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SpoT governs *Legionella pneumophila* differentiation in host macrophages

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Summary

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

Introduction

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

Legionella pneumophila is ubiquitous in aquatic environments where it resides either in biofilms or within freshwater protozoa. When humans inhale aerosols of contaminated water, this opportunistic pathogen can infect alveolar macrophages. Upon phagocytosis, transmissive *L. pneumophila* resist phagosome acidification and maturation and instead become sequestered within a protective, ER-derived vacuole (Shin and Roy, 2008). Favourable metabolic conditions within the vacuole prompt repression of transmissive genes and induction of genes required for protein synthesis and replication (Molofsky and Swanson, 2003; Sauer *et al*., 2005; Bruggemann *et al*., 2006). Following multiple rounds of cell division that presumably exhaust host cell nutrients, the bacteria differentiate back to the transmissive form, which can subsequently develop into a 'mature intracellular form' suited for prolonged viability in the environment (Garduño *et al*., 2008). In broth cultures, lysosome evasion, type IV secretion, flagella expression, sodium sensitivity and cytotoxicity are co-ordinately induced in stationary phase (Byrne and Swanson, 1998; Alli *et al*., 2000; Molofsky and Swanson, 2004); since each trait is predicted to enhance spread from one cell to another, we refer to this as the transmissive phase. In both broth and amoebae, *L. pneumophila* exhibits a biphasic gene expression pattern that corresponds to the phenotypic switch observed (Bruggemann *et al*., 2006). Thus, the transition between the exponential (E) phase and the post-exponential (PE) phase in broth cultures reproduces in large part the differentiation from the replicative to the transmissive phase observed in host cells.

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In broth cultures, the *L. pneumophila* E to PE phase transition is concomitant with accumulation of guanosine tetraphosphate or ppGpp (Hammer and Swanson, 1999; Zusman *et al*., 2002), an alarmone that acts as a general signal of bacterial starvation and stress (reviewed by Magnusson *et al*., 2005; Braeken *et al*., 2006; Potrykus and Cashel, 2008; Srivatsan and Wang, 2008). The ppGpp alarmone is thought to alter the interaction of RNA polymerase with specific promoters to either activate or inhibit transcription. A regulatory factor termed DksA for *dnaK* suppressor protein, potentiates ppGpp regulation (Potrykus and Cashel, 2008). The global transcriptional changes that ppGpp and DksA instruct are collectively referred to as the stringent response (Potrykus and Cashel, 2008). In *Escherichia coli*, ribosomal and amino acid biosynthetic operons, flagellar- and chemotaxis-related genes are all under stringent control, as are key enzymes involved in fatty acid biosynthesis (FAB) (Durfee *et al*., 2008; Traxler *et al*., 2008).

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

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

SpoT can also generate ppGpp in response to a variety of stresses, including carbon source deprivation, phosphate starvation, iron starvation and FAB inhibition (Potrykus and Cashel, 2008). For *E. coli* to respond to perturbations in FAB, SpoT interacts with acyl-carrier protein (ACP), a protein critical for FAB (Battesti and Bouveret, 2006). Point mutations within the C-terminal TGS domain of SpoT not only disrupt its interaction with ACP, but also alter its activity such that ppGpp synthesis is favoured over hydrolysis (Battesti and Bouveret, 2006). Phosphate starvation results in SpoT-dependent ppGpp accumulation that requires SpoT hydrolase activity, not synthetase activity (Spira and Yagil, 1998; Bougdour and Gottesman, 2007). It remains to be determined if other starvation conditions that elicit ppGpp accumulation alter the SpoT–ACP interaction or result from either an inhibition of ppGpp hydrolysis or an increase in synthesis.

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

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

Results

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

To rigorously test the hypothesis that the stringent response controls *L. pneumophila* virulence expression, we constructed and analysed *relA* and *relA spoT* mutant strains (Table 1). In the stationary phase, *L. pneumophila* requires RelA to produce pyomelanin, a secreted pigmented molecule important for iron metabolism (Zusman *et al*., 2002; Chatfield and Cianciotto, 2007). Therefore, to verify that *relA* was disrupted, we assayed the extracellular pigment of stationary-phase cultures. As expected, our *relA* insertion mutant was completely defective for pyomelanin production (data not shown).

As observed for other Gram-negative bacteria, we and others were unable to generate *spoT* null mutant strains of *L. pneumophila*, and attempts to construct a conditional *spoT* mutant were also unsuccessful (Zusman *et al*., 2002). Therefore, to analyse the contribution of SpoT to the *L. pneumophila* life cycle, we constructed a *relA spoT* double mutant in which 136 amino acids near the N-terminus, including the consensus His–Asp (HD) motif required for ppGpp hydrolysis (Potrykus and Cashel, 2008) and a significant portion of the putative

Table 1. Bacterial strains, plasmids and primers.

