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

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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.

Keywords

flagella; DksA; FliA; ppGpp; *Legionella*

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).

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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; 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). 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 repressing 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; Sharma & Payne, 2006; Aberg et al, 2008). 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 (Magnusson et al, 2007, Lyzen

et al, 2009). Therefore, we exploited *L. pneumophila* differentiation and its flagellar cascade to investigate the functional relationship between ppGpp and DksA.

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. 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. S1). 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 1 and 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 S1). 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 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 (Nagai et al, 2002; Tiaden et al, 2007; Rasis & Segal, 2009), 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* (Bachman & Swanson, 2001; Hovel-Miner et al, 2009), the transmission trait enhancer *letE* (Bachman & Swanson, 2004), and the regulatory RNA *rsmZ* (Rasis & Segal, 2009; Sahr et

al, 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. 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. 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 × 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. 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. 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 (Vogel et al, 1996; Byrne & Swanson, 1998). Since ppGpp activated several loci implicated in particular transmisson 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. 3A), frequently degraded within 2 h of infection (Fig. 3B), non-cytotoxic to macrophages (Fig. 3C), and sodium resistant (Fig. 3D). Induction of plasmid-borne *dksA* restored macrophage infectivity and cytotoxicity of the mutants (Fig. 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. 4). The yield of *dksA* and ppGpp^0 mutants was less than that of WT at each time analyzed (Fig. 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. 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; Sturgill-Koszycki & Swanson, 2000; Alli et al, 2000; Molofsky & Swanson, 2003; 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 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. 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), 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. 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. 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. 4) and transmission in macrophages (Fig. 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 (Schreiber et al, 1991;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. 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. 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 (Zusman et al, 2002; Chatfield & Cianciotto, 2007). 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. 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 (*gfp*; 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. 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. 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. 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. 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. 7A, bottom). In addition, ~ 20% of alarmone deficient bacteria avoided degradation, another intermediate response (Fig. 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. 7A, bottom) or remain intact in macrophages (Fig. 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 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. 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. 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. 8). In contrast, $relA^{E.c.}$ induction did not restore flagellin synthesis, cytotoxicity or motility to *dksA* mutants (Fig. 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 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;Albert-Weissenberger et al, 2010). 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 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 2;Albert-Weissenberger et al, 2010). 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 *flgB* (Albert-Weissenberger et al, 2010). 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. S3), 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. 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. 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. 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; Dalebroux 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} (Class IV; Bruggemann et al, 2006; Heuner, 2007; Albert-Weissenberger et al, 2010). The alarmone rapidly increases the level of *fleQ*, *fleR* and several Class II flagellar transcripts, including *flgB* (Table 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 (Fig. 9B; 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;Albert-Weissenberger et al, 2010). 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. 7). When plasmid-borne *dksA* expression is induced, $ppGpp^0$ bacteria initiate flagellar biosynthesis, motility and cytotoxicity to macrophages (Fig. 8). Furthermore, DksA mediates basal *fliA* promoter activity during E phase (Fig. 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 (Lyzen et al, 2009;Merrikh et al, 2009). 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 repress 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. 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. 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 and 7A). Moreover, *dksA* induction fails to rescue pigmentation (Fig. 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; Hammer et al, 2002). In contrast, ppGpp-dependent factors are critical for *L. pneumophila* transmission from lysosomal vacuoles of permissive mouse macrophages (Sturgill-Koszycki & Swanson, 2000;Dalebroux et al, 2009). Perhaps in host cells, ppGpp induces *L. pneumophila* pathways that promote infection of *A. castellanii* (Fig. 4) and spread between macrophages (Fig. 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 S1). 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; Tiaden et al, 2007; Rasis & Segal, 2009), 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 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 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 S1). 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 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 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.

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. 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* (Datsenko & Wanner, 2000; Yu et al, 2000). pGEM-*dksA* was generated by amplifying the *dksA* region (*lpg2338*) using primers dksA1 and 2 (Table S2). 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).

