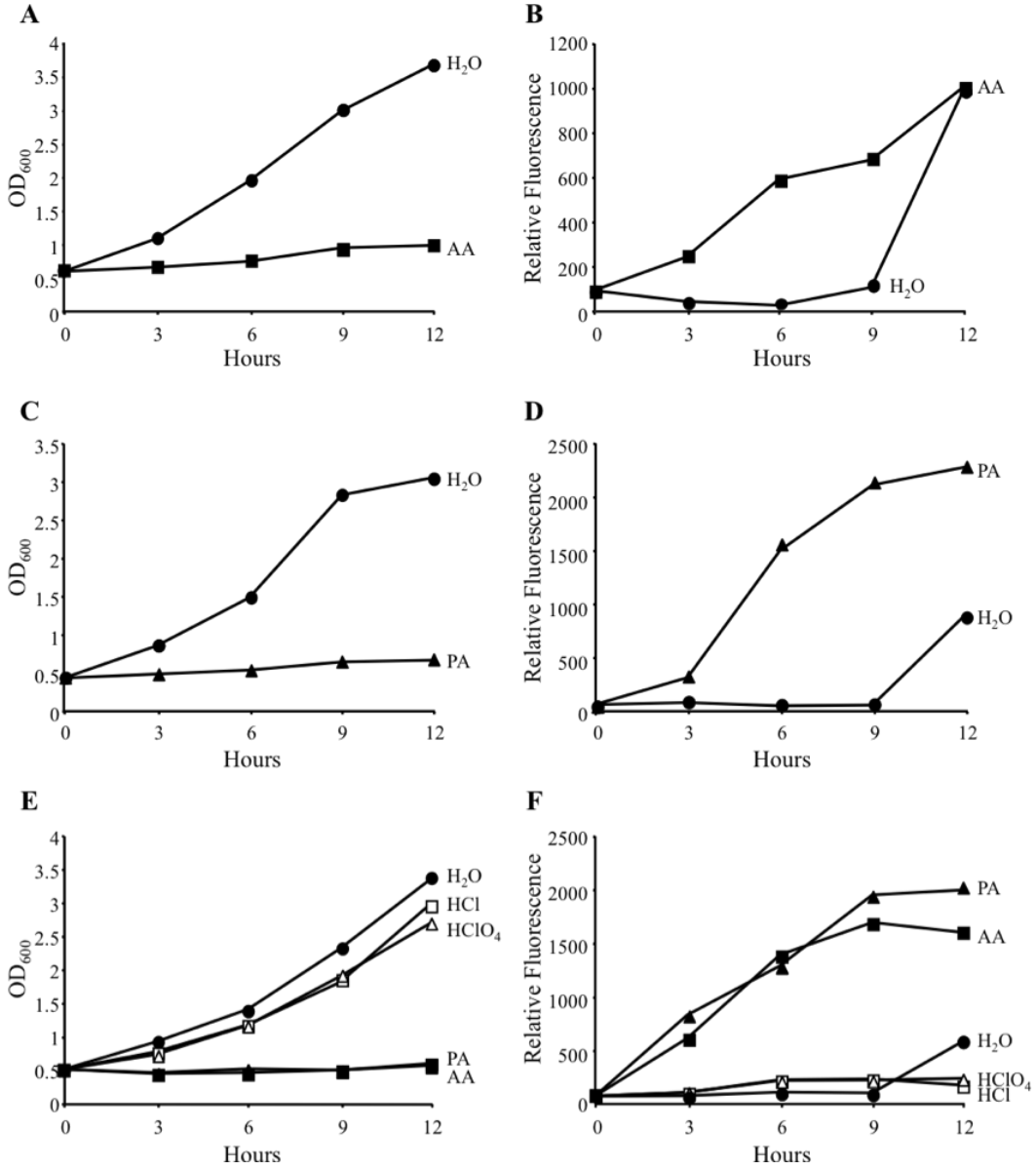

Figure A.1. Growth inhibition and the premature expression of motility are specific to fatty acid addition.
WT *L. pneumophila* carrying the *flaAgfp* reporter construct were cultured to the E phase and then supplemented with 10 mM acetic acid (AA; closed squares), propionic acid (PA; closed triangles), hydrochloric acid (HCl; open squares) or perchloric acid ($HClO_4$; open triangles). At the times indicated, the optical density (A, C, E) and relative fluorescence (B, D, F) of samples were analyzed. For all experiments, E cultures supplemented with water (H_2O; closed circles) served as a negative control. Shown are representative graphs from three or more independent experiments in which the mean fold change in fluorescence at 9 h ± SEM when compared to H_2O control was: acetic acid = 15.7 ± 3.1; propionic acid = 50.5 ± 15.2; HCl = 0.87 ± 0.48; $HClO_4$ = 1.0 ± 1.0.

Figure A.2.

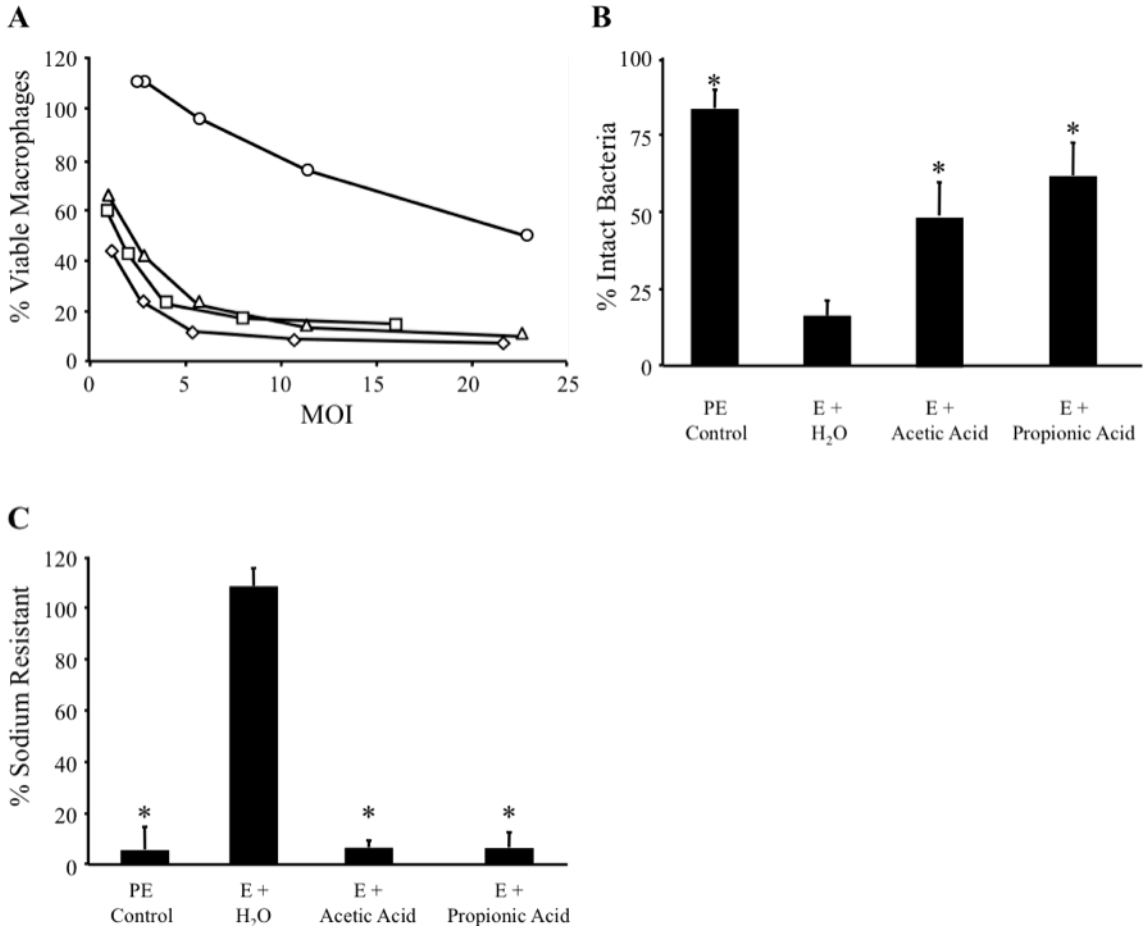

Figure A.2. Fatty acid supplementation of WT *L. pneumophila* induces the early expression of multiple transmissive phase phenotypes.
(A) Macrophage viability was assessed by alamarBlue™ reduction following a 1 h incubation with PE (diamonds) or E cultures supplemented with water (circles), acetic acid (squares) or propionic acid (triangles). Shown is a representative graph from three independent experiments preformed in triplicate. (B) Lysosome evasion following a 2 h incubation was quantified by fluorescence microscopy as the percent of intact bacteria. Displayed are the means from duplicate samples in three independent experiments. Error bars indicate SD and asterisks designate significant differences ($P < 0.01$) when compared to water control. (C) The mean percentage of sodium resistant bacteria ± SD was calculated from duplicate samples in three independent experiments. Asterisks denote statistically significant differences ($P < 0.01$) when compared to water control.

