DksA and ppGpp make distinct contributions to *Legionella pneumophila* differentiation

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As my honors thesis stems from the work that Zach has done, I would like to fully disclose exactly what bench work I did in this thesis. Before I arrived in the lab, Zach created the *ppGpp*^{0} mutant used in this study. I helped him create the *dksA* mutant. The experiments reflected in Figures 1, 2, 4, and 5 were done collaboratively between Zach and myself. Those four figures appear in our 2010 paper. The experiments in figures 3 and 6 were designed and implemented completely by myself. This includes designing and constructing the four transcription reporter plasmids used herein. In terms of writing this thesis, I borrowed from the Methods and Materials section of the 2010 publication to describe some of the assays used in this project. All other writing is my own.

I hope that you enjoy reading and thinking about this research as much as I have. I feel blessed and privileged to have the rich experience I had doing this work. Writing this has felt like the last three years of my life have gone to good work. To all of the people I have listed above (and if I have forgotten anyone else), I thank you, and I will always be indebted to you.

-Brian Yagi

DksA and ppGpp make distinct contributions to *Legionella pneumophila* differentiation

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Abstract

***Legionella pneumophila* is a Gram-negative intracellular pathogen of amoebae and human alveolar macrophages that cycles between two distinct states: replicative and transmissive. When replicative *L. pneumophila* exhaust local nutrients, the signaling molecule guanosine tetraphosphate (ppGpp) accumulates and bacteria become motile, resistant to environmental stress and equipped to escape a depleted host cell to invade a naïve one. Here, this paradigm is expanded to include DksA, a protein that in many bacteria potentiates the effect of ppGpp on RNA polymerase in addition to functioning independently and even oppositely of ppGpp to regulate gene transcription. Upon starvation in broth, *L. pneumophila* requires both DksA and ppGpp for differentiation to the transmissive form, as mutants lacking either ppGpp ($ppGpp^o$) or *dksA* were unable to survive stationary phase stress, exhibit cytotoxicity toward macrophages, or evade degradation by macrophage lysosomes. DksA can also regulate flagellar biosynthesis independently of ppGpp, as over-expressed *dksA* partially rescued motility and cytotoxicity of $ppGpp^o$ mutant *L. pneumophila*. Although DksA was critical in broth, it was dispensable for transmission between macrophages. Unlike $ppGpp^o$ *L. pneumophila*, which are degraded following the primary replication period in macrophages, *dksA* mutants survive in macrophages and replicate to wild-type levels, as judged by immunofluorescence microscopy and viable counts. The use of transcription reporter plasmids revealed that ppGpp and DksA can exert a variety of regulatory patterns. At some promoters, ppGpp and DksA are both required for activation. At other promoters, DksA is sufficient for basal levels of transcription. ppGpp can also activate some promoters without DksA. All promoters tested, however, required both ppGpp and DksA in order to be fully induced to wild type levels. Monitoring these reporters during a macrophage infection reveals that ppGpp and DksA both regulate flagellar genes *in vivo*. Thus, *L. pneumophila* utilizes a diverse interaction between DksA and ppGpp in order to differentiate and survive.**

Introduction

Legionella pneumophila is a Gram-negative gamma-proteobacteria known to be the causative agent of Legionnaires' Disease. In nature, the bacteria are ubiquitous in water reservoirs where they reside in biofilms or inside protozoa. When humans inhale aerosols that contain *L. pneumophila*, the bacteria are able to infect the host's macrophages located in the alveoli of the lung. Growth and proliferation of these bacteria cause the acute pneumonic symptoms of Legionnaires' Disease. *L. pneumophila* can be cultured in rich broth, has several observable virulence phenotypes (Byrne & Swanson, 1998), and displays interesting host-pathogen interactions, making it an ideal tool to study the regulation of bacterial pathogenesis.

Unlike other pathogens, such as *Mycobacterium tuberculosis*, *L. pneumophila* does not spread from person to person; however, in the absence of a robust immune response, it can exploit macrophages as a host for replication. *L. pneumophila* transmission is characterized by lysis of one host cell and subsequent invasion of a new one. Inside of host macrophages, the bacteria differentiate between two life forms: the replicative and transmisive (Molofsky & Swanson, 2004; Bruggemann et al, 2006) . After phagocytosis, *L. pneumophila* in its transmissive form manipulates the host cell's vesicular trafficking pathway; it evades degradation by the host's lysosomes and instead traffics to a vacuole that is derived from the host's endoplasmic reticulum (Isberg, et al, 2009). Once safely inside this vacuole, the bacterium represses its transmissive traits and begins expressing replicative traits such as ribosomal genes (Sauer, et al, 2005; Bruggeman et al, 2006). Then, the bacterium replicates to high numbers inside of this vacuole. When host resources have been depleted, *L. pneumophila* activates a universal stress pathway known as the stringent response, triggering differentiation of the bacterium into the transmissive phase. In its transmissive form, the bacterium becomes motile, breaks out of its replicative vacuole, lyses its host cell and infects a new host. Once inside the new host, the bacterium re-enters the replicative phase, and the life cycle is repeated (Molofsky & Swanson, 2004). During growth in rich broth, cultures in the exponential (E) phase are in the replicative form, while

cultures in the post exponential (PE) phase are in the transmissive form.

When *L. pneumophila* differentiates from the replicative form to the transmissive form, over half of the genes in its genome are either up-regulated or down-regulated (Brugemann, et al, 2006). This global change in gene expression of the stringent response is mediated by the alarmone guanasine tetraphosphate (ppGpp). Cellular pools of ppGpp are generated by the ppGpp synthase RelA and by the bifunctional synthase/hydrolase SpoT. RelA responds to amino acid starvation by associating with bacterial ribosomes and generating ppGpp in response to uncharged tRNA (Potrykus & Cashel, 2008). In a similar fashion, SpoT responds to fatty acid starvation by associating with acyl-carrier protein (Battesti & Bouveret, 2009). ppGpp up-regulates the transcription of transmissive genes such as flagellar genes, Dot/Icm Type IV Secretion System components, and regulatory RNAs including rsmZ. Conversely, ppGpp accumulation triggers down-regulation of replication-related genes such as ribosomal genes, genes involved in membrane modification, and others involved in ATP synthesis (Dalebroux, et al, 2010). *L. pneumophila* lacking ppGpp (*ppGpp^0*) are unable to differentiate to the transmissive form while grown in broth and are also unable to establish a successful infection of host macrophages (Dalebroux, et al, 2009).