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). 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), 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. 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. 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 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 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. pending.

Quantitative Real-time PCR

To validate the microarray data sets (Tables 1, 2 and S1), bacteria were cultured as described for *Microarray analysis*. To assess whether *dksA* contributes to ppGpp-dependent transcript accumulation (Fig. 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*$^{E.c.}$ (Table 1 and Table S1) and *dksA*/WT PE phase (Table 2) microarray data sets, *letE, rpoS, flgB, fliA, flaA, fliD, ndk, lag-1* and *lpg0260* (Table S2) were targeted (Figs. S1 and S2). To test the contribution of *dksA* to ppGpp dependent expression of flagellar genes (Fig. 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 5×10^5 to 1.0×10^6 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 flasks 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. 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. 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. 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 constituitive ppGpp synthesis could restore $ppGpp^0$ and *dksA* mutant cytotoxicity (Fig. 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. 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).

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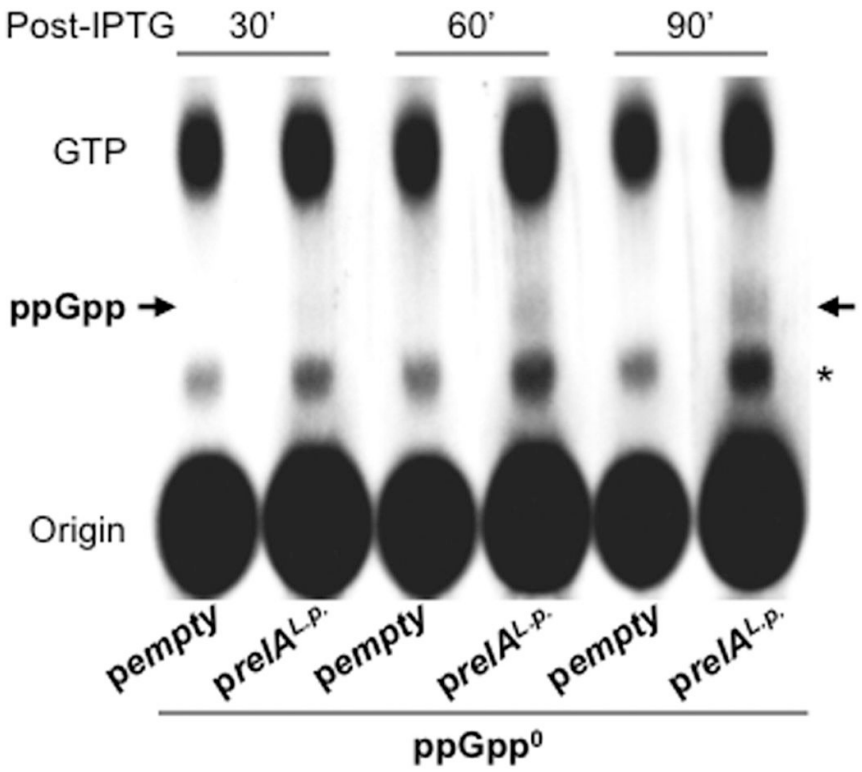

Figure 1. Kinetics of ppGpp accumulation after p*relA*$^{L.p}$ induction

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.