Figure A.3.

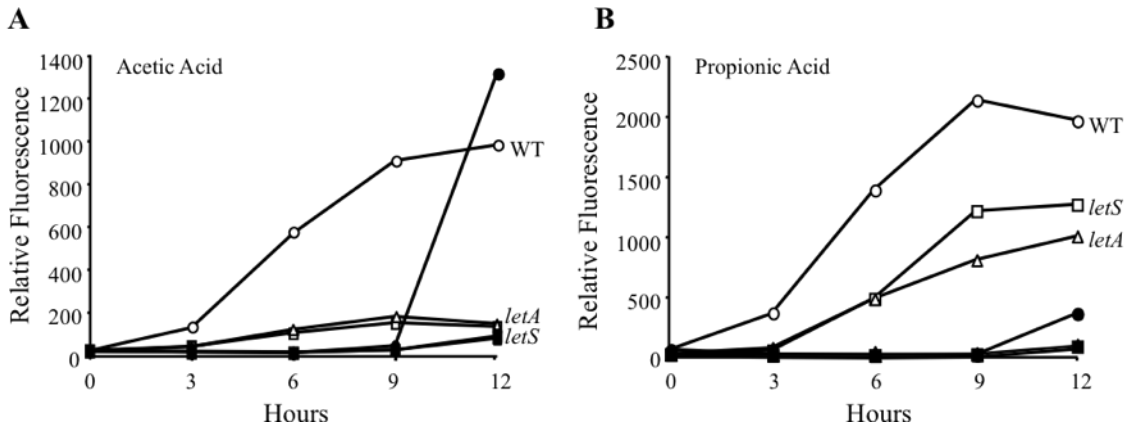

Figure A.3. The LetA/LetS signal transduction system is required for full induction of premature motility.

E phase WT (circles), *letA* (triangles) or *letS* (squares) *L. pneumophila* containing the *flaAgfp* reporter were supplemented with water (closed symbols), 10 mM acetic acid (open symbols; Fig. 3A) or 10 mM propionic acid (open symbols; Fig. 3B), and their fluorescence analyzed at the times indicated. Shown are representative graphs from three independent experiments in which the mean fold change in fluorescence at 6 h ± SEM when compared to H_2O control was: WT + acetic acid = 39.7 ± 15.7; *letA* + acetic acid = 6.2 ± 2.5; *letS* + acetic acid = 6.7 ± 2.3; WT + propionic acid = 77.0 ± 8.5; *letA* + propionic acid = 59.0 ± 10.0; *letS* + propionic acid = 52.2 ± 13.6.

Figure A.4.

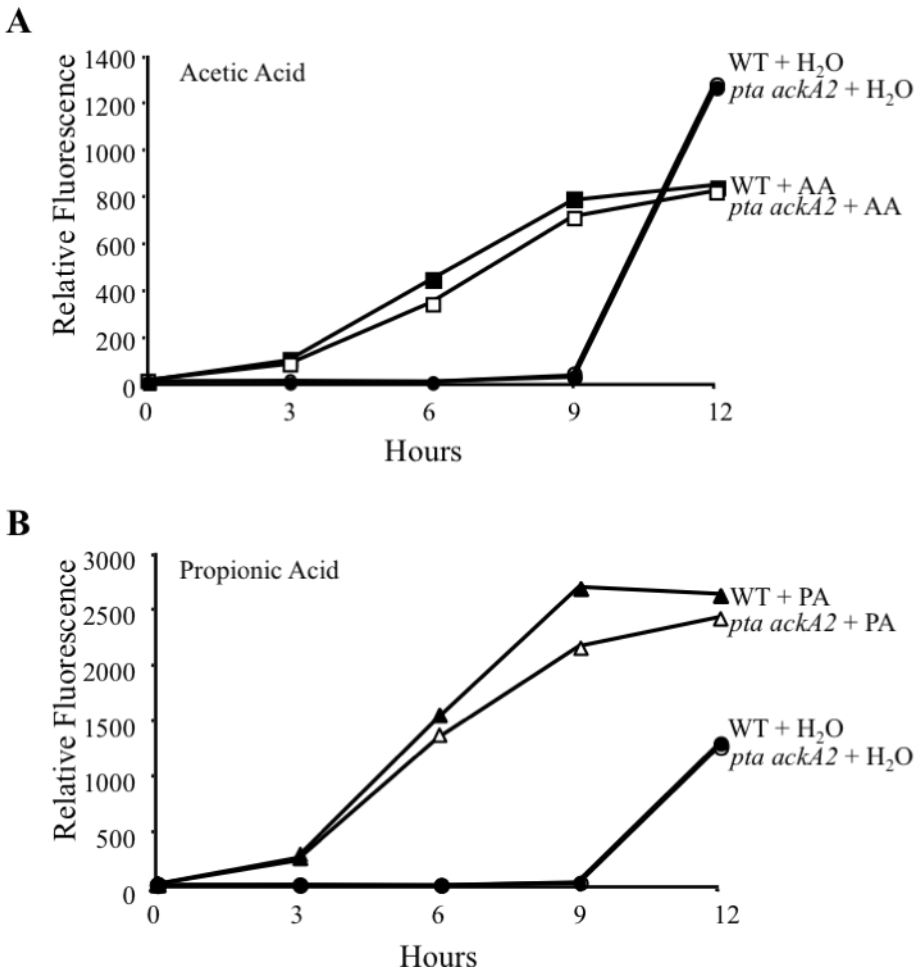

Figure A.4. Induction of motility by fatty acid addition is independent of *pta ackA2*. E phase broth cultures of WT (closed symbols) or *pta ackA2* (open symbols) *L. pneumophila* containing pflaG were supplemented with water (H_2O; circles), 10 mM acetic acid (A; AA; squares), or 10 mM propionic acid (B; PA; triangles), and their relative fluorescence assessed by fluorometry at 3 h intervals. Shown are representative graphs from three experiments in which the mean fold change in fluorescence at 9 h ± SEM when compared to H_2O control was: WT + acetic acid = 11.9 ± 4.6; *pta ackA2* + acetic acid = 11.9 ± 5.1; WT + propionic acid = 42.0 ± 17.0; *pta ackA2* + propionic acid = 37.5 ± 14.1.

Figure A.5. Alterations in fatty acid biosynthesis induce *L. pneumophila* differentiation.
(A) Schematic of fatty acid metabolism indicating where TOFA and cerulenin inhibitors act. (B and C) Inhibition of the conversion of acetyl-CoA to malonyl-CoA abrogates the early differentiation that is triggered by fatty acid supplementation. **(**B) E cultures containing pflaG were supplemented with 10 mM acetic acid (AA; squares) with (open shapes) or without (closed shapes) the acetyl-CoA carboxylase inhibitor TOFA (5 µg/ml**)**, and the fluorescence was monitored over time. Identical cultures supplemented with water (closed circles) or DMSO (vehicle control, data not shown) were analyzed as controls. The graph shown is representative of three independent experiments in which the mean fold change in fluorescence at 9 h ± SEM when compared to H_2O control was: acetic acid = 10.2 ± 4.1; TOFA = 2.4 ± 1.4; acetic acid + TOFA = 1.6 ± 0.4. **(**C) E cultures containing *flaAgfp* were supplemented with 10 mM propionic acid (PA; triangles) with (open shapes) or without (closed shapes) TOFA (5 µg/ml**)** and the fluorescence was monitored over time. Identical cultures supplemented with water (closed circles) or DMSO (vehicle control, data not shown) were analyzed as controls. The graph shown is representative of four independent experiments in which the mean fold change in fluorescence at 6 h ± SEM when compared to H_2O control was: propionic acid = 73.8 ± 16.4; TOFA = 5.3 ± 1.1; propionic acid + TOFA = 21.4 ± 6.2. (D) E cultures of WT *L. pneumophila* carrying the *flaAgfp* plasmid were supplemented with the fatty acid biosynthesis inhibitor, cerulenin (Cer, 0.5 µg/ml; open circles) or vehicle control (DMSO, closed circles), and the relative fluorescence monitored over time. The graph shown is representative of four independent experiments in which the mean fold change in fluorescence at 6 h ± SEM when compared to DMSO control was: Cer = 65.0 ± 8.1. (E) E phase cultures of WT *L. pneumophila* containing pflaG were supplemented with cerulenin (Cer, 0.5 µg/ml; closed squares) or cerulenin plus TOFA (open squares). Identical cultures treated with water (H_2O; closed circles) or TOFA alone (open circles) are shown as controls. In three separate experiments, the mean fold change in fluorescence at 6 h ± SEM when compared to H_2O control was: Cer = 39.8 ± 10.1; TOFA = 3.9 ± 1.1; Cer + TOFA = 6.8 ± 2.0.