Strain/plasmid	Relevant genotype/phenotype	Reference
Strain		
E. coli		
DH5α	F-endA1 *hsdR17* (r– m+) *supE44 thi-1 recA1 gyrA* (Nal^r) *relA1* Δ(*lacZYA-argF*)$_{U169}$Φ80dLacZΔM15λpirRK6	Laboratory collection
L. pneumophila		
MB110	Lp02 wild type, Str^R, Thy–, HsdR–	
MB695	Lp02 *relA::gent* mutant	This work
MB696	Lp02 *relA::kan* mutant	This work
MB697	Lp02 *relA::gent spoT::kan* double mutant	This work
MB698	Lp02 p206-empty, vector control strain	This work
MB687	Lp02 *relA::gent spoT::kan* double mutant p*relA*i	This work
MB688	Lp02 *relA::gent spoT::kan* double mutant p*spoT*i	This work
MB689	Lp02 *relA::gent spoT::kan* double mutant p*spoT-A413E*	This work
MB355	Lp02 p*flaA*gfp td(Δ)I	Hammer and Swanson (1999)
MB684	Lp02 *relA::kan* mutant p*flaA*gfp td(Δ)I	This work
MB685	Lp02 *relA::gent spoT::kan* double mutant p*flaA*gfp td(Δ)I	This work
Plasmids		
pGEMT-Easy	MCS within coding region of β-lactamase α-fragment linearized with single-T overhanges, Amp^R	Promega
pUC4K	Source of 1.3 kb kanamycin resistance cassette GenBlock	Pharmacia
pGEM-Gent	pGEMT-Easy with 1.7 kb gentamicin cassette clone MCS source of 1.7 kb gentamicin resistance cassette, Amp^R, Gent^R	Molofsky *et al.* (2005)
pGEM-*relA*	pGEMT-Easy with 3.2 kb PCR-amplified *relA* chromosomal region ligated into T overhangs, Amp^R	This work
pGEM-*relA*i	pGEMT-Easy with 2.7 kb PCR-amplified *relA* chromosomal region ligated into T overhangs, Amp^R	This work
pGEM-*relA::gent*	pGEM-*relA* with 1.7 kb gentamicin cassette from pUCGent bluntly ligated into the SnaBI site ~1.0 kb from transcriptional start site of *relA* ORF, Amp^R, Gent^R	This work
pGEM-*relA::kan*	pGEM-*relA* with 1.3 kb kanamycin cassette from pUC4K bluntly ligated into the SnaBI site ~1.0 kb from transcriptional start site of *relA* ORF, Amp^R, Kan^R	This work
pGEM-*spoT*	pGEMT-Easy with 2.9 kb PCR-amplified *spoT* chromosomal region ligated into T overhangs, Amp^R	This work
pGEM-*spoT*i	pGEMT-Easy with 2.5 kb PCR-amplified *spoT* chromosomal region ligated into T overhangs, Amp^R	This work
pGEM-*spoT::kan*	pGEM-*spoT* with 489 bp HindIII/HindIII SpoT ORF fragment deleted and a 1.3 kb kanamycin cassette from pUC4K bluntly ligated between the HindIII sites, Amp^R, Kan^R	This work
pGEMT-*spoT-A413E*	pGEM-*spoT*i with a GCC to GAA change, Amp^R	This work
pMMB206-Δmob	pMMB66EH derivative, Δmob, lacI^q, P$_{taclacUV5}$, Cam^R	Morales *et al.* (1991)
p*relA*i	pMMB206-Δmob with 2.7 kb SalI fragment from pGEM-*relA*i ligated at the SalI site in the MCS, colinear with the P$_{taclacUV5}$ promoter, lacI^q, inducible RelA expression, Cam^R	This work
p*spoT*i	pMMB206-Δmob with 2.5 kb SalI/HindIII fragment from pGEM-*spoT*i ligated between the SalI/HindIII sites in the MCS, colinear with the P$_{taclacUV5}$ promoter, lacI^q, inducible SpoT expression, Cam^R	This work
p*spoT-A413E*	pMMB206-Δmob with 2.5 kb SalI/HindIII fragment from pGEM-*spoT-A413E* ligated between the SalI/HindIII sites in the MCS, colinear with the P$_{taclacUV5}$ promoter, lacI^q, inducible *spoT-A413E* expression, Cam^R	This work
p*flaA*gfp	150 bp *flaA* promoter fragment fused to GFPmut3 in pKB5 with P$_{tac}$ and lacI^q removed, td(Δ)I	Hammer and Swanson (1999)

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

ppGpp synthetase domain (Gentry and Cashel, 1996), were replaced by a kanamycin resistance cassette. As the cultures entered late PE phase, the *relA spoT* double mutant cells adopted a hyperfilamentous cellular morphology (data not shown), a phenotype reminiscent of that described for *relA spoT* mutants of *E. coli* (Xiao *et al.*, 1991; Traxler *et al.*, 2008). Other than hyperfilamentation in late PE phase, in rich medium the growth

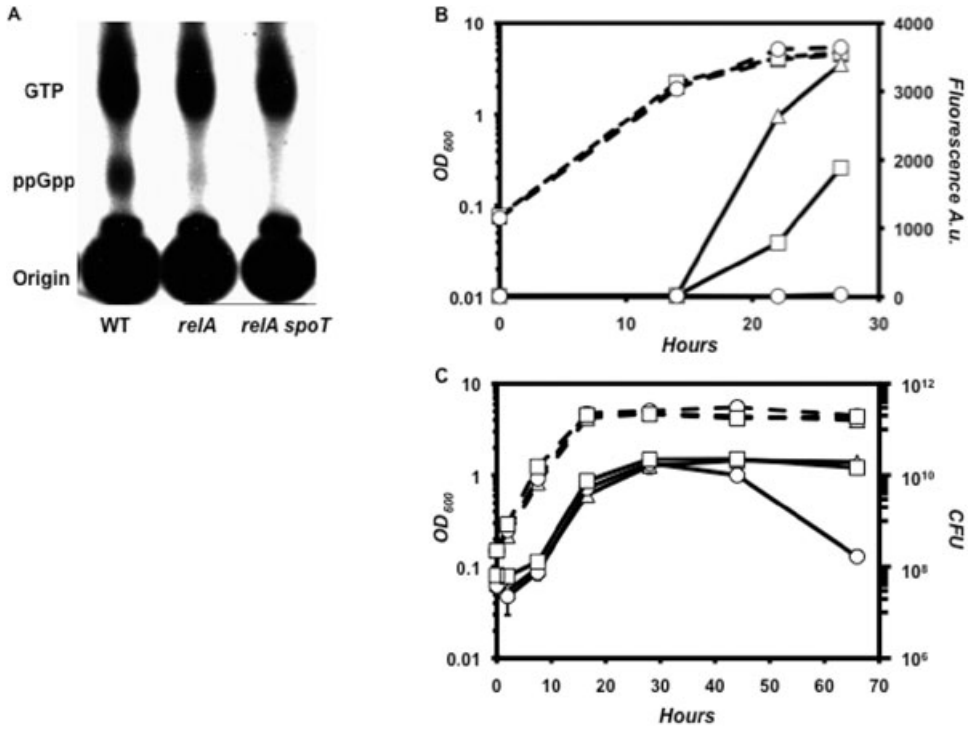

Fig. 1. RelA and SpoT contribute to ppGpp accumulation, motility and survival in PE phase.
A. To evaluate ppGpp accumulation in PE-phase, mid-E-phase AYET broth cultures of WT, *relA* and *relA spoT* bacteria were incubated for 6 h with ^{32}P phosphoric acid (approximately two generation times), a period sufficient for the cells to enter the PE phase. At this time, cell extracts were prepared, and nucleotides were separated by PEI-TLC. The autoradiogram shown represents one of two separate experiments.
B. To monitor *flaA* expression (solid lines), WT (triangles), *relA* (squares) and *relA spoT* (circles) strains transformed with p*flaAgfp* were cultured in broth, then at the times indicated bacterial density was quantified by measuring OD_{600} (dashed lines), and green fluorescence protein (GFP) accumulation was quantified by fluorometry (symbols, solid lines). Shown is a growth curve beginning in early E phase that is representative of multiple cultures in more than five independent experiments.
C. To evaluate survival of PE-phase stress, early E-phase cultures of WT (triangles), *relA* (squares) and *relA spoT* (circles) bacteria were diluted to an OD_{600} of 0.1, then at the times indicated bacterial density was quantified by measuring OD_{600} (dashed lines), and viability was assessed by enumerating colony-forming units (cfu) ml^{-1} on CYET (solid lines). Shown are mean cfu ± SE from duplicate samples, and the data represent one of three independent experiments. The difference in the mean cfu values calculated in three independent experiments for WT and *relA spoT* mutant bacteria at 66 h was statistically significant by a two-tailed Student's *t*-test ($P < 0.01$).

kinetics of E-phase *relA spoT* mutant cells were identical to WT bacteria, as judged by optical density and colony-forming units (cfu) (Fig. 1C). Therefore, when *relA* is absent, *spoT* is not essential for *L. pneumophila* to replicate in rich bacteriological medium. Why previous attempts to isolate a *relA spoT* double mutant were unsuccessful is difficult to assess since the allele construction was not described (Zusman *et al.*, 2002). However, *spoT* mutations that eliminate hydrolase but not synthetase activity would be lethal.