From mechanistic work conducted in *Escherichia coli*, ppGpp is known to control gene expression by interacting with RNA polymerase (RNAP). ppGpp's interaction with RNAP is cooperative with the protein DksA, which binds to the secondary channel of RNAP (Haugen, et al, 2008). By binding to RNAP, DksA modifies kinetic properties of specific promoters by sensitizing them to changes in ppGpp concentration (Paul, et al, 2004). DksA and ppGpp can cooperate to up- or down-regulate a gene based on its promoter sequence. *In vitro* transcription studies have shown that DksA and ppGpp directly up-regulate several virulence regulators (Nakanishi et al, 2006; Sharma & Payne, 2006; Aberg et al, 2008). DksA and ppGpp can also inhibit the transcription of flagellar promoters in *E. coli* which, opposite of *L. pneumophila*, loses its flagellum in the PE phase (Lemke, et al, 2009).

ppGpp and DksA can also regulate gene expression indirectly through their interactions with RNAP. In the vegetative state of *E coli* growth, the housekeeping sigma factor, σ^{70}, is associated with RNAP and is frequently bound to ribosomal RNA promoters (Bremer & Dennis, 1996). Upon activation of the stringent response, however, ppGpp and DksA bind RNAP and dissociate it from σ^{70}, freeing the core polymerase to access alternate sigma factors such as σ^{54} (Gummesson, et al, 2009; Bernardo, et al, 2006). Subsequently, RNAP is directed to stress response-related genes by these alternative sigma factors (Szalewska-Palasz et al, 2007). Thus, many genes under the control of an alternate sigma factor may be regulated by ppGpp and/or DksA by an indirect mechanism.

DksA and ppGpp can act as cofactors when binding to RNAP and subsequently cooperate in regulating gene expression. It appears, however, that the interplay between DksA and ppGpp may be more complicated: DksA and ppGpp can perform certain functions in the complete absence of the other. When *dksA* is over-expressed in *E. coli* mutants lacking ppGpp, transcription from several promoters is revived from the depressed levels of the ppGpp^0 mutant without over-expressed *dksA* (Potrykus & Cashel, 2008). Moreover, over-expressing DksA rescues several virulence phenotypes that are lost in *ppGpp^0* mutant bacteria with basal levels of DksA. On top of that, ppGpp and DksA have opposing effects on the adhesion phenotype of *E. coli* (Magnusson, et al, 2007) as well as the transcription of the bacteriophage lambda promoter pR (Lyzen, et al, 2009). The purpose of this research was to elucidate the contributions of ppGpp and DksA to the differentiation of *L. pneumophila*. I investigated whether the two factors could control virulence phenotypes independently. Additionally, to determine the functions of ppGpp and DksA at the level of transcription, promoter activity reporter plasmids were constructed and analyzed in broth and macrophage cultures.

Results

DksA is required for L. pneumophila *to survive into the stationary phase.*

To assess the contribution of DksA in the life cycle of *L. pneumophila,* mutant bacteria with a deletion-insertion in the *dksA* gene were grown in rich broth (Fig. 1). When compared to wild-type (WT) *L. pneumophila,* the *dksA* mutants showed no defect in replicating to high densities during exponential (E) phase growth. Upon entry into the post-exponential (PE) phase, however, the mutants were unable to survive, as judged by the quantity of viable colony forming units (CFU). PE phase viability was rescued when *dksA* mutants were complemented with a plasmid that was

Figure 1.

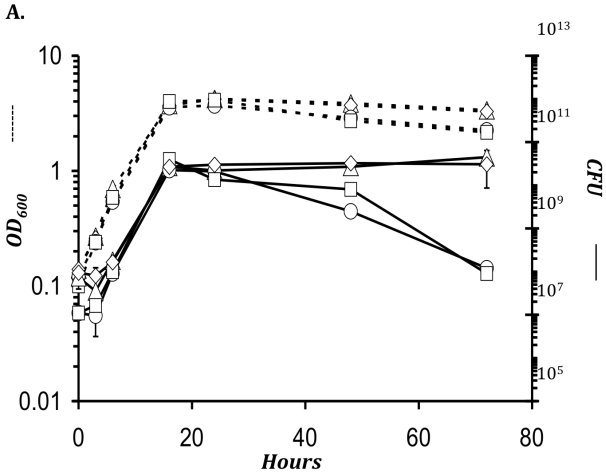

Figure 1. *Legionella* requires ppGpp and DksA to survive stationary phase stress in broth.
Early E phase AYET broth cultures of WT (triangles) and ppGpp^0 (circles) carrying empty vector and *dksA* mutants transformed with empty vector (squares) or p*dksA* (diamonds) were diluted to OD600 of 0.15, and bacteria were treated with 25 mM IPTG. At the times indicated, culture density and viability were quantified by reading OD600 (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.

induced to express *dksA* throughout growth. Additionally, WT bacteria became coccoid and motile at ~24 h whereas the *ppGpp^0* and *dksA* mutant cultures displayed increased filamentation and did not gain motility (data not shown). The *dksA* mutants' inability to survive stationary-phase stress was similar to the phenotype of mutant bacteria lacking ppGpp (*ppGpp^0*), which has been previously described (Dalebroux, et al, 2009). The defective morphology and viability of *dksA* mutants in PE phase supports the hypothesis that DksA and ppGpp contribute to differentiation in *L. pneumophila*. For subsequent experiments that required *dksA* or *ppGpp^0* mutant bacteria in the PE phase, the cultures analyzed did not significantly differ in viable counts from WT (corresponding to the 18 – 24 h time points in Fig. 1).

DksA regulates L. pneumophila *stationary phase virulence traits along with ppGpp.*

L. pneumophila displays a number of measurable virulence traits upon differentiation to the transmissive phase including the ability to: infect macrophages, avoid degradation inside macrophages, kill macrophages (cytotoxicity), and arrest growth in the presence of sodium (Byrne & Swanson, 1998). Sodium sensitivity is a phenotype associated with Type IV secretion, which is activated in *L. pneumophila* in the PE-phase of growth (Vogel, et al., 1996). Since *L. pneumophila* induces its virulence traits upon entry into PE phase in response to ppGpp, the contribution of DksA to these hallmark virulence traits was assessed. Bacteria lacking *dksA* were grown to the PE-phase in broth, at which point they were poorly infectious toward macrophages (Fig. 2A), were readily degraded once inside macrophages (Fig. 2B), were non-cytotoxic toward host macrophages (Fig. 2C), and did not become sodium-sensitive (Fig. 2D). These phenotypes were all similar to WT bacteria in the E phase as well as *ppGpp^0* mutants that had been grown to the PE phase. Again, *dksA* mutant phenotypes were complemented by inducing the expression of plasmid-borne *dksA* (Fig. 2A and 2C). These results further indicate that, along with ppGpp, DksA contributes to *L. pneumophila* differentiation.