Figure A.5.

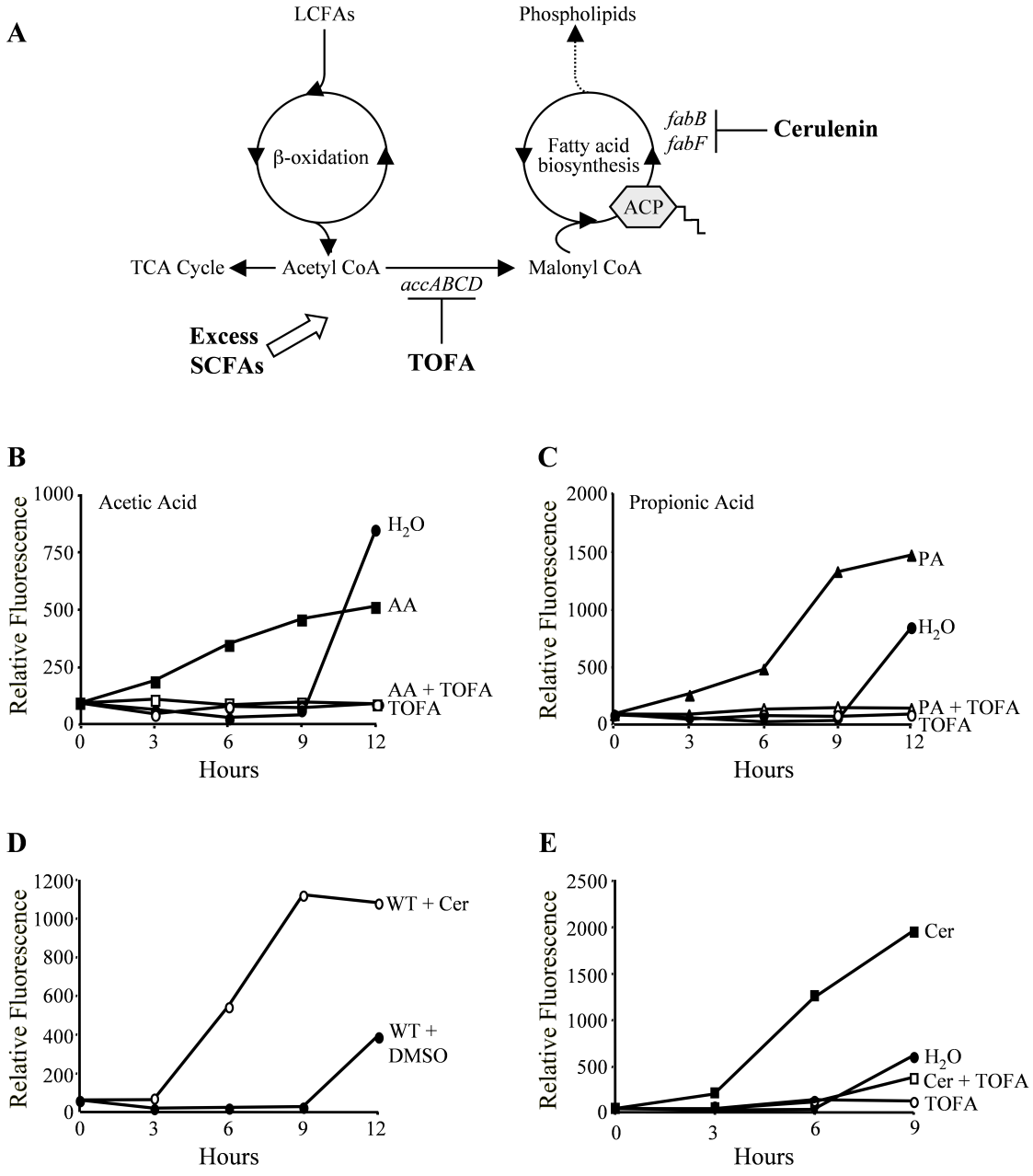
A
LCFAs
Phospholipids
β-oxidation
Fatty acid biosynthesis
fabB
fabF
Cerulenin
ACP
TCA Cycle
Acetyl CoA
Malonyl CoA
accABCD
Excess SCFAs
TOFA
B
Acetic Acid
Relative Fluorescence
Hours
H2O
AA
AA + TOFA
TOFA
C
Propionic Acid
PA
H2O
PA + TOFA
TOFA
D
WT + Cer
WT + DMSO
E
Cer
H2O
Cer + TOFA
TOFA

Figure A.6.

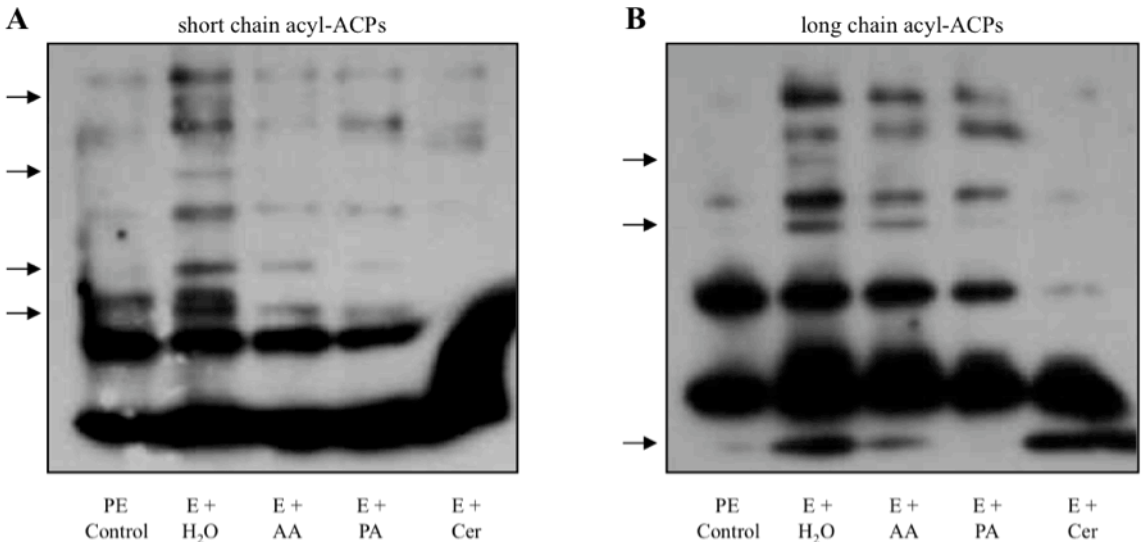

Figure A.6. Perturbations in fatty acid biosynthesis alter *L. pneumophila* acyl-ACP profiles.

After incubating E phase *L. pneumophila* for 3 h with water (H_2O), acetic acid (AA), propionic acid (PA), or cerulenin (Cer), acyl-ACPs were purified, separated on 13% short chain fatty acid (A) or long chain fatty acid (B) native polyacrylamide gels, and then detected by western analysis. Also shown are acyl-ACP pools from PE bacteria. Arrows denote protein bands that differ between the control and experimental samples. A film representative of three independent experiments is displayed.

Figure A.7.