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

To assess in greater detail the contributions of RelA and SpoT to *L. pneumophila* biology, we tested the ability of *relA* and *relA spoT* mutants to accumulate ppGpp in stationary phase when cultured in rich broth containing yeast extract. After radiolabelling the *L. pneumophila* guanosine nucleotide pools for 6 h (two doubling times), ppGpp levels were analysed by thin-layer chromatography (TLC). WT *L. pneumophila* cultured into PE phase accumulated a pool of ppGpp, much of which was dependent on *relA* (Fig. 1A). The *relA* mutants also accumulated a small but appreciable pool of ppGpp that was not observed in the *relA spoT* double mutant (Fig. 1A). Thus, when *L. pneumophila* are cultured in rich broth to the PE phase, RelA synthetase activity accounts for the majority of ppGpp, whereas SpoT synthetase activity contributes modestly. Since *L. pneumophila* obtains carbon and energy from amino acids, not sugars (Tesh and Miller, 1981; Tesh *et al.*, 1983), the predominant role for RelA in these yeast extract broth cultures is not surprising.

To test the biological consequences of the small amount of *spoT*-dependent ppGpp that accumulates in PE phase, we assayed *flaA* expression and motility,

two hallmarks of stationary-phase *L. pneumophila*. The *relA* and *relA spoT* mutants were transformed with a plasmid containing a *flaA:gfp* transcriptional fusion that enables promoter activity of *flaA*, the primary flagellar subunit of *L. pneumophila*, to be analysed by fluorometry (Hammer and Swanson, 1999). As expected, the *flaA* promoter was inactive during E phase in WT, *relA* and *relA spoT* cultures (Hammer and Swanson, 1999). Upon entry into PE phase, both WT and *relA* mutant *L. pneumophila* activated the *flaA* promoter; however, in *relA* mutant cultures the *flaA* promoter activity was decreased relative to WT, consistent with a previous report (Zusman *et al*., 2002). In contrast, *relA spoT* mutant cultures failed to activate *flaA* expression upon entry into PE phase, indicating that residual *flaA* promoter activity observed in *relA* mutants is dependent on SpoT (Fig. 1B). Consistent with their *flaA* expression, the *relA* mutants also exhibited a 30–50% decrease in the fraction of motile bacteria per field relative to WT cultures, while *relA spoT* mutants failed to express motility at all PE-phase time points analysed (data not shown). Therefore, both RelA and SpoT contribute to *flaA* expression by PE *L. pneumophila*, and the small *spoT*-dependent ppGpp pool that accumulates in PE phase (Fig. 1A) is nevertheless sufficient to activate partial *flaA* expression and motility by *L. pneumophila*.

To investigate the importance of the stringent response to survival in the PE phase, we assessed the culture density and viability of WT, *relA* and *relA spoT* cultures over time. Both parameters were similar for all strains between 2 and 30 h. However, by 44 h, or ~20 h after entry into PE phase, *relA spoT* cells appeared hyperfilamentous relative to WT and *relA*, which had each adopted the more coccoid-like morphology that is characteristic of PE-phase *L. pneumophila* (Molofsky and Swanson, 2004). Concomitant with filamentation, by 44 h *relA spoT* cultures began to exhibit a loss in cfu relative to WT and *relA* (Fig. 1C). This decrease in viability of *relA spoT* cells was even more pronounced after 66 h. Therefore, as reported for other bacteria, including *C. jejuni* and *Helicobacter pylori* (Gaynor *et al*., 2005; Mouery *et al*., 2006), *L. pneumophila* requires the stringent response for differentiation and survival in stationary phase.

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

To verify that, like *E. coli*, *L. pneumophila* relies on RelA to initiate the stringent response following amino acid starvation, we subjected WT and mutant cultures to treatment with serine hydroxamate. An analogue of the amino acid serine, serine hydroxamate, inhibits the attachment of serine to transfer RNA, and it has been used extensively to characterize in *E. coli* the mechanism by which RelA senses accumulation of uncharged tRNAs at the ribosome (Tosa and Pizer, 1971; Magnusson *et al*., 2005). Unlike mock-treated cultures, WT cells subjected to serine hydroxamate treatment accumulated an appreciable pool of ppGpp (Fig. 2A). In contrast, neither *relA* nor *relA spoT* mutant *L. pneumophila* appeared to respond to serine hydroxamate treatment.

To determine the physiological relevance of the ppGpp pool detected, we asked if the *relA*-dependent ppGpp that accumulated after serine hydroxamate treatment was sufficient to activate *flaA* expression. Indeed, the pattern of *flaA* expression in serine hydroxamate-treated cultures (Fig. 2B, top) mimicked that observed for ppGpp production (Fig. 2A). Whereas WT cultures activated *flaA* as early as 3 h post treatment, neither *relA* nor *relA spoT* treated with serine hydroxamate activated *flaA* throughout the time-course analysed (Fig. 2B, top). Mock-treated cultures activated *flaA* with kinetics similar to that of control PE-phase cultures (Fig. 1B). Serine hydroxamate treatment did partially inhibit growth of all strains relative to mock-treated control samples (Fig. 2B, bottom), an effect that occurred independently of appreciable ppGpp accumulation (Fig. 2A). Together these observations verify that, in response to amino acid starvation, RelA synthesizes adequate ppGpp to activate *flaA* in *L. pneumophila*.

SpoT-dependent ppGpp accumulation activates flaA *when FAB is inhibited*

In *E. coli*, RelA responds to amino acid starvation, whereas SpoT responds to carbon, phosphate and iron starvation and FAB inhibition (Magnusson *et al*., 2005). Therefore, we tested whether *L. pneumophila* RelA and SpoT also respond to distinct metabolic cues. For this purpose we used the antibiotic cerulenin, a specific inhibitor of FAB in bacteria that acts on the 3-ketoacyl-[acyl carrier-protein (ACP)] synthases I and II, FabB and FabF (Seyfzadeh *et al*., 1993). Appreciable amounts of ppGpp were detected in WT and *relA* cultures treated with cerulenin, whereas *relA spoT* mutant *L. pneumophila* failed to accumulate detectable ppGpp under these conditions (Fig. 2C). The response was specific to the antibiotic, since cultures receiving the DMSO vehicle did not exhibit ppGpp.