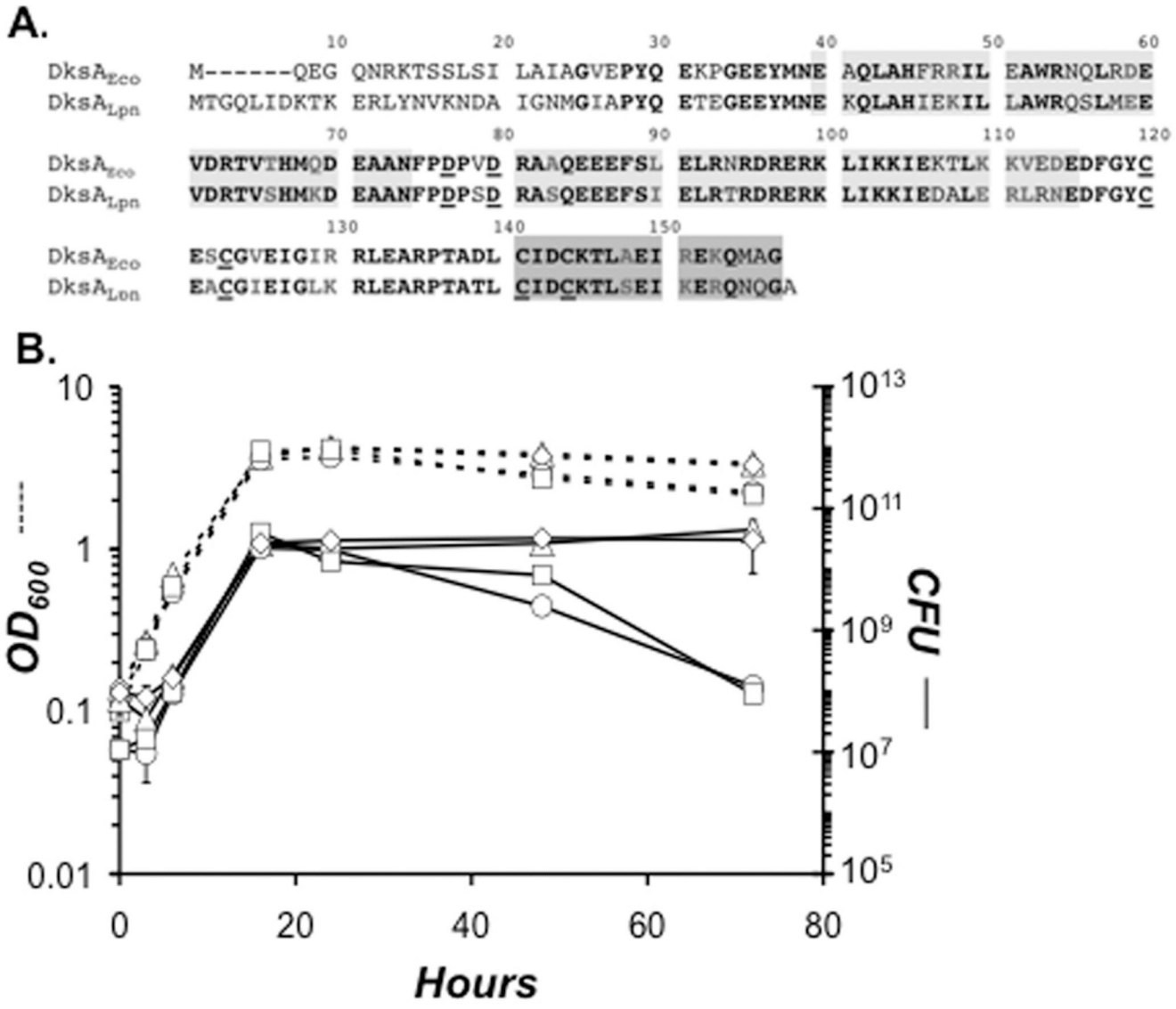

Figure 2. To survive stationary phase stress *L. pneumophila* requires DksA and ppGpp

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.