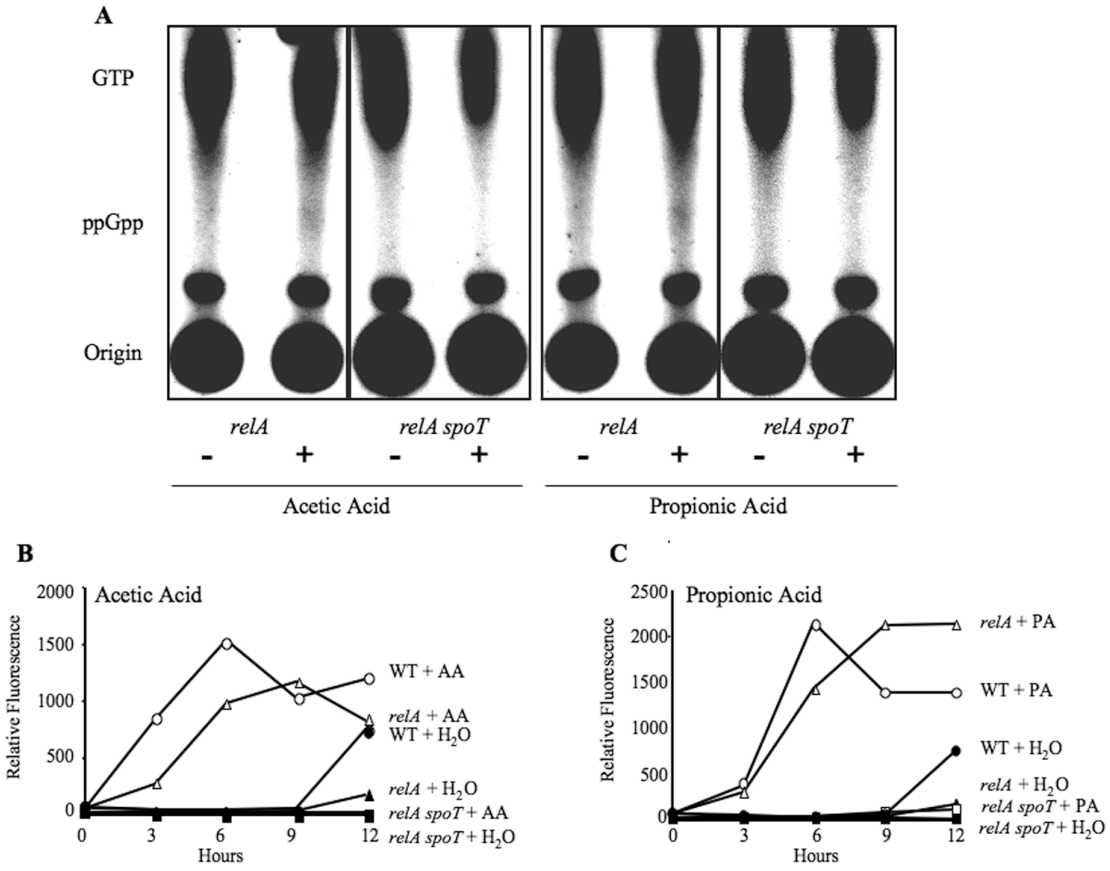

Figure A.7. *L. pneumophila* employs the stringent response to induce differentiation when fatty acid biosynthesis is altered.
(A) Perturbations in fatty acid biosynthesis trigger ppGpp production in the E phase. After labeling nucleotide pools with ^{32}P, E cultures of *relA* and *relA spoT* mutant *L. pneumophila* were supplemented with water or 10 mM SCFAs for 1.5 hours to stimulate ppGpp synthesis. Since ppGpp is synthesized from GTP and low levels of ppGpp are difficult to detect by TLC, the GTP pools are also indicated for each sample. Representative chromatograms from 2 or more independent experiments are shown for each condition. (B and C) *L. pneumophila* requires SpoT to sense SCFAs. E phase cultures of WT (circles), *relA* (triangles) or *relA spoT* (squares) *L. pneumophila* containing the *flaAgfp* reporter were supplemented with water (closed symbols), 10 mM acetic acid (AA) (B; open symbols) or 10 mM propionic acid (PA) (C; open symbols), and their fluorescence analyzed at the times indicated. Shown are representative graphs from at least three independent experiments in which the mean fold change in fluorescence at 6 h ± SEM when compared to H_2O controls was: WT + AA = 23.6 ± 5.9; *relA* + AA = 30.7 ± 10.2; *relA spoT* + AA = 3.4 ± 1.9; WT + PA = 48.5 ± 16.3; *relA* + PA = 47.5 ± 16.0; *relA spoT* + PA = 4.7 ± 2.6.

Figure A.8.

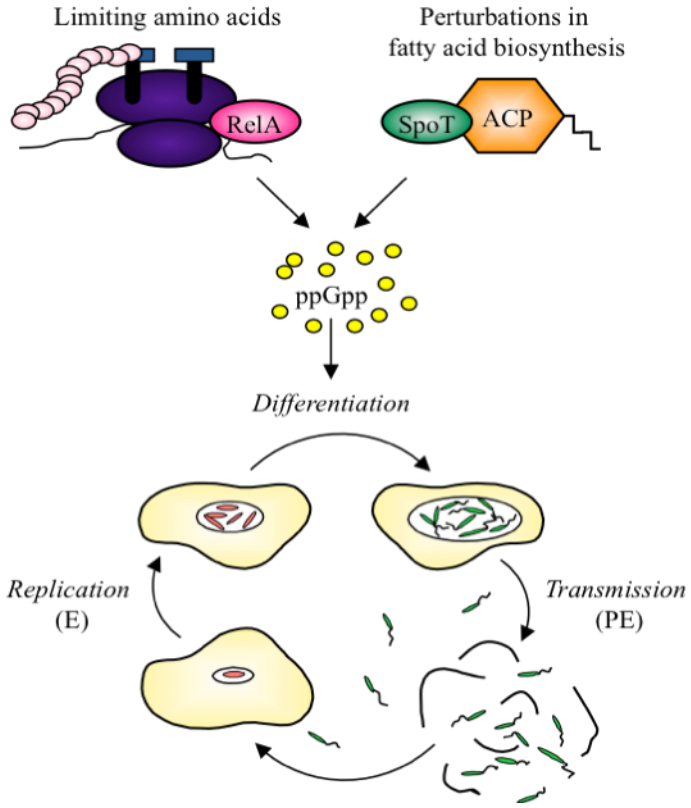

Figure A.8. ***L. pneumophila*** **monitors flux in fatty acid biosynthesis to coordinate differentiation.** (See text for description)

BIBLIOGRAPHY

Abdelrahman, Y. M. & R. J. Belland, (2005) The chlamydial developmental cycle. *FEMS Microbiol Rev* **29**: 949-959.

Aberg, A., J. Fernandez-Vazquez, J. D. Cabrer-Panes, A. Sanchez & C. Balsalobre, (2009) Similar and divergent effects of ppGpp and DksA deficiencies on transcription in *Escherichia coli*. *J Bacteriol* **191**: 3226-3236.

Aberg, A., V. Shingler & C. Balsalobre, (2006) (p)ppGpp regulates type 1 fimbriation of *Escherichia coli* by modulating the expression of the site-specific recombinase FimB. *Mol Microbiol* **60**: 1520-1533.

Aberg, A., V. Shingler & C. Balsalobre, (2008) Regulation of the *fimB* promoter: a case of differential regulation by ppGpp and DksA in vivo. *Mol Microbiol* **67**: 1223-1241.

Abranches, J., A. R. Martinez, J. K. Kajfasz, V. Chavez, D. A. Garsin & J. A. Lemos, (2009) The molecular alarmone (p)ppGpp mediates stress responses, vancomycin tolerance, and virulence in *Enterococcus faecalis*. *J Bacteriol* **191**: 2248-2256.

Abu-Zant, A., R. Asare, J. E. Graham & Y. Abu Kwaik, (2006) Role for RpoS but not RelA of *Legionella pneumophila* in modulation of phagosome biogenesis and adaptation to the phagosomal microenvironment. *Infect Immun* **74**: 3021-3026.

Albert-Weissenberger, C., T. Sahr, O. Sismeiro, J. Hacker, K. Heuner & C. Buchrieser, Control of flagellar gene regulation in *Legionella pneumophila* and its relation to growth phase. *J Bacteriol* **192**: 446-455.

Alli, O. A., L. Y. Gao, L. L. Pedersen, S. Zink, M. Radulic, M. Doric & Y. Abu Kwaik, (2000) Temporal pore formation-mediated egress from macrophages and alveolar epithelial cells by *Legionella pneumophila*. *Infect Immun* **68**: 6431-6440.

Archuleta, R. J., P. Yvonne Hoppes & T. P. Primm, (2005) *Mycobacterium avium* enters a state of metabolic dormancy in response to starvation. *Tuberculosis (Edinb)* **85**: 147-158.