To investigate the phenotypic consequences of the modest SpoT-dependent ppGpp pool, we assayed the ability of WT and mutant cultures to activate *flaA* following cerulenin treatment. As observed after the serine hydroxamate treatment, the pattern of *flaA* expression reflected the size of the ppGpp pool in each strain (Fig. 2D, top). Cerulenin-treated WT and *relA* mutant *L. pneumophila* activated *flaA*, but *relA spoT* cells did not (Fig. 2D, top). As

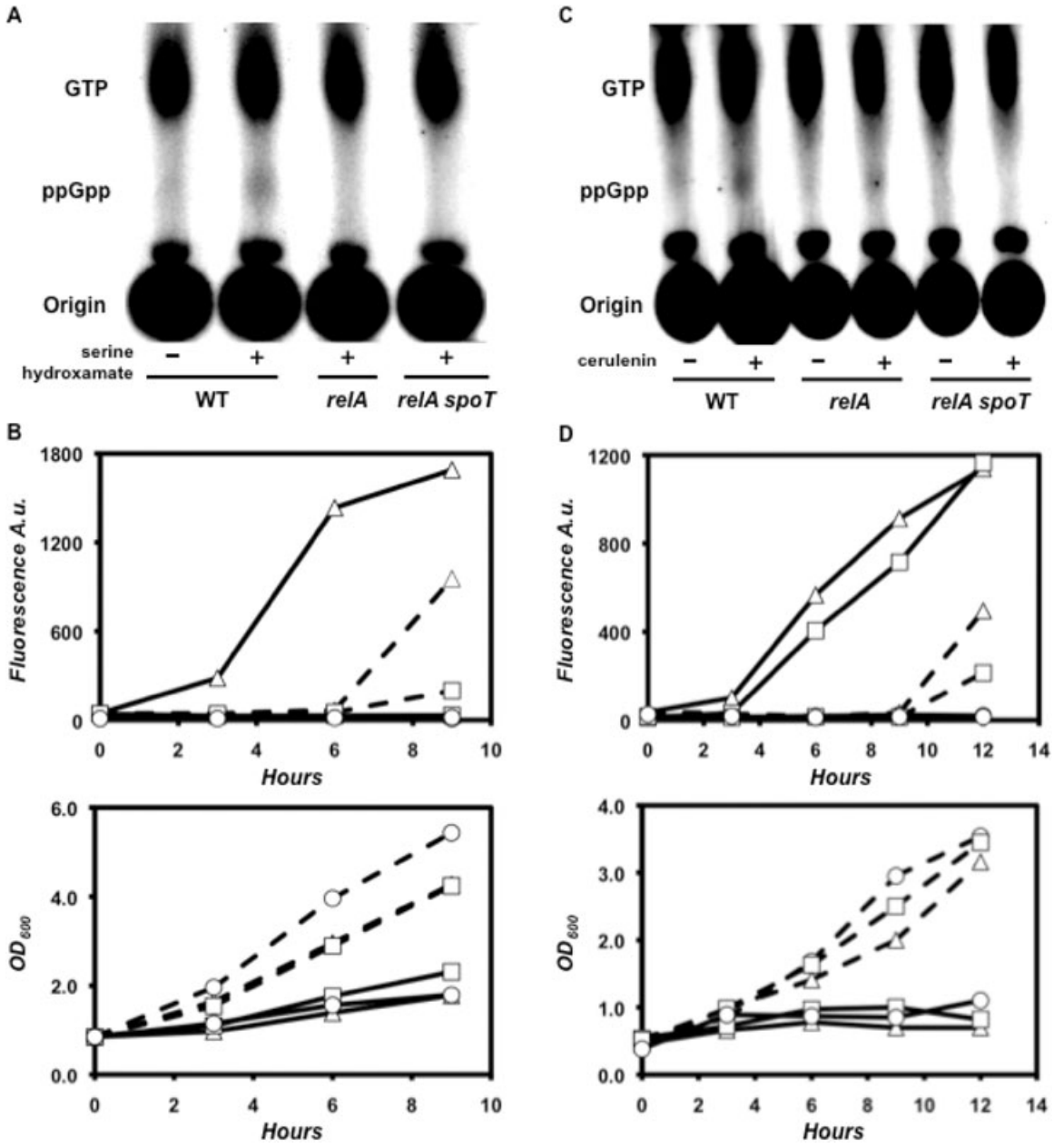

Fig. 2. *L. pneumophila* requires RelA and SpoT for ppGpp accumulation and *flaA* expression in response to distinct metabolic cues.
A. To analyse ppGpp accumulation in response to amino acid starvation, early E-phase AYET broth cultures were labelled with ^{32}P phosphoric acid for two generations before addition of either 1 mM serine hydroxamate (+) or water (–). After an additional 1.5 h incubation period, cell extracts were prepared, and nucleotides were separated by PEI-TLC. The autoradiogram represents one of two independent experiments.
B. To quantify *flaA* expression in response to amino acid starvation, mid-E-phase AYE cultures of WT (triangles), *relA* (squares), *relA spoT* (circles) strains harbouring p*flaAgfp* were diluted to early E phase before addition of either 1 mM serine hydroxamate (solid lines) or water (dashed lines), then cultures were sampled at 3 h intervals until WT and *relA* reference samples entered PE phase (OD_{600}, bottom) and exhibited *flaA* promoter activity fluorescence (9 h; top). These data represent one of two independent experiments. In the second experiment, the actual fluorescence values at 9 h post serine hydroxamate addition were WT, 1098 A.u.; *relA*, 22 A.u.; and *relA spoT*, 11 A.u.
C. To analyse ppGpp accumulation in response to inhibition of fatty acid biosynthesis, mid-E-phase AYET cultures of WT, *relA*, *relA spoT* were incubated for 1 h with cerulenin at 0.5 μg ml^{-1} (+) or DMSO (–), then cell extracts were prepared and analysed by PEI-TLC. Shown is one autoradiogram representing one of three independent experiments.
D. To quantify *flaA* expression in response to fatty acid biosynthesis inhibition, mid-E-phase cultures of WT (triangles), *relA* (squares) or *relA spoT* (circles) bacteria were treated with cerulenin (solid lines) or DMSO (dashed lines), then samples were collected at 3 h intervals to measure bacterial density (bottom) and fluorescence (top) until WT and *relA* reference cultures entered PE phase and exhibited fluorescence (12 h). These data represent one of two independent experiments. In the second experiment, the actual fluorescence values at 12 h post cerulenin addition were WT, 1617 A.u.; *relA*, 1082 A.u.; and *relA spoT*, 21 A.u.

observed for cultures treated with serine hydroxamate, cerulenin treatment also inhibited growth independently of ppGpp accumulation in the strain (Fig. 2D, bottom). Control cultures treated with DMSO were not inhibited for growth and activated fluorescence only upon entry into PE phase. Thus, *L. pneumophila* is equipped to differentiate in response to amino acid starvation via RelA, and inhibition of FAB using SpoT.