DksA and ppGpp induce several genes that are activated in the PE phase.

Since DksA and ppGpp are both known to regulate the global change in gene expression during the stringent response (Lemke, et al., 2009), reporter vectors were constructed to assay the promoter activity of four genes that *L. pneumophila* activates in PE phase (Bruggemann, et al., 2006). *fliA* is a Class III gene of the flagellar regulon that encodes an alternative sigma factor (σ^{28}) that directly regulates Class IV genes including *flaA* (Heuner et al., 2002). *flaA*, a Class IV gene, codes for flagellin, the monomeric unit of the flagella. *hfq* encodes a protective chaperone for mRNAs (reviewed in Brennan & Link, 2007). *rsmZ* is a small regulatory RNA that, along with *rsmY,* binds to CsrA, thereby relieving CsrA-mediated repression of mRNAs (Sahr, et al., 2009). The promoters for these four genes were each cloned into a plasmid directly upstream of the gene encoding green fluorescent protein (*gfp*) as previously described (Hammer & Swanson, 1999). I next used these tools to inspect how the promoters were activated throughout the life cycle of WT *L. pneumophila* and to determine whether their activation required either ppGpp or DksA.

Figure 2.

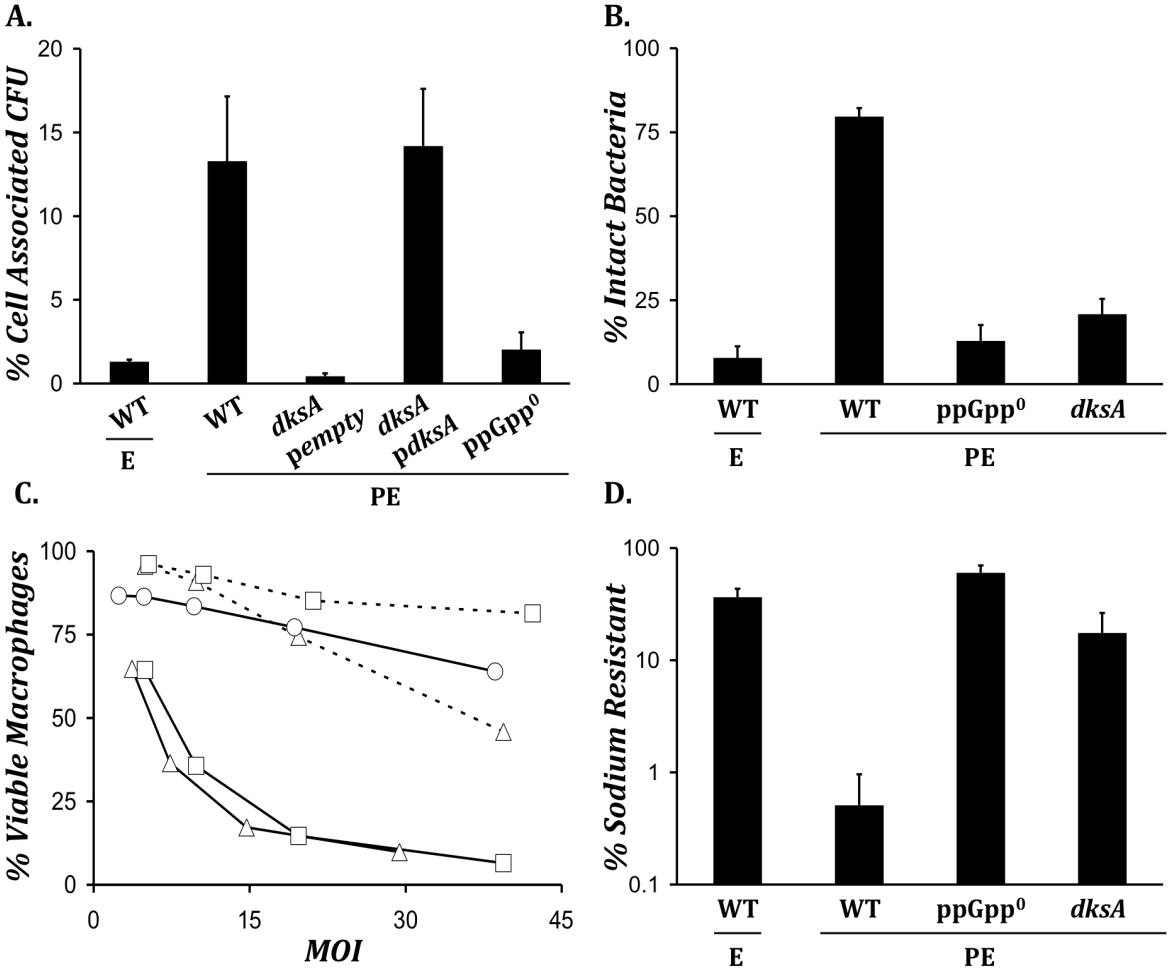

Figure 2. To activate PE phase transmission traits, *L. pneumophila* requires DksA and ppGpp.

A. To analyse 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 mM IPTG. Graphed are the mean per cent 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.

DksA and ppGpp controlled transcription of the four reporter constructs studied, though their degree of control varied. When cultured in rich broth, DksA was required for basal levels of *fliA* activation in the E phase, as judged by the 3 h time point in Fig. 3A. Upon entry into the stationary phase, however, both DksA and ppGpp were required for *fliA* expression, as neither *dksA* nor *ppGpp^0* mutants activated the *fliA* promoter to WT levels beyond 6 h. *L. pneumophila* requires both ppGpp and DksA to activate *flaA* (Fig. 3B). Likewise, *hfq* activation required both ppGpp and DksA (Fig. 3C). *rsmZ* promoter activity was independent of ppGpp and DksA during the first 6

Figure 3.