Babitzke, P. & T. Romeo, (2007) CsrB sRNA family: sequestration of RNA-binding regulatory proteins. *Curr Opin Microbiol* **10**: 156-163.

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

Bachman, M. A. & M. S. Swanson, (2004a) Genetic evidence that *Legionella pneumophila* RpoS modulates expression of the transmission phenotype in both the exponential phase and the stationary phase. *Infect Immun* **72**: 2468-2476.

Bachman, M. A. & M. S. Swanson, (2004b) The LetE protein enhances expression of multiple LetA/LetS-dependent transmission traits by *Legionella pneumophila*. *Infect Immun* **72**: 3284-3293.

Bandyopadhyay, P., B. Byrne, Y. Chan, M. S. Swanson & H. M. Steinman, (2003) *Legionella pneumophila* catalase-peroxidases are required for proper trafficking and growth in primary macrophages. *Infect Immun* **71**: 4526-4535.

Banerji, S., P. Aurass & A. Flieger, (2008) The manifold phospholipases A of *Legionella pneumophila* - identification, export, regulation, and their link to bacterial virulence. *Int J Med Microbiol* **298**: 169-181.

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

Battesti, A. & E. Bouveret, (2009) Bacteria possessing two RelA/SpoT-like proteins have evolved a specific stringent response involving the acyl carrier protein-SpoT interaction. *J Bacteriol* **191**: 616-624.

Berger, K. H. & R. R. Isberg, (1993) Two distinct defects in intracellular growth complemented by a single genetic locus in *Legionella pneumophila*. *Mol Microbiol* **7**: 7-19.

Berger, K. H., J. J. Merriam & R. R. Isberg, (1994) Altered intracellular targeting properties associated with mutations in the *Legionella pneumophila dotA* gene. *Mol Microbiol* **14**: 809-822.

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

Blankschien, M. D., K. Potrykus, E. Grace, A. Choudhary, D. Vinella, M. Cashel & C. Herman, (2009) TraR, a homolog of a RNAP secondary channel interactor, modulates transcription. *PLoS Genet* **5**: e1000345.

Bohnhoff, M., C. P. Miller & W. R. Martin, (1964) Resistance of the Mouse's Intestinal Tract to Experimental *Salmonella* Infection. Ii. Factors Responsible for Its Loss Following Streptomycin Treatment. *J Exp Med* **120**: 817-828.

Boucher, P. E., F. D. Menozzi & C. Locht, (1994) The modular architecture of bacterial response regulators. Insights into the activation mechanism of the BvgA transactivator of *Bordetella pertussis*. *J Mol Biol* **241**: 363-377.

Bougdour, A. & S. Gottesman, (2007) ppGpp regulation of RpoS degradation via anti-adaptor protein IraP. *Proc Natl Acad Sci U S A* **104**: 12896-12901.

Braeken, K., M. Moris, R. Daniels, J. Vanderleyden & J. Michiels, (2006) New horizons for (p)ppGpp in bacterial and plant physiology. *Trends Microbiol* **14**: 45-54.

Bremer, H., and P. P. Dennis (1996) Modulation of Chemical Composition and Other Paramters of the Cell by Growth Rate. In: *Escherichia coli* and *Salmonella*: Cellular and Molecular Biology, 2 ed. R. Curtiss III, J. Ingraham, E. C., C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed). Washington, D.C.: ASM Press, pp. 1553-1569.

Brencic, A., K. A. McFarland, H. R. McManus, S. Castang, I. Mogno, S. L. Dove & S. Lory, (2009) The GacS/GacA signal transduction system of *Pseudomonas aeruginosa* acts exclusively through its control over the transcription of the RsmY and RsmZ regulatory small RNAs. *Mol Microbiol* **73**: 434-445.

Brown, L., D. Gentry, T. Elliott & M. Cashel, (2002) DksA affects ppGpp induction of RpoS at a translational level. *J Bacteriol* **184**: 4455-4465.

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

Buglino, J., V. Shen, P. Hakimian & C. D. Lima, (2002) Structural and biochemical analysis of the Obg GTP binding protein. *Structure* **10**: 1581-1592.

Buttke, T. M. & L. O. Ingram, (1978) Inhibition of unsaturated fatty acid synthesis in *Escherichia coli* by the antibiotic cerulenin. *Biochemistry* **17**: 5282-5286.

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

Cashel, M., (1994) Detection of (p)ppGpp accumulation patterns in *Escherichia coli* mutants. In: Methods in Molecular Genetics, Molecular Microbiology Techniques, Part A. K. W. Adolph (ed). New York: Academic Press, pp. 341-356.

Cashel, M. & J. Gallant, (1969) Two compounds implicated in the function of the RC gene of Escherichia coli. *Nature* **221**: 838-841.

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

Charity, J. C., L. T. Blalock, M. M. Costante-Hamm, D. L. Kasper & S. L. Dove, (2009) Small molecule control of virulence gene expression in *Francisella tularensis*. *PLoS Pathog* **5**: e1000641.

Charpentier, X., S. P. Faucher, S. Kalachikov & H. A. Shuman, (2008) Loss of RNase R induces competence development in *Legionella pneumophila*. *J Bacteriol* **190**: 8126-8136.

Chatfield, C. H. & N. P. Cianciotto, (2007) The secreted pyomelanin pigment of *Legionella pneumophila* confers ferric reductase activity. *Infect Immun* **75**: 4062-4070.

Chen, D. E., S. Podell, J. D. Sauer, M. S. Swanson & M. H. Saier, Jr., (2008) The phagosomal nutrient transporter (Pht) family. *Microbiology* **154**: 42-53.

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

Chevance, F. F. & K. T. Hughes, (2008) Coordinating assembly of a bacterial macromolecular machine. *Nat Rev Microbiol* **6**: 455-465.

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

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

Cook, G. A., M. T. King & R. L. Veech, (1978) Ketogenesis and malonyl coenzyme A content of isolated rat hepatocytes. *J Biol Chem* **253**: 2529-2531.

Costanzo, A., H. Nicoloff, S. E. Barchinger, A. B. Banta, R. L. Gourse & S. E. Ades, (2008) ppGpp and DksA likely regulate the activity of the extracytoplasmic stress factor sigmaE in *Escherichia coli* by both direct and indirect mechanisms. *Mol Microbiol* **67**: 619-632.

Dahl, J. L., C. N. Kraus, H. I. Boshoff, B. Doan, K. Foley, D. Avarbock, G. Kaplan, V. Mizrahi, H. Rubin & C. E. Barry, 3rd, (2003) The role of RelMtb-mediated adaptation to stationary phase in long-term persistence of *Mycobacterium tuberculosis* in mice. *Proc Natl Acad Sci U S A* **100**: 10026-10031.

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

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

Das, B., R. R. Pal, S. Bag & R. K. Bhadra, (2009) Stringent response in *Vibrio cholerae*: genetic analysis of *spoT* gene function and identification of a novel (p)ppGpp synthetase gene. *Mol Microbiol* **72**: 380-398.

Dasgupta, N., M. C. Wolfgang, A. L. Goodman, S. K. Arora, J. Jyot, S. Lory & R. Ramphal, (2003) A four-tiered transcriptional regulatory circuit controls flagellar biogenesis in *Pseudomonas aeruginosa*. *Mol Microbiol* **50**: 809-824.

Datsenko, K. A. & B. L. Wanner, (2000) One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc Natl Acad Sci U S A* **97**: 6640-6645.

DiRusso, C. C. & T. Nystrom, (1998) The fats of Escherichia coli during infancy and old age: regulation by global regulators, alarmones and lipid intermediates. *Mol Microbiol* **27**: 1-8.

Durfee, T., A. M. Hansen, H. Zhi, F. R. Blattner & D. J. Jin, (2008) Transcription profiling of the stringent response in *Escherichia coli*. *J Bacteriol* **190**: 1084-1096.

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

Erickson, D. L., J. L. Lines, E. C. Pesci, V. Venturi & D. G. Storey, (2004) *Pseudomonas aeruginosa relA* Contributes to Virulence in *Drosophila melanogaster*. *Infect. Immun.* **72**: 5638-5645.

Faulkner, G., S. G. Berk, E. Garduno, M. A. Ortiz-Jimenez & R. A. Garduno, (2008) Passage through *Tetrahymena tropicalis* triggers a rapid morphological differentiation in *Legionella pneumophila*. *J Bacteriol* **190**: 7728-7738.