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

In addition to their response to FAB inhibition (Fig. 2), *L. pneumophila* that are exposed to excess short-chain fatty acids (SCFA) also activate transmissive phenotypes in a SpoT-dependent manner (R.L. Edwards, unpublished). Like cerulenin treatment, addition of either 10 mM acetic or propionic acid to E-phase *L. pneumophila* triggers expression of several transmission traits, including lysosome evasion, *flaA* expression and motility, as well as cytotoxicity (R.L. Edwards, unpublished). In *E. coli*, SpoT monitors and responds to perturbations in fatty acid metabolism through a specific interaction with ACP (Battesti and Bouveret, 2006). Accordingly, we tested the hypothesis that a SpoT–ACP interaction is essential for *L. pneumophila* to activate transmissive phenotypes when FAB is perturbed by excess SCFA. To do so, we designed plasmids for the inducible expression of either WT *spoT* or *spoT-A413E*, an allele encoding an enzyme predicted to be defective for ACP interaction (Battesti and Bouveret, 2006). Within the ~60-amino-acid TGS domain in which the critical alanine residue resides, *L. pneumophila* SpoT exhibits 67% identity and 86% similarity to the *E. coli* protein, and the seven amino acids C-terminal to the alanine residue are identical. In particular, we asked whether expression of plasmid-borne *spoT* or *spoT-A413E* was sufficient to restore flagellin expression by *relA spoT* bacteria exposed to 10 mM SCFA. Since similar origins of replication and antibiotic resistance markers for the relevant plasmids complicated use of the *flaA* reporter, we instead measured cytotoxicity, a phenotype dependent on flagellin as well as type IV secretion (Molofsky and Swanson, 2004).

Wild-type or mutant *spoT* expression was first induced for 1 h with 25 μM isopropyl-beta-D-thiogalactopyranoside (IPTG) before the cells were cultured for 3 h with 10 mM SCFA and 25 mM IPTG. When exposed to propionic acid, WT *spoT* restored *relA spoT* mutant cytotoxicity towards macrophages, but *spoT-A413E* did not (Fig. 3A). Likewise, only WT *spoT* rescued *relA spoT* cytotoxicity after acetic acid or cerulenin addition (data not shown). The plasmid-encoded *spoT-A413E* allele was functional: when the IPTG concentration was doubled to 50 μM, *relA spoT* mutants carrying the *spoT-A413E* plasmid were even more cytotoxic than those harbouring either WT *spoT* or the empty vector (Fig. 3B). Furthermore, the enhanced cytoxicity occurred independently of SCFA treatment. Thus, the *spoT-A413E* mutation appears to render the enzyme insensitive to perturbations in fatty acid metabolism and also increases the ppGpp pools, presumably by disrupting the balance between ppGpp synthesis and hydrolysis (Battesti and Bouveret, 2006).

The stringent response activates transmissive traits

Legionella pneumophila co-ordinates entry into stationary phase with expression of not only motility but also virulence-associated phenotypes like sodium sensitivity, cytotoxicity and lysosome avoidance (Byrne and Swanson, 1998). Accordingly, to assess the contributions of RelA and SpoT to virulence trait expression in PE *L. pneumophila*, we assayed WT and mutant bacteria for sodium sensitivity and lysosome evasion. As predicted, unlike WT, *relA spoT* mutants remained sodium resistant even when cultured into PE phase (Fig. 4A). PE-phase *relA* mutant *L. pneumophila*, which accumulate low amounts of ppGpp, activate *flaA* and become motile (Fig. 1), also become more sensitive to sodium. Likewise, whereas PE WT and *relA* resisted phagosome–lysosome fusion and remained intact following macrophage phagocytosis, E-phase WT and PE-phase *relA spoT* were degraded (Fig. 4B). Therefore, even the modest SpoT-dependent pool of ppGpp is sufficient to induce significant sodium sensitivity and to protect *L. pneumophila* from lysosomal degradation.

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

Since *L. pneumophila* requires ppGpp for *flaA* expression, motility, sodium sensitivity and lysosome avoidance (Figs 1 and 4) we predicted that, during PE-phase starvation, induction of either synthetase would equip replicative *relA spoT* mutants to enter the transmissive phase. Indeed, IPTG induction of plasmid-borne *relA*, *spoT* or *spoT-A413E* suppressed both the flagellar and the cell-remodelling defect of *relA spoT* mutant bacteria, as judged by the motility of coccoid bacteria. In contrast, *relA spoT* bacteria carrying only the vector remained amotile and elongated (data not shown). Furthermore, when either *relA*, *spoT* or *spoT-A413E* was induced, *relA spoT L. pneumophila* killed more macrophages than either *relA spoT* mutant bacteria carrying vector alone or E-phase WT (Fig. 5A). Induction of *relA*, *spoT* and *spoT-A413E* also increased the infectivity of *relA spoT* bacteria: when harbouring plasmids encoding either ppGpp synthetase and cultured with IPTG to the PE phase, significantly

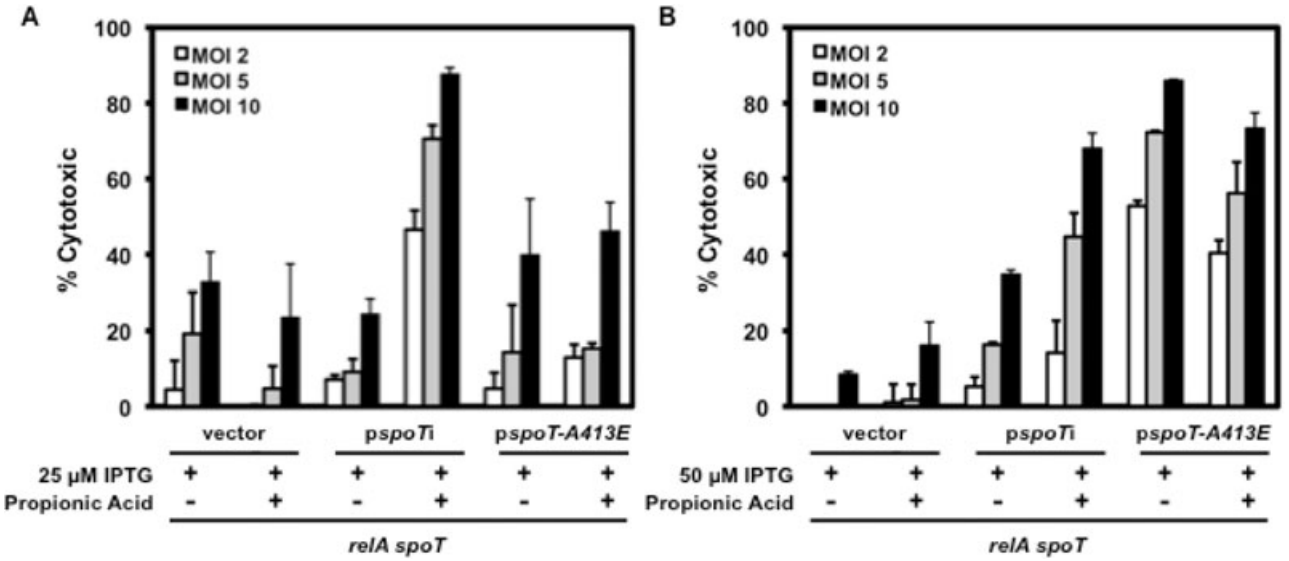

Fig. 3. SpoT mutation abrogates transmission trait expression following perturbations in fatty acid metabolism.
A. E-phase cells normalized to an OD_{600} of 0.5–0.6 were incubated with 25 µM IPTG for 1 h. Then cultures were split and 10 mM propionic acid was added to one half, while the other half was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