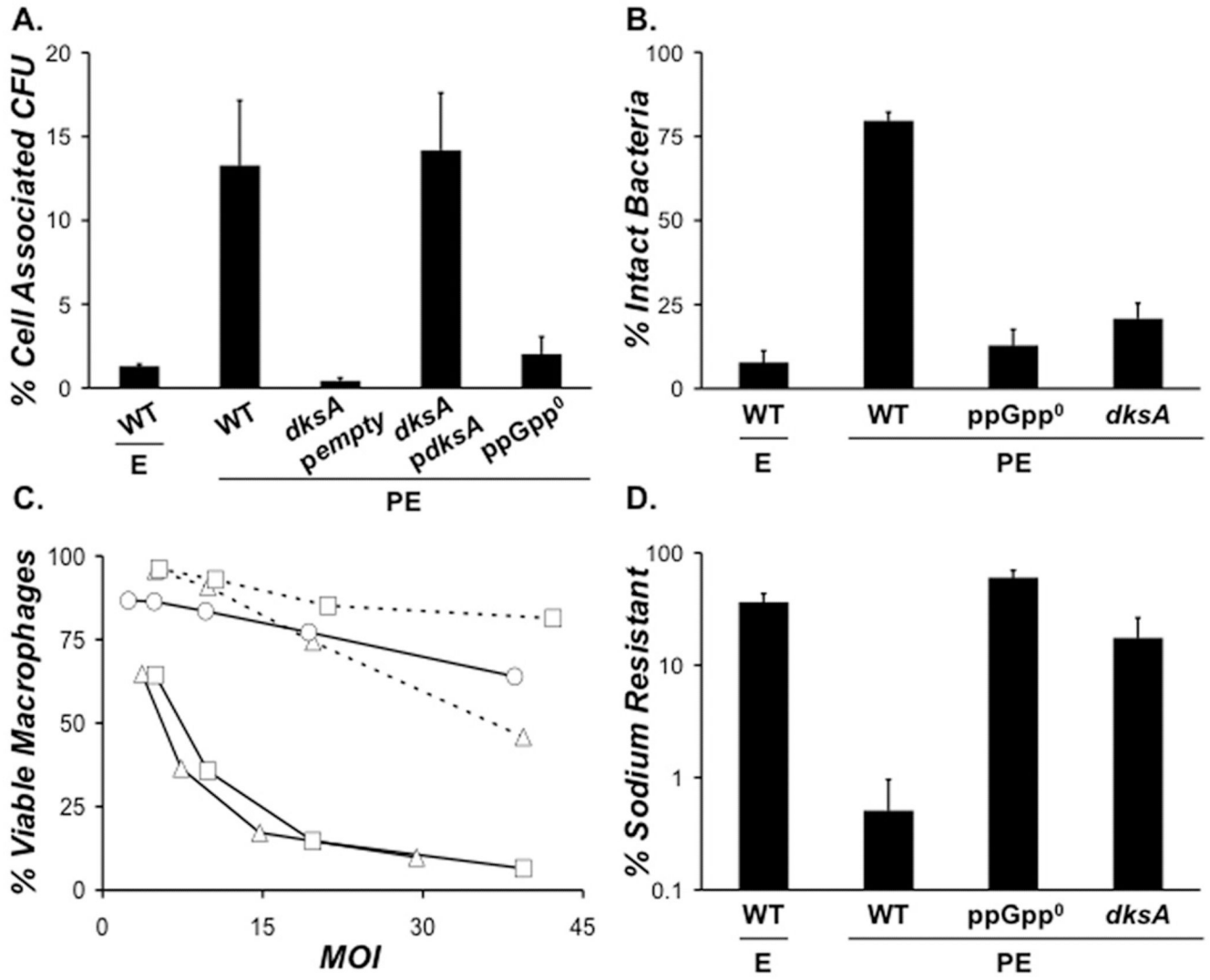

Figure 3. To activate PE phase transmission traits *L. pneumophila* requires DksA and ppGpp

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.