Faulkner, G. & R. A. Garduno, (2002) Ultrastructural analysis of differentiation in *Legionella pneumophila*. *J Bacteriol* **184**: 7025-7041.

Fernandez-Moreira, E., J. H. Helbig & M. S. Swanson, (2006) Membrane vesicles shed by *Legionella pneumophila* inhibit fusion of phagosomes with lysosomes. *Infect Immun* **74**: 3285-3295.

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

Fields, B. S., R. F. Benson & R. E. Besser, (2002) *Legionella* and Legionnaires' disease: 25 years of investigation. *Clin Microbiol Rev* **15**: 506-526.

Flieger, A., S. Gongab, M. Faigle, H. A. Mayer, U. Kehrer, J. Mussotter, P. Bartmann & B. Neumeister, (2000) Phospholipase A secreted by *Legionella pneumophila* destroys alveolar surfactant phospholipids. *FEMS Microbiol Lett* **188**: 129-133.

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

Gallant, J., J. Irr & M. Cashel, (1971) The mechanism of amino acid control of guanylate and adenylate biosynthesis. *J Biol Chem* **246**: 5812-5816.

Gaynor, E. C., D. H. Wells, J. K. MacKichan & S. Falkow, (2005) The C*ampylobacter jejuni* stringent response controls specific stress survival and virulence-associated phenotypes. *Mol Microbiol* **56**: 8-27.

Gentry, D. R. & M. Cashel, (1996) Mutational analysis of the *Escherichia coli spoT* gene identifies distinct but overlapping regions involved in ppGpp synthesis and degradation. *Mol Microbiol* **19**: 1373-1384.

Gokhale, R. S., P. Saxena, T. Chopra & D. Mohanty, (2007) Versatile polyketide enzymatic machinery for the biosynthesis of complex mycobacterial lipids. *Nat Prod Rep* **24**: 267-277.

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

Grabner, R. & W. Meerbach, (1991) Phagocytosis of surfactant by alveolar macrophages in vitro. *Am J Physiol* **261**: L472-477.

Gudapaty, S., K. Suzuki, X. Wang, P. Babitzke & T. Romeo, (2001) Regulatory interactions of Csr components: the RNA binding protein CsrA activates *csrB* transcription in Escherichia coli. *J Bacteriol* **183**: 6017-6027.

Gummesson, B., L. U. Magnusson, M. Lovmar, K. Kvint, O. Persson, M. Ballesteros, A. Farewell & T. Nystrom, (2009) Increased RNA polymerase availability directs resources towards growth at the expense of maintenance. *EMBO J* **28**: 2209-2219.

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

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

Haugen, S. P., W. Ross & R. L. Gourse, (2008) Advances in bacterial promoter recognition and its control by factors that do not bind DNA. *Nat Rev Microbiol* **6**: 507-519.

Heath, R. J., S. Jackowski & C. O. Rock, (1994) Guanosine tetraphosphate inhibition of fatty acid and phospholipid synthesis in *Escherichia coli* is relieved by overexpression of glycerol-3-phosphate acyltransferase (*plsB*). *J Biol Chem* **269**: 26584-26590.

Heinzen, R. A., T. Hackstadt & J. E. Samuel, (1999) Developmental biology of *Coxiella burnettii*. *Trends Microbiol* **7**: 149-154.

Hengge, R., (2009) Proteolysis of sigmaS (RpoS) and the general stress response in *Escherichia coli*. *Res Microbiol* **160**: 667-676.

Heuner, K., and C. Albert-Weissenberger, (2007) The flagellar regulon of *Legionella pneumophila* and the expression of virulence traits. In: *Legionella* Molecular Microbiology. K. Heuner, and M. Swanson (ed). Norfolk, United Kingdom: Caister Academic Press, pp. 101-121.

Heuner, K., C. Dietrich, C. Skriwan, M. Steinert & J. Hacker, (2002) Influence of the alternative sigma(28) factor on virulence and flagellum expression of *Legionella pneumophila*. *Infect Immun* **70**: 1604-1608.

Hood, M. A., J. B. Guckert, D. C. White & F. Deck, (1986) Effect of nutrient deprivation on lipid, carbohydrate, DNA, RNA, and protein levels in *Vibrio cholerae*. *Appl Environ Microbiol* **52**: 788-793.

Hovel-Miner, G., S. Pampou, S. P. Faucher, M. Clarke, I. Morozova, P. Morozov, J. J. Russo, H. A. Shuman & S. Kalachikov, (2009) SigmaS controls multiple pathways associated with intracellular multiplication of *Legionella pneumophila*. *J Bacteriol* **191**: 2461-2473.

Inaoka, T. & K. Ochi, (2002) RelA protein is involved in induction of genetic competence in certain *Bacillus subtilis* strains by moderating the level of intracellular GTP. *J Bacteriol* **184**: 3923-3930.

Isberg, R. R., T. J. O'Connor & M. Heidtman, (2009) The *Legionella pneumophila* replication vacuole: making a cosy niche inside host cells. *Nat Rev Microbiol* **7**: 13-24.

Jackowski, S. & C. O. Rock, (1983) Ratio of active to inactive forms of acyl carrier protein in *Escherichia coli*. *J Biol Chem* **258**: 15186-15191.

Jacobi, S., R. Schade & K. Heuner, (2004) Characterization of the alternative sigma factor sigma54 and the transcriptional regulator FleQ of *Legionella pneumophila*, which are both involved in the regulation cascade of flagellar gene expression. *J Bacteriol* **186**: 2540-2547.

Jonas, K., H. Tomenius, U. Romling, D. Georgellis & O. Melefors, (2006) Identification of YhdA as a regulator of the *Escherichia coli* carbon storage regulation system. *FEMS Microbiol Lett* **264**: 232-237.

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

Karakousis, P. C., T. Yoshimatsu, G. Lamichhane, S. C. Woolwine, E. L. Nuermberger, J. Grosset & W. R. Bishai, (2004) Dormancy phenotype displayed by extracellular *Mycobacterium tuberculosis* within artificial granulomas in mice. *J Exp Med* **200**: 647-657.

Krasny, L. & R. L. Gourse, (2004) An alternative strategy for bacterial ribosome synthesis: *Bacillus subtilis* rRNA transcription regulation. *EMBO J* **23**: 4473-4483.

Krasny, L., H. Tiserova, J. Jonak, D. Rejman & H. Sanderova, (2008) The identity of the transcription +1 position is crucial for changes in gene expression in response to amino acid starvation in *Bacillus subtilis*. *Mol Microbiol* **69**: 42-54.

Lapouge, K., M. Schubert, F. H. Allain & D. Haas, (2008) Gac/Rsm signal transduction pathway of gamma-proteobacteria: from RNA recognition to regulation of social behaviour. *Mol Microbiol* **67**: 241-253.

Lawhon, S. D., R. Maurer, M. Suyemoto & C. Altier, (2002) Intestinal short-chain fatty acids alter *Salmonella typhimurium* invasion gene expression and virulence through BarA/SirA. *Mol Microbiol* **46**: 1451-1464.

Lemke, J. J., T. Durfee & R. L. Gourse, (2009) DksA and ppGpp Directly Regulate Transcription of the *Escherichia coli* Flagellar Cascade. *Mol Microbiol* **74**: 1368-1379.

Lemos, J. A., V. K. Lin, M. M. Nascimento, J. Abranches & R. A. Burne, (2007) Three gene products govern (p)ppGpp production by *Streptococcus mutans*. *Mol Microbiol* **65**: 1568-1581.

Liu, M., G. M. Conover & R. R. Isberg, (2008) *Legionella pneumophila* EnhC is required for efficient replication in tumour necrosis factor alpha-stimulated macrophages. *Cell Microbiol* **10**: 1906-1923.

Lopez, J. M., A. Dromerick & E. Freese, (1981) Response of guanosine 5'-triphosphate concentration to nutritional changes and its significance for *Bacillus subtilis* sporulation. *J Bacteriol* **146**: 605-613.

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

Lyzen, R., M. Kochanowska, G. Wegrzyn & A. Szalewska-Palasz, (2009) Transcription from bacteriophage {lambda} pR promoter is regulated independently and antagonistically by DksA and ppGpp. *Nucleic Acids Res*.

Magnuson, K., S. Jackowski, C. O. Rock & J. E. Cronan, Jr., (1993) Regulation of fatty acid biosynthesis in *Escherichia coli*. *Microbiol Rev* **57**: 522-542.