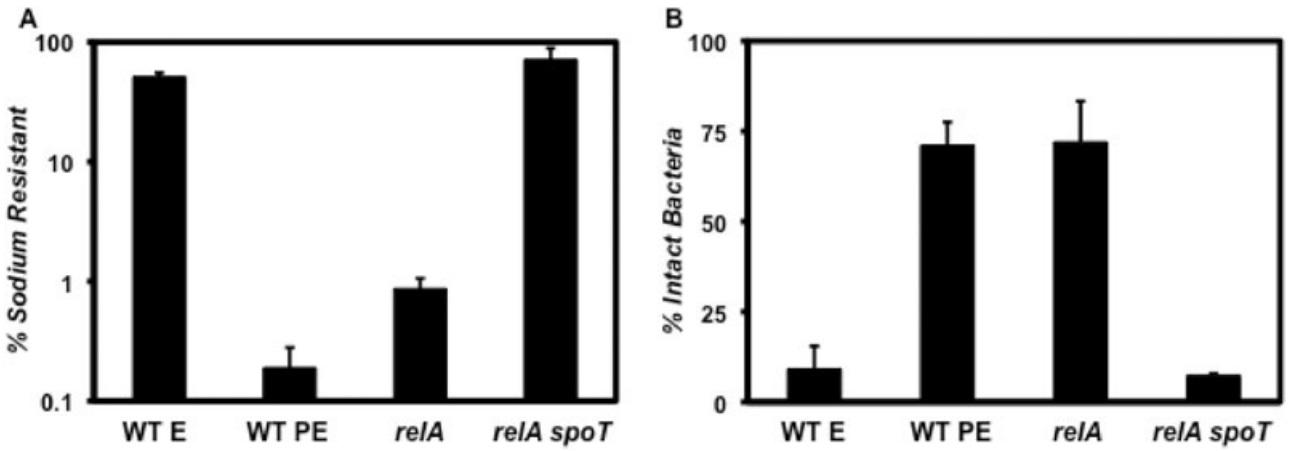

Fig. 4. The stringent response governs *L. pneumophila* transmission traits in broth.
A. To quantify sodium resistance, E- or PE-phase cultures of the strains shown were plated on medium with or without 100 mM NaCl, then efficiency of colony formation was calculated as [(cfu on CYET + 100 mM NaCl)/(cfu on CYET)] × 100. Shown are mean percentages ± SE from duplicate samples, and the data represent one of three independent experiments.
B. The ability of bacteria to bind, enter and survive ingestion by macrophages was quantified by fluorescence microscopy by scoring the per cent of bacteria that were intact 2 h after infection. Infected macrophages were double-labelled with the DNA stain DAPI to visualize macrophage nuclei and intact bacteria and with *L. pneumophila*-specific antibody to visualize both intact and degraded *L. pneumophila*. A total of 100 intracellular bacteria were counted per coverslip. Shown are mean percentages ± SE from duplicate coverslips, and the data represent one of three independent experiments.

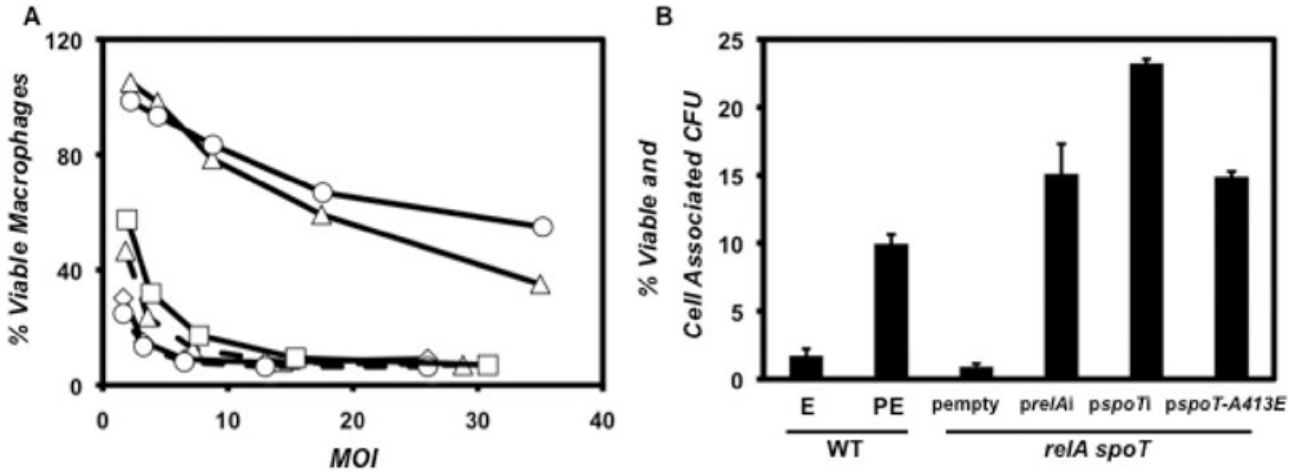

Fig. 5. Induction of either ppGpp synthetase rescues transmission trait expression of *relA spoT* mutants.
A. To determine the contribution of RelA and SpoT to *L. pneumophila* cytotoxicity, *relA spoT* mutants transformed with vector (circles), p*relA*i (squares), p*spoT*i (diamonds) or p*spoT-A413E* (circles, dashed line) were cultured from mid-E phase to PE phase with 200 μM IPTG. Bacteria were added to triplicate wells of macrophages at the moi shown. WT cells carrying vector and cultured to either E phase (triangles) or PE phase (triangles, dashed line) were added as controls. After 1 h incubation, cytotoxicity was measured. The values plotted represent the mean ± standard error for triplicate samples determined in one of three similar experiments.
B. Macrophages were infected at an moi of ~1.0 for 2 h with either E- or PE-phase WT carrying empty vector, *relA spoT* mutants transformed with empty vector, p*relA*i, p*spoT*i or p*spoT-A413E* cultured from mid-E phase to PE phase with 200 μM IPTG, as described above. Shown are mean per cent of cell-associated cfu ± SE from duplicate wells in one of three independent experiments.

more *relA spoT* mutants were infectious than those carrying the empty vector (Fig. 5B). Thus, expression of either *relA*, *spoT* or *spoT-A413E* was sufficient to trigger replicative-phase *relA spoT* mutants to enter the transmissive phase. These results not only verify that the phenotypic defects of *relA spoT* mutants were not due to secondary site mutations or polar effects, but also indicate that, regardless of its source, ppGpp co-ordinates the replicative to transmissive phase transition in broth.

The stringent response is essential during macrophage infection

As in broth, *L. pneumophila* cycles between replicative and transmissive phases in host cells (Byrne and Swanson, 1998; Hammer and Swanson, 1999; Alli *et al.*, 2000; Bruggemann *et al.*, 2006). Since ppGpp is essential for activation of the transmission phenotype *in vitro* (Figs 1A, 4 and 5), we postulated that RelA and SpoT are also required for transmission between host cells.