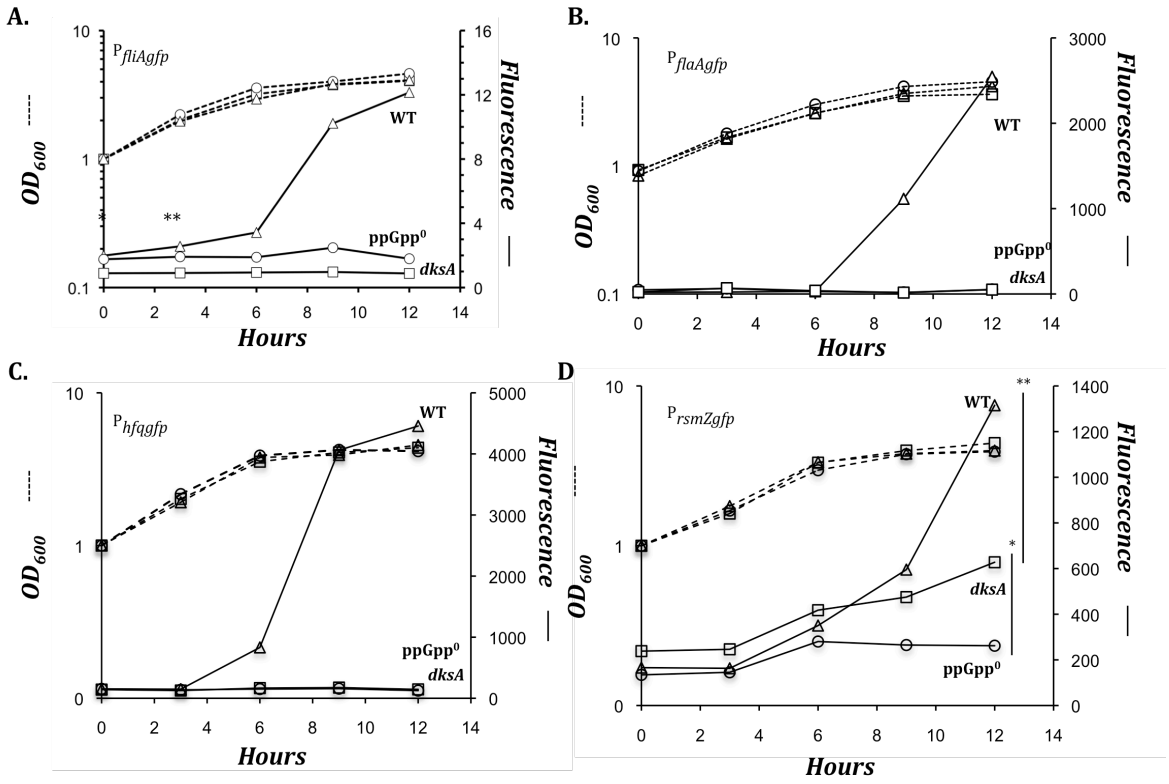

Figure 3. ppGpp and DksA make distinct contributions to the activation of *L. pneumophila* virulence factors *fliA, flaA, rsmZ,* and *hfq* during growth in rich broth.

To monitor promoter activity at the E to PE phase transition, mid-E phase WT (triangles), ppGpp^0 (circles), or *dksA* (squares) bacteria harboring the promoter-*gfp* reporter plasmid were diluted to an OD_{600} of 1.0. Cell density (dashed lines) and promoter activity (solid lines) was quantified by monitoring GFP fluorescence along with OD_{600} until bacteria entered stationary phase. The data represent one of three independent experiments that each showed similar patterns.

A. Basal transcription of *fliA* requires DksA, as the difference between WT and *dksA* and *ppGpp^0* and *dksA* were statistically different by a two-tailed paired student t-test (**p=0.04 and p=0.006 respectively). PE phase activation of *fliA* requires both ppGpp and DksA.

B. Expression of *flaA* requires both ppGpp and DksA.

C. Expression of *hfq* requires both ppGpp and DksA.

D. E phase expression of *rsmZ* is repressed by DksA while activation of PE phase is dependent on both ppGpp and, to a lesser extent, DksA. * indicates that the values of ppGpp^0 and *dksA* were statistically significantly different (two-tailed paired students t-test yielded p=0.01). ** indicates a p-value of 0.002. between the WT and *dksA* data points at 12 hours.

h of growth (Fig. 3D). However, upon bacterial entry into the PE phase, mutants lacking ppGpp or *dksA* were unable to activate the *rsmZ* promoter to WT levels. There was a statistically significant difference between the expression levels in the *ppGpp^0* mutants and the *dksA* mutants, however, indicating that ppGpp is capable of partially activating *rsmZ* in the absence of DksA. Therefore, a diversity of transcriptional control is exerted by ppGpp and DksA. For the *flaA* and *hfq* promoters, both stringent response factors were completely required for transcription, whereas ppGpp and DksA showed non-overlapping and independent activity at the *fliA* and *rsmZ* promoters.

DksA is dispensable for L. pneumophila *growth and survival in host macrophages.*

During a *L. pneumophila* infection of host macrophages, the bacteria differentiate between the replicative form (analogous to E phase cultures) and the transmissive form (analogous to PE phase cultures). To understand how DksA and ppGpp regulate differentiation during a macrophage infection, murine bone marrow-derived macrophages were infected with WT bacteria

grown to the E or PE phase or mutants lacking ppGpp or *dksA* grown to the PE phase. As previously seen in Fig. 2A, the *ppGpp⁰* and *dksA* mutants were inefficient at infecting macrophages within 2 h. Those that successfully entered, however, replicated for 24 h (Fig. 4A). By 24 h post infection, *L. pneumophila* differentiates from the replicative form to the transmissive form, lyses its primary host macrophage and subsequently infects a second naïve macrophage (Byrne & Swanson, 1998; Hammer & Swanson, 1999; Molofsky & Swanson, 2003). After 24 h, mutants lacking *dksA* continued to replicate to WT levels as measured by CFU. The yield of *ppGpp⁰* mutants, on the other hand, did not increase beyond 24 h. Therefore, during a macrophage infection, DksA is dispensable to *L. pneumophila*. The bacteria can express factors necessary for macrophage transmission with ppGpp alone.

To further test the contribution of DksA to the survival of bacteria residing inside of macrophages, the percentage of intact bacteria was scored 18 h after infecting host macrophages. *L. pneumophila* lacking ppGpp were degraded by the lysosome more frequently than the mutants lacking *dksA* (Fig. 4B). These microscopy results further support the hypothesis that ppGpp alone is required for *L. pneumophila* differentiation during a macrophage infection and underscore the first major phenotypic difference between ppGpp and DksA: DksA is dispensable for *L. pneumophila* to survive and differentiate inside of macrophages.

DksA and ppGpp can induce* L. pneumophila *virulence phenotypes independently of each other.

To understand the mechanism that accounts for the differential contributions of ppGpp and DksA illustrated in Fig. 4, the ability of ppGpp or DksA to work independently of the other was tested. To over-express *dksA* in the absence of ppGpp, the *dksA* gene was placed on a plasmid and transferred to the *ppGpp⁰* mutant strain (*ppGpp⁰* p*dksA*). To generate excess ppGpp in the absence of DksA, the gene encoding a constitutively active *E. coli* RelA cloned under inducible control on a plasmid and transferred to the *dksA* mutant strain (*dksA* p*relA*$^{E.c.}$). Since RelA is a ppGpp synthase, ppGpp accumulates to high levels upon *relA* induction (Hammer & Swanson, 1999).