Magnusson, L. U., A. Farewell & T. Nystrom, (2005) ppGpp: a global regulator in *Escherichia coli*. *Trends Microbiol* **13**: 236-242.

Magnusson, L. U., B. Gummesson, P. Joksimovic, A. Farewell & T. Nystrom, (2007) Identical, independent, and opposing roles of ppGpp and DksA in *Escherichia coli*. *J Bacteriol* **189**: 5193-5202.

McCleary, W. R., J. B. Stock & A. J. Ninfa, (1993) Is acetyl phosphate a global signal in *Escherichia coli*? *J Bacteriol* **175**: 2793-2798.

McCune, S. A. & R. A. Harris, (1979) Mechanism responsible for 5-(tetradecyloxy)-2-furoic acid inhibition of hepatic lipogenesis. *J Biol Chem* **254**: 10095-10101.

McNealy, T. L., V. Forsbach-Birk, C. Shi & R. Marre, (2005) The Hfq homolog in *Legionella pneumophila* demonstrates regulation by LetA and RpoS and interacts with the global regulator CsrA. *J Bacteriol* **187**: 1527-1532.

Merrikh, H., A. E. Ferrazzoli, A. Bougdour, A. Olivier-Mason & S. T. Lovett, (2009a) A DNA damage response in *Escherichia coli* involving the alternative sigma factor, RpoS. *Proc Natl Acad Sci U S A* **106**: 611-616.

Merrikh, H., A. E. Ferrazzoli & S. T. Lovett, (2009b) Growth phase and (p)ppGpp control of IraD, a regulator of RpoS stability, in *Escherichia coli*. *J Bacteriol* **191**: 7436-7446.

Milohanic, E., P. Glaser, J. Y. Coppee, L. Frangeul, Y. Vega, J. A. Vazquez-Boland, F. Kunst, P. Cossart & C. Buchrieser, (2003) Transcriptome analysis of *Listeria monocytogenes* identifies three groups of genes differently regulated by PrfA. *Mol Microbiol* **47**: 1613-1625.

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

Molofsky, A. B., L. M. Shetron-Rama & M. S. Swanson, (2005) Components of the *Legionella pneumophila* flagellar regulon contribute to multiple virulence traits, including lysosome avoidance and macrophage death. *Infect Immun* **73**: 5720-5734.

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

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

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

Mouery, K., B. A. Rader, E. C. Gaynor & K. Guillemin, (2006) The stringent response is required for *Helicobacter pylori* survival of stationary phase, exposure to acid, and aerobic shock. *J Bacteriol* **188**: 5494-5500.

Nagai, H., J. C. Kagan, X. Zhu, R. A. Kahn & C. R. Roy, (2002) A bacterial guanine nucleotide exchange factor activates ARF on *Legionella* phagosomes. *Science* **295**: 679-682.

Nakanishi, N., H. Abe, Y. Ogura, T. Hayashi, K. Tashiro, S. Kuhara, N. Sugimoto & T. Tobe, (2006) ppGpp with DksA controls gene expression in the locus of enterocyte effacement (LEE) pathogenicity island of enterohaemorrhagic *Escherichia coli* through activation of two virulence regulatory genes. *Mol Microbiol* **61**: 194-205.

Nanamiya, H., K. Kasai, A. Nozawa, C. S. Yun, T. Narisawa, K. Murakami, Y. Natori, F. Kawamura & Y. Tozawa, (2008) Identification and functional analysis of novel (p)ppGpp synthetase genes in *Bacillus subtilis*. *Mol Microbiol* **67**: 291-304.

Okada, Y., S. Makino, T. Tobe, N. Okada & S. Yamazaki, (2002) Cloning of rel from *Listeria monocytogenes* as an osmotolerance involvement gene. *Appl Environ Microbiol* **68**: 1541-1547.

Panek, E., G. A. Cook & N. W. Cornell, (1977) Inhibition by 5-(tetradecyloxy)-2-furoic acid of fatty acid and cholesterol synthesis in isolated rat hepatocytes. *Lipids* **12**: 814-818.

Paredes, C. J., K. V. Alsaker & E. T. Papoutsakis, (2005) A comparative genomic view of clostridial sporulation and physiology. *Nat Rev Microbiol* **3**: 969-978.

Perederina, A., V. Svetlov, M. N. Vassylyeva, T. H. Tahirov, S. Yokoyama, I. Artsimovitch & D. G. Vassylyev, (2004) Regulation through the secondary channel--structural framework for ppGpp-DksA synergism during transcription. *Cell* **118**: 297-309.

Persky, N. S., D. J. Ferullo, D. L. Cooper, H. R. Moore & S. T. Lovett, (2009) The ObgE/CgtA GTPase influences the stringent response to amino acid starvation in *Escherichia coli*. *Mol Microbiol* **73**: 253-266.

Pizarro-Cerda, J. & K. Tedin, (2004) The bacterial signal molecule, ppGpp, regulates *Salmonella* virulence gene expression. *Mol Microbiol* **52**: 1827-1844.

Pizer, E. S., J. Thupari, W. F. Han, M. L. Pinn, F. J. Chrest, G. L. Frehywot, C. A. Townsend & F. P. Kuhajda, (2000) Malonyl-coenzyme-A is a potential mediator of cytotoxicity induced by fatty-acid synthase inhibition in human breast cancer cells and xenografts. *Cancer Res* **60**: 213-218.

Post-Beittenmiller, D., J. G. Jaworski & J. B. Ohlrogge, (1991) In vivo pools of free and acylated acyl carrier proteins in spinach. Evidence for sites of regulation of fatty acid biosynthesis. *J Biol Chem* **266**: 1858-1865.

Potrykus, K. & M. Cashel, (2008) (p)ppGpp: still magical? *Annu Rev Microbiol* **62**: 35-51.

Primm, T. P., S. J. Andersen, V. Mizrahi, D. Avarbock, H. Rubin & C. E. Barry, 3rd, (2000) The stringent response of *Mycobacterium tuberculosis* is required for long-term survival. *J Bacteriol* **182**: 4889-4898.

Purevdorj-Gage, B., W. J. Costerton & P. Stoodley, (2005) Phenotypic differentiation and seeding dispersal in non-mucoid and mucoid *Pseudomonas aeruginosa* biofilms. *Microbiology* **151**: 1569-1576.

Rasis, M. & G. Segal, (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.

Ren, T., D. S. Zamboni, C. R. Roy, W. F. Dietrich & R. E. Vance, (2006) Flagellin-deficient *Legionella* mutants evade caspase-1- and Naip5-mediated macrophage immunity. *PLoS Pathog* **2**: e18.

Rock, C. O. & J. E. Cronan, Jr., (1981) Acyl carrier protein from *Escherichia coli*. *Methods Enzymol* **71 Pt C**: 341-351.

Rowbotham, T. J., (1986) Current views on the relationships between amoebae, Legionellae and man. *Isr J Med Sci* **22**: 678-689.

Sahr, T., H. Bruggemann, M. Jules, M. Lomma, C. Albert-Weissenberger, C. Cazalet & C. Buchrieser, (2009) Two small ncRNAs jointly govern virulence and transmission in *Legionella pneumophila*. *Mol Microbiol*.

Sauer, J. D., M. A. Bachman & M. S. Swanson, (2005) The phagosomal transporter A couples threonine acquisition to differentiation and replication of *Legionella pneumophila* in macrophages. *Proc Natl Acad Sci U S A* **102**: 9924-9929.

Schreiber, G., S. Metzger, E. Aizenman, S. Roza, M. Cashel & G. Glaser, (1991) Overexpression of the *relA* gene in *Escherichia coli*. *J Biol Chem* **266**: 3760-3767.

Schujman, G. E., S. Altabe & D. de Mendoza, (2008) A malonyl-CoA-dependent switch in the bacterial response to a dysfunction of lipid metabolism. *Mol Microbiol* **68**: 987-996.

Seyfzadeh, M., J. Keener & M. Nomura, (1993) *spoT*-dependent accumulation of guanosine tetraphosphate in response to fatty acid starvation in *Escherichia coli*. *Proc Natl Acad Sci U S A* **90**: 11004-11008.

Sharma, A. K. & S. M. Payne, (2006) Induction of expression of *hfq* by DksA is essential for *Shigella flexneri* virulence. *Mol Microbiol* **62**: 469-479.

Shin, S. & C. R. Roy, (2008) Host cell processes that influence the intracellular survival of *Legionella pneumophila*. *Cell Microbiol* **10**: 1209-1220.