As observed previously, *relA* was dispensable for *L. pneumophila* growth and survival in macrophages (Fig. 6A; Zusman *et al.*, 2002; Abu-Zant *et al.*, 2006). In contrast, *relA spoT* mutants infected with a decreased efficiency relative to PE-phase WT, a phenotype similar to WT in E phase (Fig. 6A; 2 h time point). Those *relA spoT* and E-phase WT bacteria that survived the initial attack moved on to replicate efficiently between 2 and 24 h. However, after 24 h, E-phase WT cfu increased to values equivalent to PE-phase WT, but *relA spoT* mutants exhibited no further increase in cfu over the remainder of the period analysed (Fig. 6A).

Since 24 h corresponds to the period of flagellar expression and initiation of secondary infection (Byrne and Swanson, 1998), we postulated that *relA spoT* mutants were defective for either escape from an exhausted host or entry into a naïve one. To distinguish between these two hypotheses, we performed immunofluorescence microscopy. At 16 h, both WT and *relA spoT* bacteria occupied replication vacuoles (data not shown); however, between 18 and 24 h, a unique class of infected macrophages emerged in *relA spoT* cultures. Intact bacteria were no longer observed; instead, degraded *L. pneumophila* particles were dispersed throughout the macrophage (Fig. 6B). In addition, there was scant evidence of secondary infection by *relA spoT* mutants at time points beyond 18 h (data not shown). Thus, the majority of mutant bacteria were apparently destroyed following the replicative growth period, prior to escape. In the rare macrophages that contained one or two bacteria at time points beyond 18 h, the *relA spoT* mutants were also degraded, likely due to their inability to avoid phagosome–lysosome fusion (Fig. 6B). At 18 h, the percentage of infected macrophages that harboured either single or multiple degraded *relA spoT* mutant bacteria was nearly 80%, approximately three times greater than the ~25% of infected macrophages containing degraded WT bacteria (Fig. 6C). Therefore, the stringent response is required for

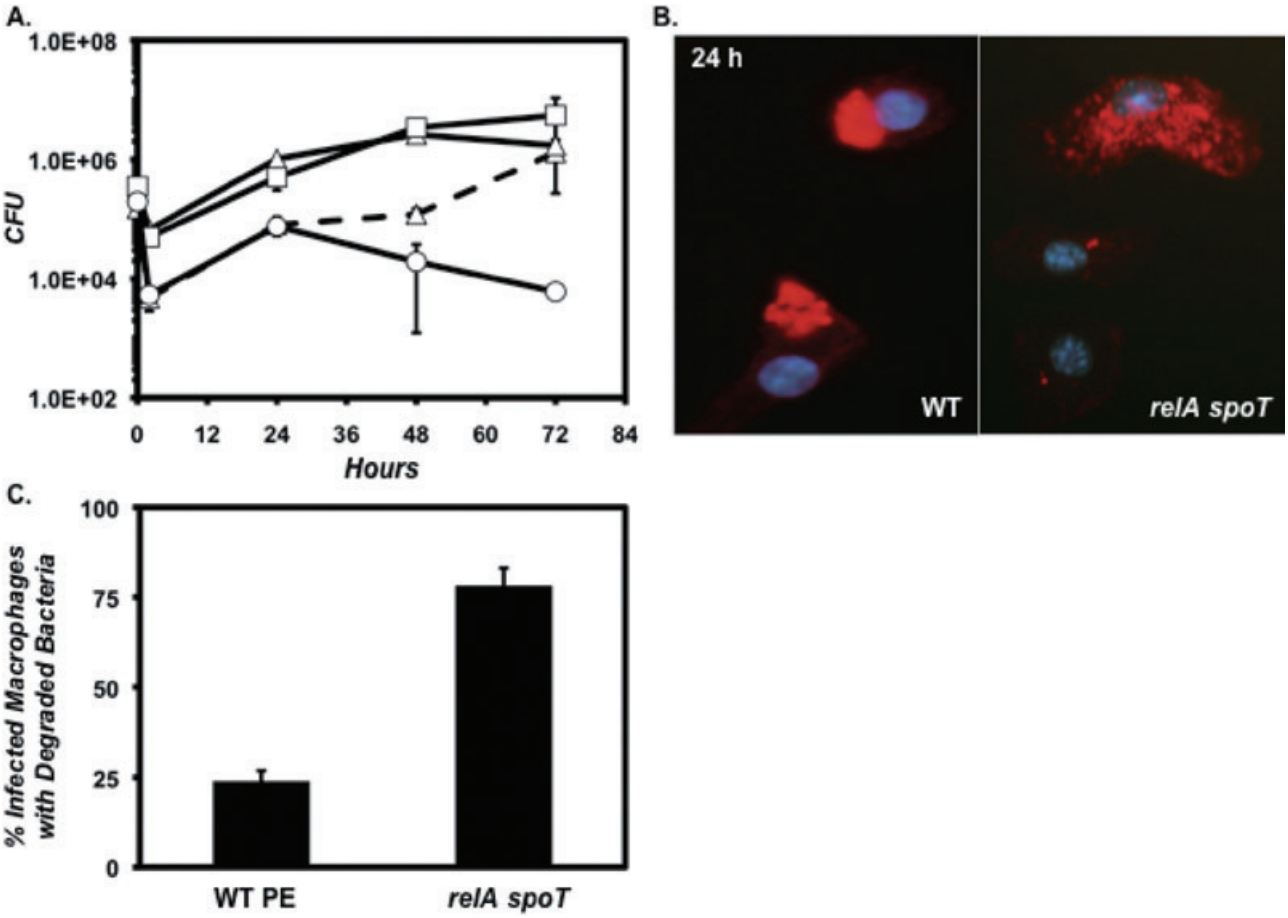

Fig. 6. The stringent response is essential for *L. pneumophila* transmission in macrophages.
A. Macrophages were infected at an moi of ~1.0 with E-phase WT (triangles, dashed line), PE-phase WT (triangles, solid line), PE-phase *relA* (squares) or PE-phase *relA spoT* mutants (circles) for the periods shown, and then the number of viable bacteria per well was determined. Shown are mean cfu ± SE from duplicate samples in one of three independent experiments. The difference in the mean cfu values calculated from three independent experiments for WT and *relA spoT* mutant bacteria at 72 h was statistically significant by a two-tailed Student's *t*-test ($P < 0.02$).
B. Macrophages were infected at an moi of ~1.0 with PE-phase WT or PE-phase *relA spoT* mutant bacteria and coverslips were fixed at 24 h. Cells were labelled blue with DAPI and red with anti-*L. pneumophila* antibody, and fluorescence microscopy was performed. The image of the *relA spoT* mutant infected macrophages represents the categories of degradation scored in (C).
C. Macrophages were infected as in (B), coverslips were fixed at 18 h and fluorescence microscopy was performed as described above. A total of 100 infected macrophages were scored on duplicate coverslips. Shown are the mean percentages of macrophages containing single, or multiple degraded bacteria from duplicate samples in one of two independent experiments.