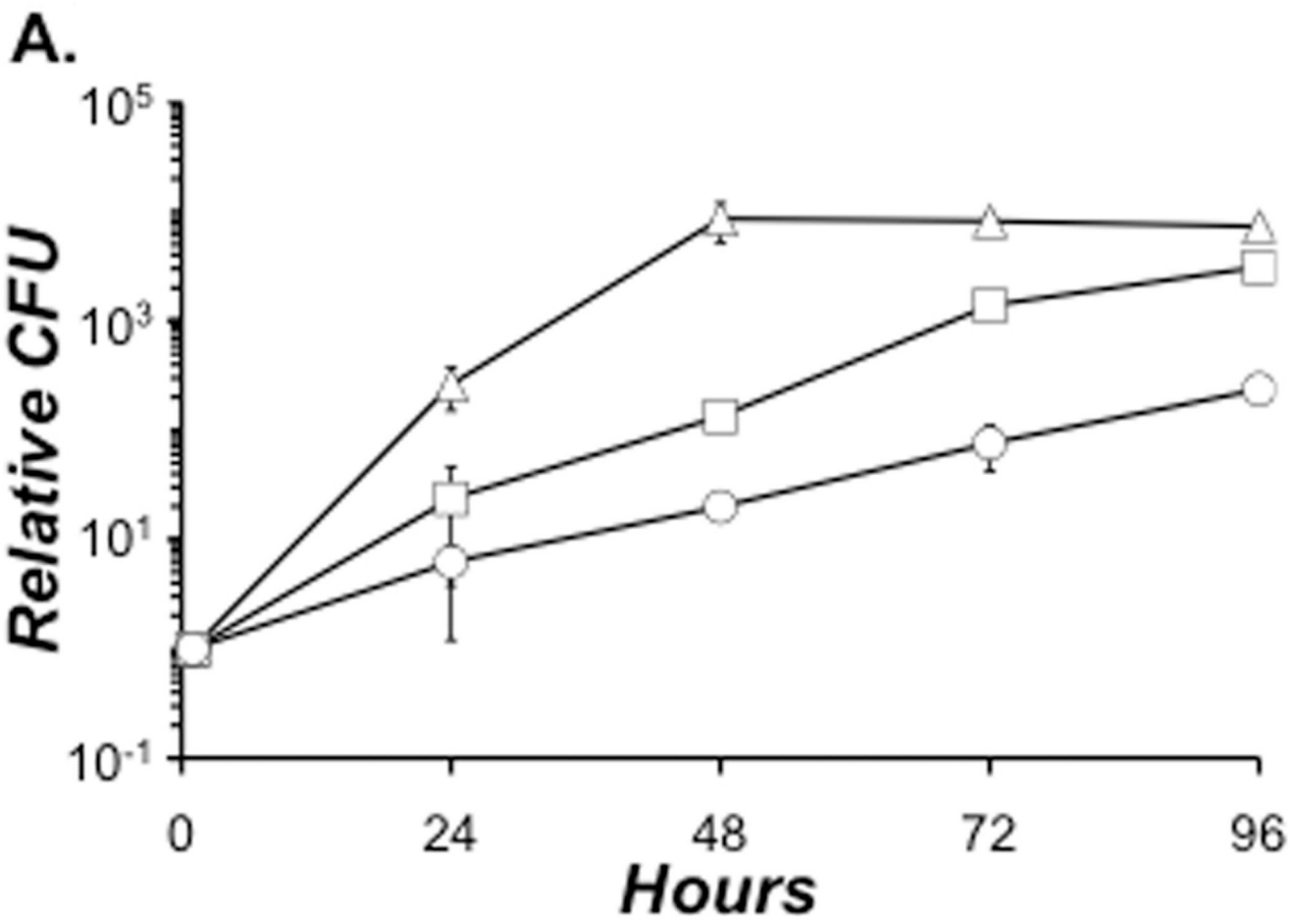

Figure 4. For optimal growth in *A. castellanii L. pneumophila* requires both DksA and ppGpp

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.