DksA controlled the expression of FlaA independently of ppGpp (Fig. 5A). After growth in broth to the PE phase, only the strains containing DksA synthesized flagellin, as judged by western blot analysis (Fig. 5A top panel). The *ppGpp⁰* strain carrying an empty vector control produced low levels of flagellin most likely caused by the physiological levels of DksA that were still present. When *dksA* was over-expressed by the *ppGpp⁰* mutants, more flagellin was produced. Moreover, DksA bypassed the *ppGpp⁰* mutant

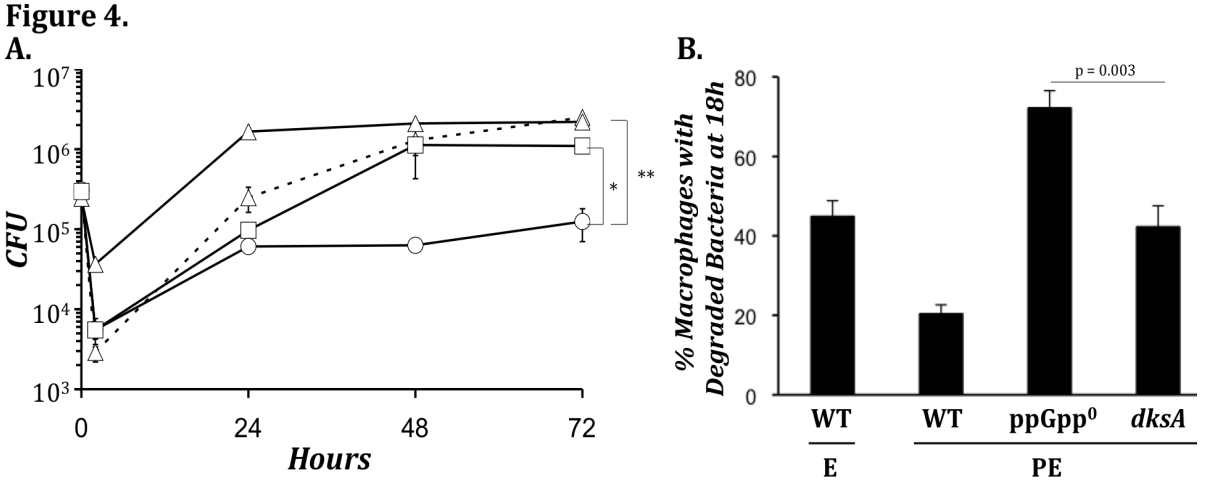

Figure 4. *L. pneumophila* requires ppGpp for macrophage transmission; DksA is less critical.
A. Macrophages were infected at an moi of ~1 with E phase WT (triangles, dashed lines), PE phase WT (triangles, solid line), ppGpp^0 (circles), or *dksA* (squares) bacteria. The number of viable bacteria was determined at the time points shown. Depicted are the mean cfu +/- SE from duplicate samples in one of four independent experiments. The symbol '*' indicates that the difference in the mean cfu values calculated from four independent experiments for WT and ppGpp0 mutant bacteria at 72 h was statistically significant by a paired, two-tailed Student's *t*-test (P = 0.047). The symbol '**' indicates that the difference between ppGpp0 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 % +/- SE of macrophages with degraded bacteria at 18 h from three independent experiments.

Figure 5.

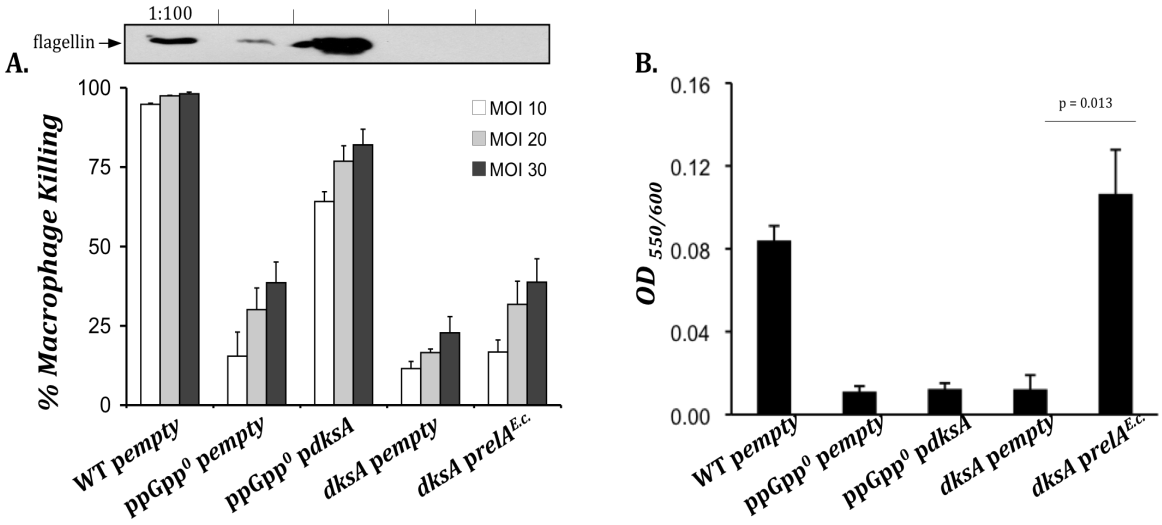

Figure 5. ppGpp and DksA can regulate *L. pneumophila* virulence traits independently of the other.
A. To assess levels of flagellin and macrophage cytotoxicity, bacterial cells were pelleted and harvested at 9 h post IPTG (indicated in (A) by '*'). Levels of cell-associated flagellin were assessed by Western analysis (upper image). At 9 h post IPTG, bacteria were also added to triplicate wells of macrophages at the moi 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.
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 OD550 by OD_{600}. The graph depicts the mean OD_{550}/OD_{600} values +/- SE from three independent experiments.

defect in cytotoxicity, a host response to flagellin (Molofsky, et al., 2005). The *ppGpp⁰* mutant became increasingly cytotoxic when *dksA* was overexpressed (Fig. 5A bottom panel). Taken together, these experiments show that DksA is sufficient to induce *flaA* expression in the bacteria, even in the complete absence of ppGpp.

On the other hand, ppGpp controlled other *L. pneumophila* virulence phenotypes independently of DksA. During late PE phase growth in broth, *L. pneumophila* secrete a pyomelanin pigment into the media (Zusman et al, 2002; Chatfield & Cianciotto, 2007). This PE phase phenotype is dependent on both ppGpp and DksA, as the mutant strains harboring the empty vector control did not secrete pigment (Fig. 5B). When *relA* was induced in the *dksA* mutant strain, however, pigment production was rescued. Restoration of pigmentation was not seen in the *ppGpp⁰* p*dksA* strain, however, indicating that ppGpp can induce pigment production in the absence of DksA, but not vice versa.