Song, M., H. J. Kim, E. Y. Kim, M. Shin, H. C. Lee, Y. Hong, J. H. Rhee, H. Yoon, S. Ryu, S. Lim & H. E. Choy, (2004) ppGpp-dependent stationary phase induction of genes on *Salmonella* pathogenicity island 1. *J Biol Chem* **279**: 34183-34190.

Spira, B. & E. Yagil, (1998) The relation between ppGpp and the PHO regulon in *Escherichia coli*. *Mol Gen Genet* **257**: 469-477.

Srivatsan, A. & J. D. Wang, (2008) Control of bacterial transcription, translation and replication by (p)ppGpp. *Curr Opin Microbiol* **11**: 100-105.

Stallings, C. L., N. C. Stephanou, L. Chu, A. Hochschild, B. E. Nickels & M. S. Glickman, (2009) CarD is an essential regulator of rRNA transcription required for *Mycobacterium tuberculosis* persistence. *Cell* **138**: 146-159.

Stone, B. J. & Y. A. Kwaik, (1999) Natural competence for DNA transformation by *Legionella pneumophila* and its association with expression of type IV pili. *J Bacteriol* **181**: 1395-1402.

Sturgill-Koszycki, S. & M. S. Swanson, (2000) *Legionella pneumophila* replication vacuoles mature into acidic, endocytic organelles. *J Exp Med* **192**: 1261-1272.

Sun, W., K. L. Roland, C. G. Branger, X. Kuang & R. Curtiss, 3rd, (2009) The role of *relA* and *spoT* in *Yersinia pestis* KIM5 pathogenicity. *PLoS One* **4**: e6720.

Swanson, M. S. & B. K. Hammer, (2000) *Legionella pneumophila* pathogesesis: a fateful journey from amoebae to macrophages. *Annu Rev Microbiol* **54**: 567-613.

Swanson, M. S. & R. R. Isberg, (1995) Association of *Legionella pneumophila* with the macrophage endoplasmic reticulum. *Infect Immun* **63**: 3609-3620.

Szalewska-Palasz, A., L. U. Johansson, L. M. Bernardo, E. Skarfstad, E. Stec, K. Brannstrom & V. Shingler, (2007) Properties of RNA polymerase bypass mutants: implications for the role of ppGpp and its co-factor DksA in controlling transcription dependent on sigma54. *J Biol Chem* **282**: 18046-18056.

Taylor, C. M., M. Beresford, H. A. S. Epton, D. C. Sigee, G. Shama, P. W. Andrew & I. S. Roberts, (2002) *Listeria monocytogenes relA* and *hpt* Mutants Are Impaired in Surface-Attached Growth and Virulence. *J. Bacteriol.* **184**: 621-628.

Tesh, M. J. & R. D. Miller, (1981) Amino acid requirements for *Legionella pneumophila* growth. *J Clin Microbiol* **13**: 865-869.

Tesh, M. J., S. A. Morse & R. D. Miller, (1983) Intermediary metabolism in *Legionella pneumophila*: utilization of amino acids and other compounds as energy sources. *J Bacteriol* **154**: 1104-1109.

Thompson, A., M. D. Rolfe, S. Lucchini, P. Schwerk, J. C. Hinton & K. Tedin, (2006) The bacterial signal molecule, ppGpp, mediates the environmental regulation of both the invasion and intracellular virulence gene programs of *Salmonella*. *J Biol Chem* **281**: 30112-30121.

Tiaden, A., T. Spirig, S. S. Weber, H. Bruggemann, R. Bosshard, C. Buchrieser & H. Hilbi, (2007) The *Legionella pneumophila* response regulator LqsR promotes host cell interactions as an element of the virulence regulatory network controlled by RpoS and LetA. *Cell Microbiol* **9**: 2903-2920.

Tosa, T. & L. I. Pizer, (1971) Biochemical bases for the antimetabolite action of L-serine hydroxamate. *J Bacteriol* **106**: 972-982.

Traxler, M. F., S. M. Summers, H. T. Nguyen, V. M. Zacharia, G. A. Hightower, J. T. Smith & T. Conway, (2008) The global, ppGpp-mediated stringent response to amino acid starvation in *Escherichia coli*. *Mol Microbiol* **68**: 1128-1148.

Ulrich, A. K., D. de Mendoza, J. L. Garwin & J. E. Cronan, Jr., (1983) Genetic and biochemical analyses of *Escherichia coli* mutants altered in the temperature-dependent regulation of membrane lipid composition. *J Bacteriol* **154**: 221-230.

Vance, D., I. Goldberg, O. Mitsuhashi & K. Bloch, (1972) Inhibition of fatty acid synthetases by the antibiotic cerulenin. *Biochem Biophys Res Commun* **48**: 649-656.

Vogel, J. P., C. Roy & R. R. Isberg, (1996) Use of salt to isolate *Legionella pneumophila* mutants unable to replicate in macrophages. *Ann N Y Acad Sci* **797**: 271-272.

Voth, D. E. & R. A. Heinzen, (2007) Lounging in a lysosome: the intracellular lifestyle of *Coxiella burnetii*. *Cell Microbiol* **9**: 829-840.

Wang, J. D., G. M. Sanders & A. D. Grossman, (2007) Nutritional control of elongation of DNA replication by (p)ppGpp. *Cell* **128**: 865-875.

Warner, D. F. & V. Mizrahi, (2006) Tuberculosis chemotherapy: the influence of bacillary stress and damage response pathways on drug efficacy. *Clin Microbiol Rev* **19**: 558-570.

Warren, W. J. & R. D. Miller, (1979) Growth of Legionnaires disease bacterium (*Legionella pneumophila*) in chemically defined medium. *J Clin Microbiol* **10**: 50-55.

Watarai, M., I. Derre, J. Kirby, J. D. Growney, W. F. Dietrich & R. R. Isberg, (2001) *Legionella pneumophila* is internalized by a macropinocytotic uptake pathway controlled by the Dot/Icm system and the mouse Lgn1 locus. *J Exp Med* **194**: 1081-1096.

Wei, B. L., A. M. Brun-Zinkernagel, J. W. Simecka, B. M. Pruss, P. Babitzke & T. Romeo, (2001) Positive regulation of motility and *flhDC* expression by the RNA-binding protein CsrA of *Escherichia coli*. *Mol Microbiol* **40**: 245-256.

Weilbacher, T., K. Suzuki, A. K. Dubey, X. Wang, S. Gudapaty, I. Morozov, C. S. Baker, D. Georgellis, P. Babitzke & T. Romeo, (2003) A novel sRNA component of the carbon storage regulatory system of *Escherichia coli*. *Mol Microbiol* **48**: 657-670.

Wells, D. H. & E. C. Gaynor, (2006) *Helicobacter pylori* Initiates the Stringent Response upon Nutrient and pH Downshift. *J. Bacteriol.* **188**: 3726-3729.

Wieland, H., M. Faigle, F. Lang, H. Northoff & B. Neumeister, (2002) Regulation of the *Legionella mip*-promotor during infection of human monocytes. *FEMS Microbiol Lett* **212**: 127-132.

Wieland, H., S. Ullrich, F. Lang & B. Neumeister, (2005) Intracellular multiplication of *Legionella pneumophila* depends on host cell amino acid transporter SLC1A5. *Mol Microbiol* **55**: 1528-1537.

Wolfe, A. J., (2005) The acetate switch. *Microbiol Mol Biol Rev* **69**: 12-50.

Xiao, H., M. Kalman, K. Ikehara, S. Zemel, G. Glaser & M. Cashel, (1991) Residual guanosine 3',5'-bispyrophosphate synthetic activity of *relA* null mutants can be eliminated by *spoT* null mutations. *J Biol Chem* **266**: 5980-5990.

Yu, D., H. M. Ellis, E. C. Lee, N. A. Jenkins, N. G. Copeland & D. L. Court, (2000) An efficient recombination system for chromosome engineering in *Escherichia coli*. *Proc Natl Acad Sci U S A* **97**: 5978-5983.

Zhao, G., N. Weatherspoon, W. Kong, R. Curtiss, 3rd & Y. Shi, (2008) A dual-signal regulatory circuit activates transcription of a set of divergent operons in *Salmonella typhimurium*. *Proc Natl Acad Sci U S A* **105**: 20924-20929.

Zusman, D. R., A. E. Scott, Z. Yang & J. R. Kirby, (2007) Chemosensory pathways, motility and development in *Myxococcus xanthus*. *Nat Rev Microbiol* **5**: 862-872.

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