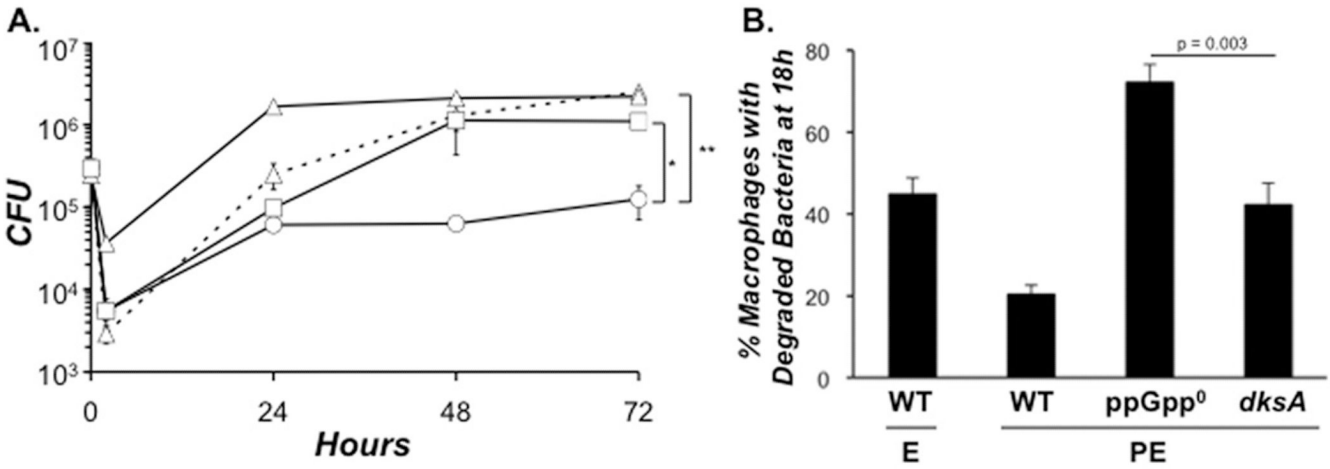

Figure 5. ***L. pneumophila*** **requires ppGpp for macrophage transmission; DksA is less critical**

A. Macrophages were infected at an MOI of ~ 1 with E phase WT (triangles, dashed lines), PE phase WT (triangles, solid line), 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. * 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.

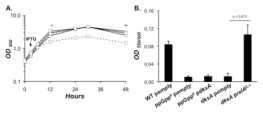

Figure 6. Constitutive ppGpp synthesis is sufficient to bypass the *dksA* requirement for cell cycle arrest and pigment production

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.