DksA and ppGpp control transcription of fliA *and* flaA *during* L. pneumophila *growth in host macrophages.*

The ability of ppGpp to trigger certain PE phase traits in the absence of DksA suggested that ppGpp can control transcription of certain genes during a macrophage infection, thereby accounting for the dispensability of DksA (See Fig. 4). To test whether transcriptional control is exerted by both ppGpp and DksA during a macrophage infection, WT, *ppGpp⁰* and *dksA* bacteria containing the $P_{fliAgfp}$ or the $P_{flaAgfp}$ reporter plasmid were cultured in macrophages. The percentage of bacteria expressing *gfp* was analyzed by microscopy while the bacteria were in the replicative phase and again while they were in the transmissive phase (Fig. 6). The *fliA* and *flaA* promoters were activated by WT bacteria in the transmissive phase, as judged by the percent of intact bacteria that were GFP positive. Mutant bacteria lacking ppGpp or *dksA*, however, were defective for activation of either the *flaA* or the *fliA* promoter at the same time points. Similar to how the two stringent response factors function in broth cultures (Fig. 3), ppGpp and DksA both control the expression of flagellar genes while *L. pneumophila* grow in macrophages. Thus, if ppGpp activates a gene during growth in macrophages that it doesn't during growth in broth, leading to the dispensability of DksA, it must be a gene other than *fliA* or *fliA.*

Figure 6.

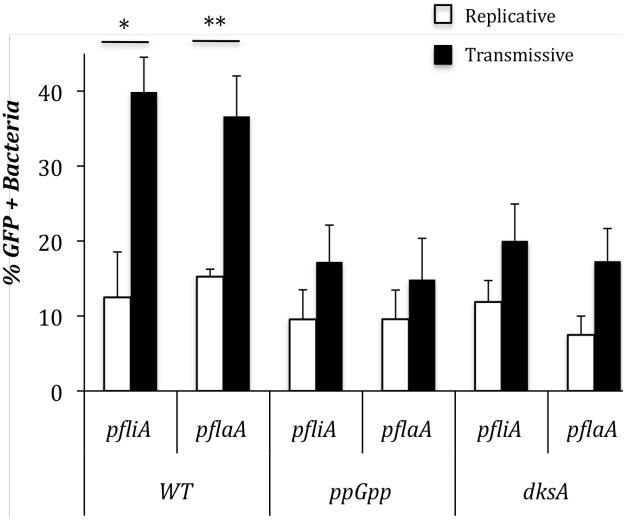

Figure 6. ppGpp and DksA control expression of flagellar genes when *L. pneumophila* differentiate during an infection of host macrophages.

Macrophages were infected with PE phase WT (moi~1), ppGpp^0 (moi~5), or *dksA* (moi~5) bacteria harboring either the $P_{fliAgfp}$ or $P_{flaAgfp}$ plasmid. At 12h, 18h, 19h or 20h, coverslips were fixed. 100 intact bacteria and intact bacterial vacuoles from duplicate coverslips were scored for positive GFP fluorescence. If the WT bacteria were greater than 20% positive for GFP, the time point was defined as the transmissive phase (solid black bars). Shown is the average total percentage of bacteria that were positive for GFP from three independent experiments +/- standard error of the mean. * indicates that WT $P_{fliAgfp}$ was statistically significantly increased from the replicative to transmissive phase (two tailed paired students t-test p=0.05). ** indicates that WT $P_{flaAgfp}$ was statistically significantly increased from the replicative to transmissive phase (two tailed paired students t-test p=0.03).

Discussion

When *L. pneumophila* senses nutrient depletion, it differentiates into the transmissive form through the canonical stringent response pathway. This global change in gene expression is coordinated by ppGpp (Dalebroux, et al, 2009), and my thesis work extends that dependence to the protein DksA. *L. pneumophila* lacking *dksA* are incapable of surviving environmental stress in the PE phase of growth (Fig. 1) and fail to display any of the hallmark virulence phenotypes of *L. pneumophila* (Fig. 2), showing that DksA is critical for differentiation to the transmissive form. Moreover, four genes induced during differentiation (*fliA, flaA, hfq,* and *rsmZ*) all require DksA to be fully expressed (Fig. 3). DksA and ppGpp are also critical for regulation of flagellar genes during the course of a macrophage infection (Fig. 6).

This work also discovered non-overlapping functions of DksA and ppGpp, highlighted by the phenotypes of WT and mutant *L. pneumophila*. ppGpp is essential for *L. pneumophila* to establish an infection of host macrophages, whereas DksA is dispensable (Fig. 4). Moreover, the over-expression of ppGpp can compensate for the loss of DksA in pigment production, whereas excess *dksA* can compensate for the loss of ppGpp in cytotoxicity (Fig. 7A). Both of these virulence phenotypes are dependent on intricate, multi-component bacterial systems: pigmentation is dependent on the Type II secretion system (Zusman et al, 2002; Chatfield & Cianciotto, 2007), while cytotoxicity is dependent on flagellin, the final product of the flagellar regulon (Molofsky, et al, 2006). These data predict that somewhere within these complex regulons, either DksA or ppGpp can bind RNAP independently of the other, activate the promoter of a gene (or genes), and ultimately drive the virulence phenotype for the bacterium.

The results of these experiments also show that *L. pneumophila's* requirements to express transmission traits depend on its environment. DksA is required for transmission from broth to macrophage (Fig. 2A), but it is not required for transmission from macrophage to macrophage (Fig. 4). Several other virulence regulators such as LetA and LetS (*L. pneumophila's* two-component response regulator) and FliA are essential to induce virulence in broth but not in macrophages (Hammer, et al, 2002). It is possible that there are environmental stresses that occur in broth that do not occur in the macrophage that signal via DksA for *L. pneumophila* to become transmissive. Results shown in Fig. 6, however, argue against this possibility: DksA activated the *fliA* and *flaA* promoters when the bacteria were cultured in broth or in macrophages, showing that it functions similarly when *L. pneumophila* is in either environment. As one approach to identify metabolic signals from the bacteria's surroundings that require DksA, a Biolog screen that compares the response of WT and *dksA* mutant bacteria could be conducted as previously described (Edwards, et al, 2009).