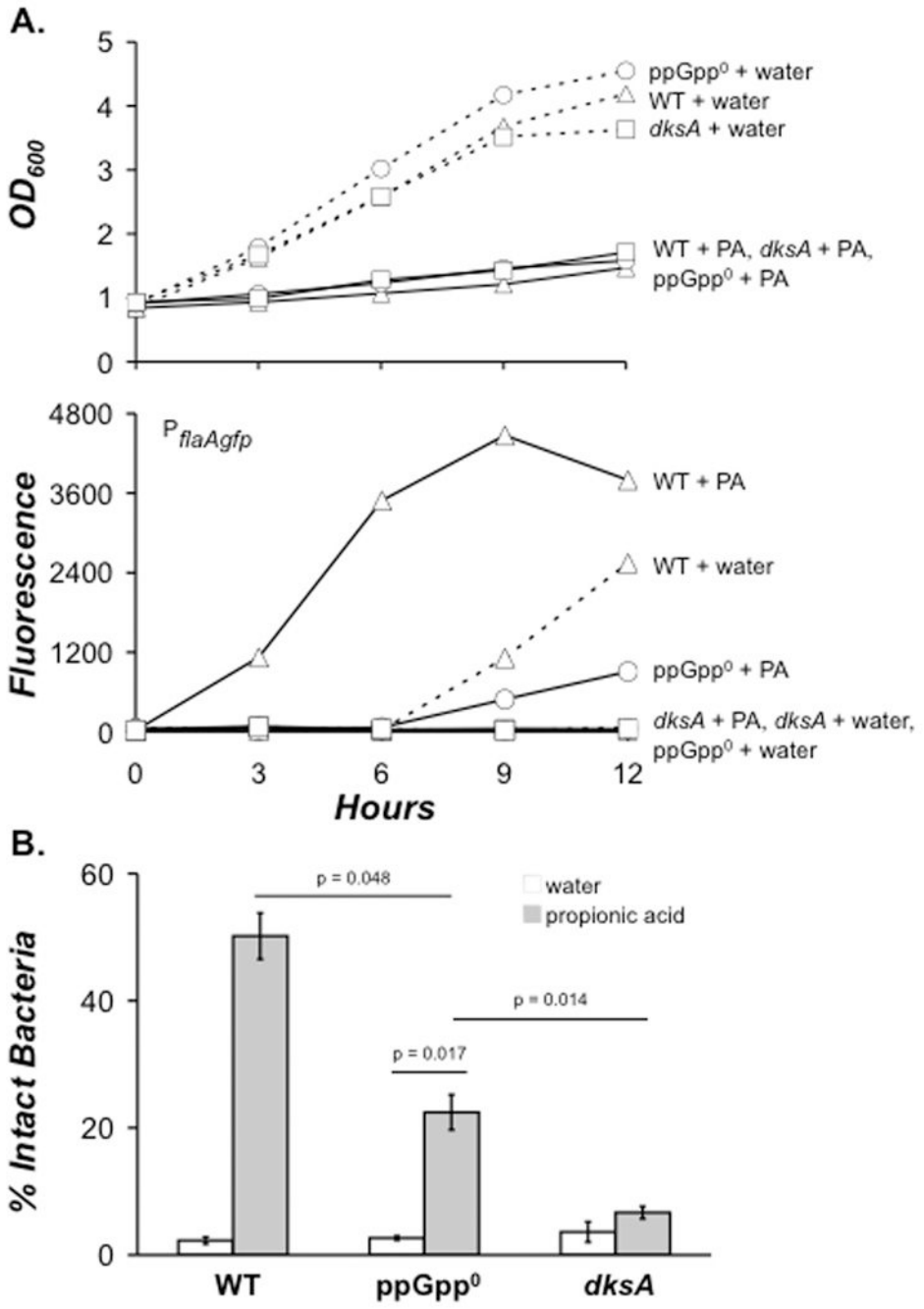

Figure 7. In response to fatty acid stress, DksA activates transmission traits with and independently of ppGpp

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 ($p = 0.005$).

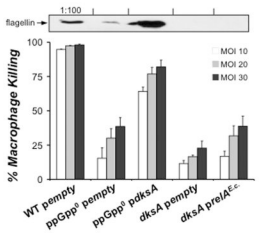

Figure 8. Induction of *dksA* is sufficient to bypass the requirement for ppGpp for flagellin-dependent phenotypes

In the strains subjected to the induction described in Figure 6A, we assessed levels of flagellin and macrophage cytotoxicity. At 9 h post-IPTG (indicated in Fig. 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.