The fact that DksA is dispensable to *L. pneumophila* cultured in macrophages but not in broth could also mean that the canonical phenotypes that measure transmission in broth (Fig. 2) are not the traits required to sustain a successful infection of host macrophages. For example, cytotoxicity is measured in Fig. 2C as a trait of transmissive bacteria. The possibility

exists, however, that *L. pneumophila* factors are not required for lysis of vacuoles or macrophages to exit their primary host and subsequently infect a second, naïve macrophage. Instead, the bacteria could continue to hijack the host's vesicular trafficking system and be exocytosed after the primary host's nutrients have been depleted. Or, macrophages might have mechanisms to disrupt pathogen vacuoles.

Alternatively, *L. pneumophila* may respond to host cells primarily through ppGpp's regulation of certain genes. It is possible that ppGpp induces genes that promote survival within macrophages (Fig. 4) but does not induce those same genes when grown in broth. Indeed, when *L. pneumophila* differentiaties in amoeba, its natural phagocytic host , it only induces 77% of the genes expressed when it differentiates in broth (Bruggemann, et al, 2006). An extension of this experiment could be utilized to determine if any genes are activated by ppGpp but not DksA during *L. pneumophila* growth in phagocytes. For example, a genome-wide microarray experiment using the *dksA* mutant could compare the genes activated in the mutants when they are cultured in broth or amoeba. The subset of genes that are activated in the *dksA* mutant during growth in amoeba but not during growth in broth could encode the factor(s) that make DksA dispensable to *L. pneumophila* during intracellular infections. This subset of genes would also be valuable for mechanistic studies to understand the cooperative and distinct roles of ppGpp and DksA in controlling transcription.

The four transcription reporter plasmids created in this study also offer interesting kinetic information about how ppGpp and DksA induce the expression of PE phase genes. Specifically, there are three distinct patterns of regulation exhibited (Fig. 7B). The first, demonstrated by the P_{hfqgfp} and the $P_{flaAgfp}$ reporters, are examples where ppGpp and DksA are absolutely required for transcription. At other promoters, demonstrated by the $P_{fliAgfp}$ reporter, DksA contributes to basal levels of *fliA* transcription, whereas ppGpp is required in addition to DksA to potentiate full induction of *fliA*. Alternatively, the $P_{rsmZgfp}$ reporter indicates that ppGpp can activate some promoters in the absence of DksA, but DksA is required for WT levels of activation. Taken in total, the reporter plasmids show that genetic regulation exerted by DksA and ppGpp is not black and white; there is interplay between the two factors that can modulate transcription at

Figure 7.

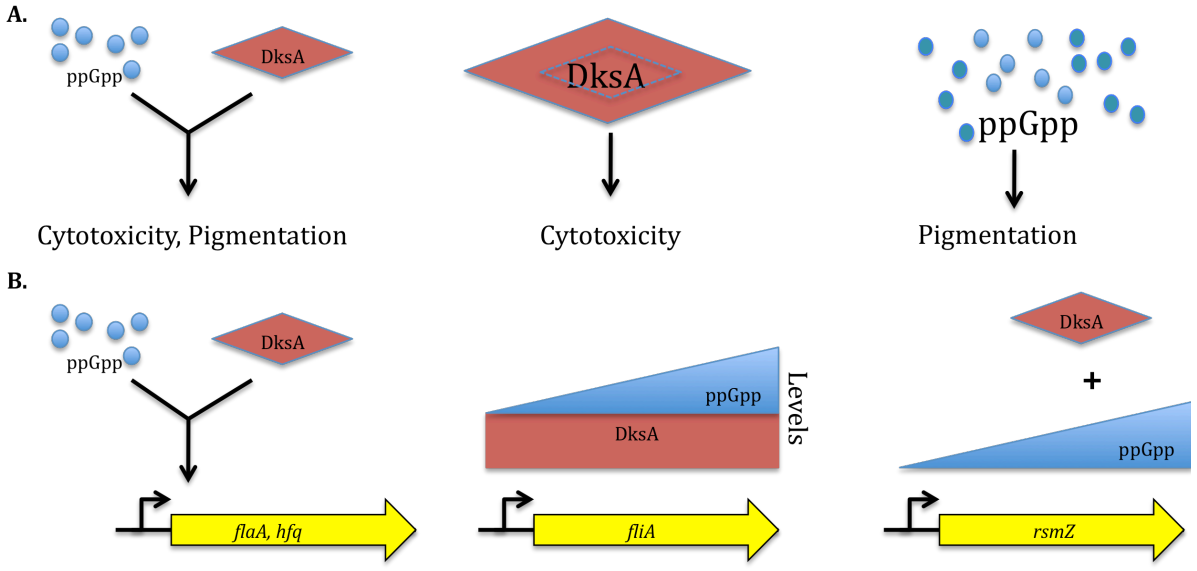

Figure 7. DksA and ppGpp show diverse patterns of regulation of promoters and phenotypes in *L. pneumophila*.
A. In WT *L. pneumophila*, both DksA and ppGpp are required for the bacteria to become cytotoxic and secrete pigment (left diagram). The absence of either ppGpp or DksA leads to the loss of cytotoxicity and pigmentation. However, over-accumulation of DksA in the absence of ppGpp can rescue cytotoxicity (middle diagram). Likewise, excess ppGpp can rescue pigmentation (right diagram).
B. At the *flaA* and *hfq* promoters, ppGpp (small, blue circles) and DksA (a maroon diamond) are both required to activate transcription (left diagram). At the *fliA* promoter, DksA activates basal transcription. When ppGpp begins to accumulate upon differentiation, *fliA* transcription increases to WT levels (center diagram). The accumulation of ppGpp is sufficient to drive the *rsmZ* promoter without DksA, but WT levels of transcription require ppGpp and DksA (right diagram).

different promoters.

Similar to the patterns observed in *E. coli*, this research has shown that the interplay between DksA and ppGpp is complex. *L. pneumophila* has developed a complicated interplay between DksA and ppGpp to more precisely control the expression of the variety of genes that are needed throughout its life cycle. For example, qRT-PCR studies have shown that the activation of *flgB*, a gene encoding the proximal rod of the flagellum, is sensitive to the amount and duration of ppGpp. When DksA is present, the accumulation of ppGpp initially creates a pulse of *flgB* transcription. Prolonged exposure to ppGpp, however, deactivates the *flgB* promoter (Dalebroux, et al, 2010). This makes sense biologically as there are only six units of FlgB that are needed to form the flagellum in *Salmonella enterica,* whose flagellum closely models *L. pneumophila* (Chevance & Hughes, 2008). Thus, the stoichiometry of FlgB is controlled by the accumulation of ppGpp such that the appropriate amount of *flgB* is expressed.