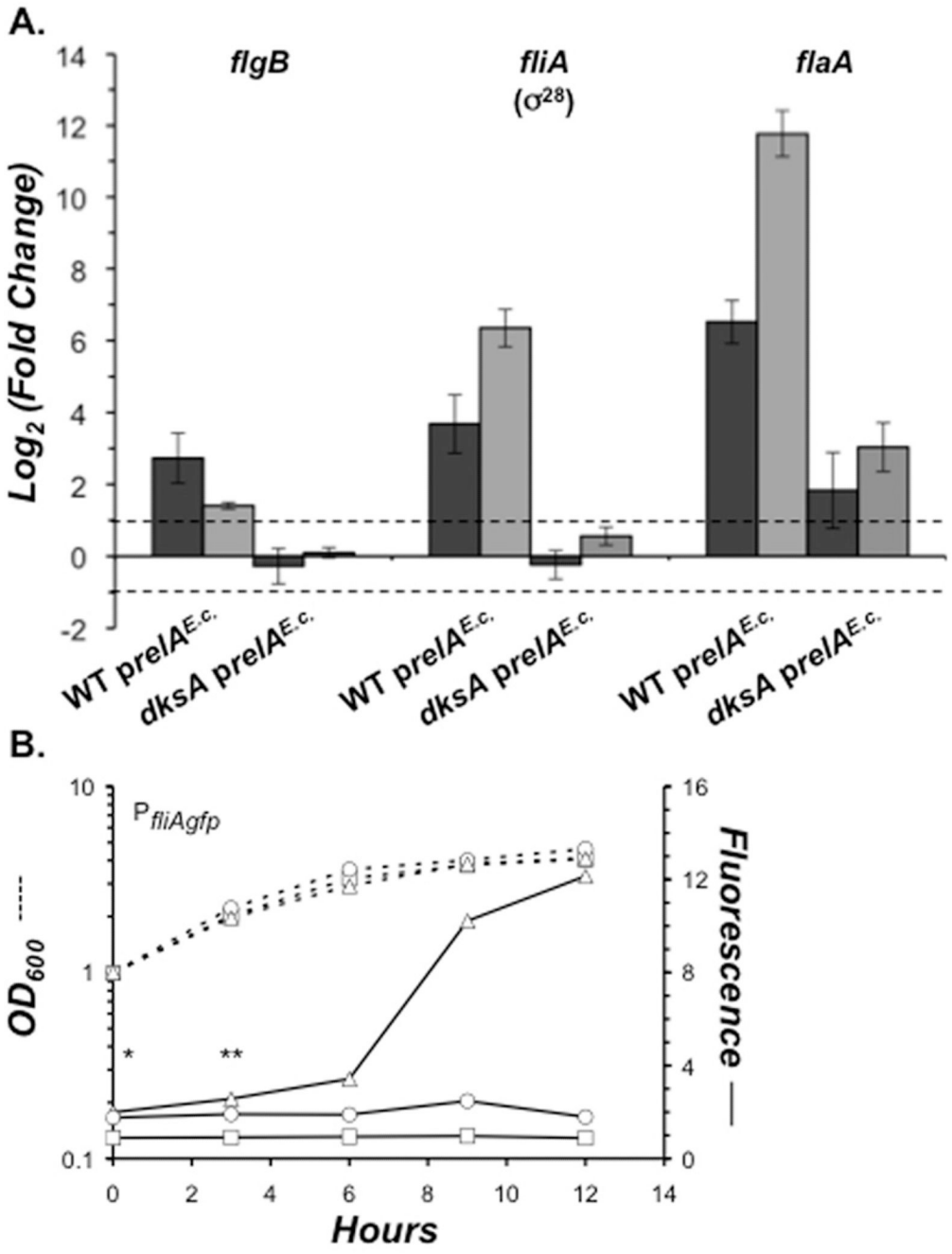

Figure 9. *L. pneumophila* uses ppGpp to modulate DksA-dependent gene expression

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 $\log_2$ (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 1000× 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**).

Table 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

Gene Name / Function	Gene.ID	Annotation	ppGpp^0 p*relA*$^{L.p.}$/p*empty*
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. S1* for qRT-PCR validation. $p < 0.01$ for all targets shown.

Table 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

Gene Name	Gene.ID	Annotation	*dksA*/WT
*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. S2* for RT-PCR validation.

$p < 0.01$ for all targets shown.

* indicates a gene regulated by *fliA* (σ^{28}) (Albert-Weissenberger et al, 2010)

Table 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*)$_{U169}$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	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 overhanges, 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, colinear 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	This work

Strain	Relevant genotype/phenotype	Reference
	from pGEM-Gent, $Gent^R$.	
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, colinear 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, 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	This work