Analysis of the flagellar promoters studied in this work (*fliA* and *flaA*) further shows that *L. pneumophila* employs the dynamic interplay between DksA and ppGpp to regulate these genes favorably for its life cycle. *fliA* is dependent on the housekeeping sigma-factor, σ^{70}. Thus, DksA can interact with the RNAP- σ^{70} holoenzyme to mediate basal levels of transcription during the E phase of growth (Fig. 3A). When *fliA* must be highly activated during the PE phase, the DksA-RNAP-σ^{70} complex is already assembled to incorporate the growing pools of ppGpp. This will allow rapid activation of *fliA*. Since the transcription of *flaA* (flagellin) requires FliA (σ^{28}) as its sigma-factor, the requirement for DksA and ppGpp is acute at the *flaA* promoter: neither the *ppGpp⁰* mutant nor the *dksA* mutant showed significant amounts of transcription (Fig. 3B). *In vitro* transcription assays on the promoter for the flagellin gene in *E. coli* has shown that DksA and ppGpp do not control the promoter directly (Lemke, et al, 2009). Thus, DksA and ppGpp likely bind to RNAP and dissociate it from σ^{70}. Subsequently, σ^{28} directs free RNAP to the *flaA* promoter and activates transcription. It is also possible that DksA alone can free RNAP upon binding the secondary chanel of the polymerase. This would account for the fact that DksA alone can result in flagellin productioin (Fig. 4A). *L. pneumophila* could utilize DksA and ppGpp first to activate transcription of σ^{28}. Subsequently, the two factors dissociate RNAP from σ^{70}, allowing σ^{28} to direct RNAP to the *flaA* promoter.

Based on this analysis of the flagellar regulon, *L. pneumophila* use DksA and/or ppGpp to detect when and how much a certain gene product is required. By utilizing both DksA and ppGpp, *L. pneumophila* has developed a complex and dynamic network to regulate its pathogenesis. At certain promoters in certain conditions, the factors cooperate as contributors to *L. pneumophila* virulence. In other circumstances, the two factors can act independently, contributing to virulence phenotypes or survival in host macrophages. *L. pneumophila* has thus acquired a dynamic system to respond to the world around it by altering its gene expression and surviving accordingly.

Materials and Methods

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. 1). 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 agar plates 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* (Datsenko & Wanner, 2000; Yu et al, 2000). pGEM-*dksA* was generated by amplifying the *dksA* region (*lpg2338*)

using primers dksA1 and 2. 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.

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. 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 by growing with IPTG to the concentrations specified.

Inducible relA expression. To generate strains in which expression of *relA* could be induced, a copy of *E. coli relA* that had been truncated at its C-terminus and was therefore constitutively active (Schriebert, et al, 1990) was cloned into pMMB66EH, a broad host range vector containing a $P_{taclacUV5}$ IPTG-inducible promoter with a gent cassette. The $relA^{E.c.}$ gene was ligated into the MCS of the plasmid immediately 3' of the $P_{taclacUV5}$ promoter as previously described (Hammer & Swanson, 1999). To induce *relA* in the absence of *dksA*, the plasmid was electroporated into MB699 to create MB702. Selection was maintained with gentamycin and *relA* was induced by adding IPTG to the concentrations specified.

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 between BamHI and XbaI restriction sites in the multiple cloning site of pKB5 directly 5'of *gfp* as described (Hammer & Swanson, 1999). This plasmid was used to transform MB110, MB697 and MB699, generating MB733, MB734 and MB735, respectively. Selection was maintained by culturing bacteria in the absence of thymidine, as the plasmid encodes *tdΔI* which restores *L. pneumopila* thymidine prototrophy.

rsmZ-gfp promoter fusion. A fragment containing 190 bp 5' of the putative σ^{38} promoter, LetA binding site and transcriptional start for *rsmZ* was amplified using primers rsmZ P1 and P2. The fragment was ligated between BamHI and Xba I restriction sites in the multiple cloning site of pKB5 directly 5'of *gfp* as described (Hammer & Swanson, 1999). This plasmid was used to transform MB110, MB697 and MB699, generating BY60, BY63 and BY65, respectively. Selection was maintained by culturing bacteria in the absence of thymidine, as the plasmid encodes *tdΔI* which restores *L. pneumopila* thymidine prototrophy.

hfq-gfp promoter fusion. A fragment containing 363 bp 5' of the *hfq* RBS and encoding the putative σ^{70} promoter and transcriptional start was amplified using primers hfq P1 and P2. The fragment was ligated between BamHI and XbaI restriction sites in the multiple cloning site of pKB5 directly 5'of *gfp* as described (Hammer & Swanson, 1999). This plasmid was used to transform MB110, MB697 and MB699, generating BY68, BY71 and BY74, respectively. Selection was maintained by culturing bacteria in the absence of thymidine, as the plasmid encodes *tdΔI* which restores *L. pneumopila* thymidine prototrophy.

Fluorometry. To monitor expression of the promoter fusion plasmids, E phase cultures of the strains harbouring the plasmid were diluted to OD_{600} =1.0 (t = 0) and cultured to stationary phase. Every three hours, growth and fluorescence were monitored. Growth was monitored by OD_{600} readings, and fluorescence was detected as described (Edwards, et al, 2009)

except that cultures were normalized to OD_{600} = 3.0.

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).

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, 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).

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, intracellular growth curves, and fluorescence microscopy studies.

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. 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).

Cytotoxicity. To measure contact-dependent cytotoxicity, *L. pneumophila* were added to macrophages at the indicated MOI, and cytotoxicity was measured using spectrophotometry as described (Molofsky et al, 2005). To determine the contribution of *dksA* to PE phase activation of *L. pneumophila* cytotoxicity (Fig. 2C), 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 constituitive ppGpp synthesis could restore ppGpp^0 and *dksA* mutant cytotoxicity (Fig. 5A), WT, ppGpp^0, and *dksA* bacteria carrying p*empty*, p*dksA*, or p*relA*$^{E.c.}$ were cultured from E to PE phase in the presence of 200 μM IPTG prior to infection.

Degradation and GFP fluorescence in macrophages. The percentage of intracellular *L. pneumophila* that remain intact after a 2 h infection was quantified by fluorescence microscopy (Bachman & Swanson, 2001). To test the GFP fluorescence of bacteria harbouring a promoter-gfp fusion plasmid, coverslips were fixed at 12, 18, 19 or 20h. These coverslips were treated with anti-Lp02 primary antibody and a rabbit IgG derived secondary antibody tagged with Texas Red. 100 intact bacteria or bacterial vacuoles were scored on duplicate coverslips. To account for unsynchronized intracellular life cycles, the time point was considered when >20% of the WT p*flaAgfp* cells were positive for GFP (See Fig. 6).

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