L. pneumophila transmission between macrophages, and SpoT is a critical component of its developmental switch.

SpoT is essential for transmission between macrophages

To investigate further the contributions of RelA and SpoT to differentiation of replicative bacteria to the transmissive state in macrophages, we tested whether induction of either *relA* or *spoT* was sufficient to rescue the intracellular *relA spoT* mutants. The *relA spoT* mutant *L. pneumophila* were cultured in the absence of IPTG to early PE phase and then added to macrophages. After ~16 h, IPTG was added to the cultures and maintained for the remainder of the infection. Like *relA spoT* bacteria harbouring empty vector, double mutants in which *relA* had been induced exhibited an ~20-fold increase in cfu between 2 and 24 h, but no appreciable increase beyond 24 h (Fig. 7). Likewise, constitutive expression of *relA* by *relA spoT* mutants in rich broth was also incompatible with replication in broth, as judged by measuring both the optical density and cfu of the cultures (Fig. S1C). In contrast, when *spoT* was induced, *relA spoT* mutants replicated similarly to WT in macrophages (Fig. 7) and in broth (Fig. S1). In mouse macrophages, the cfu increase observed after 24 h reflects secondary and tertiary infections; therefore, SpoT activity is critical for *L. pneumophila* transmission between host cells.

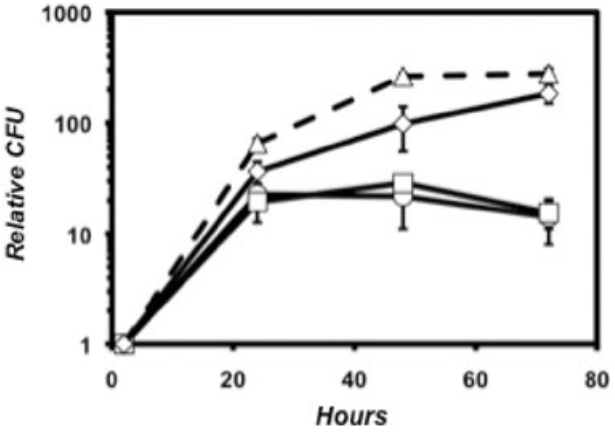

Fig. 7. SpoT, not RelA, is essential for *L. pneumophila* transmission in macrophages. Macrophages were infected at an moi ~1.0 with E-phase WT (triangles, dashed line) and PE-phase *relA spoT* harbouring: vector (circles), *prelA*i (squares) or *pspoT*i (diamonds). At 16 h post infection, 200 μM IPTG was added to the wells and maintained throughout the remainder of experiment. Relative cfu was calculated by dividing the cfu values obtained at 24, 48 and 72 h by the 2 h value. Shown are mean fold increase in cfu ± SE from duplicate wells in one of three independent experiments.

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

To investigate whether SpoT synthetase and/or hydrolase activity is critical for the life cycle of *L. pneumophila* in macrophage cultures, we tested whether induction of *relA*, *spoT* or *spoT-A413E* equipped transmissive bacteria to differentiate into the replicative phase in both macrophages and on solid medium. Broth-grown cultures of *relA spoT* bacteria carrying inducible *relA*, *spoT* or *spoT-A413E* were cultured with IPTG into PE phase to activate transmission traits. Next, transmissive bacteria were either added to macrophages in the presence of IPTG (Fig. 8A), or plated onto rich medium with or without IPTG (Fig. 8B). During macrophage infection with the *relA spoT* strains, induction of WT *spoT* resulted in a pattern of intracellular growth identical to that of PE-phase WT bacteria (Fig. 8A). In contrast, induction of neither *relA* nor *spoT-A413E* equipped *relA spoT L. pneumophila* to replicate intracellularly (Fig. 8A). Similar patterns were observed when transmissive bacteria were plated onto bacteriological medium. All the strains formed colonies when transferred to medium without IPTG (Fig. 8B, left). On medium containing IPTG, mutant *relA spoT L. pneumophila* that either expressed WT *spoT* or carried the empty vector readily formed colonies (Fig. 8B, right). In contrast, consistent with their intracellular growth defect, *relA spoT* mutants harbouring either inducible *relA* or *spoT-A413E* failed to replicate when IPTG was maintained in the medium (Fig. 8B, right). These genetic data suggest that the enzyme encoded by *spoT-A413E* favours ppGpp accumulation, more closely resembling RelA. Together, these results indicate that SpoT hydrolase activity is required for transmissive cells to re-enter the replicative phase either during macrophage infection or on bacteriological medium.

Discussion

To endure starvation and other stresses, many bacteria rely on the stringent response pathway to alter their transcriptional profiles and enhance their fitness. When nutrients become scarce in broth, *L. pneumophila* generates ppGpp to co-ordinate its transition from a replicative to a transmissive form (Hammer and Swanson, 1999; Zusman *et al*., 2002). Although *L. pneumophila* utilizes RelA to produce ppGpp upon entry into the PE phase in yeast extract broth, this enzyme is dispensable for replication in either amoeba or human macrophages (Zusman *et al*., 2002; Abu-Zant *et al*., 2006). Like several other microbes, *L. pneumophila* also encodes the bifunctional and essential enzyme SpoT, which can either synthesize or hydrolyse ppGpp depending on local cues. Analysis of broth cultures indicates that the two ppGpp synthetases confer versatility to *L. pneumophila*, enabling conversion to the transmissive state when either protein or fatty acid synthesis is compromised (Fig. 2). However, in macrophages only SpoT is required, and its role is bipartite (Figs 6A and 8A). After establishing its protective vacuole, intracellular *L. pneumophila* relies on SpoT to hydrolyse ppGpp to reduce the alarmone pool and switch from the transmissive to the replicative form. When conditions deteriorate, the intracellular progeny elicit SpoT synthetase activity to co-ordinate their conversion to the transmissive state and escape from the exhausted host (Fig. 9).

In broth, *L. pneumophila* requires ppGpp from either RelA or SpoT to initiate the replicative to transmissive transition (Fig. 9). When *L. pneumophila* is cultured in broth, a large proportion of its ppGpp pool is generated by RelA (Fig. 1A). The more minor contribution by SpoT can be observed as the residual ppGpp detected in *relA* mutants, since only two ppGpp synthetases are apparent in the *L. pneumophila* genome, and no alarmone is detectable in *relA spoT* double mutant cells (Fig. 1A). Like *E. coli* SpoT, the *L. pneumophila* enzyme may be a weak synthetase (Seyfzadeh *et al*., 1993). Alternatively, the low levels of SpoT-dependent ppGpp observed may be a consequence of experimental conditions: its fastidious character precludes use of a defined medium where competing inorganic phosphate levels can be minimized. Here *L. pneumophila* was cultured in a rich medium that contains amino acids as the sole energy source, which not only reduces the efficiency of the radiolabelling of the phosphate pool, but also favours RelA-dependent ppGpp production. Nevertheless, the weak ppGpp signal gener-

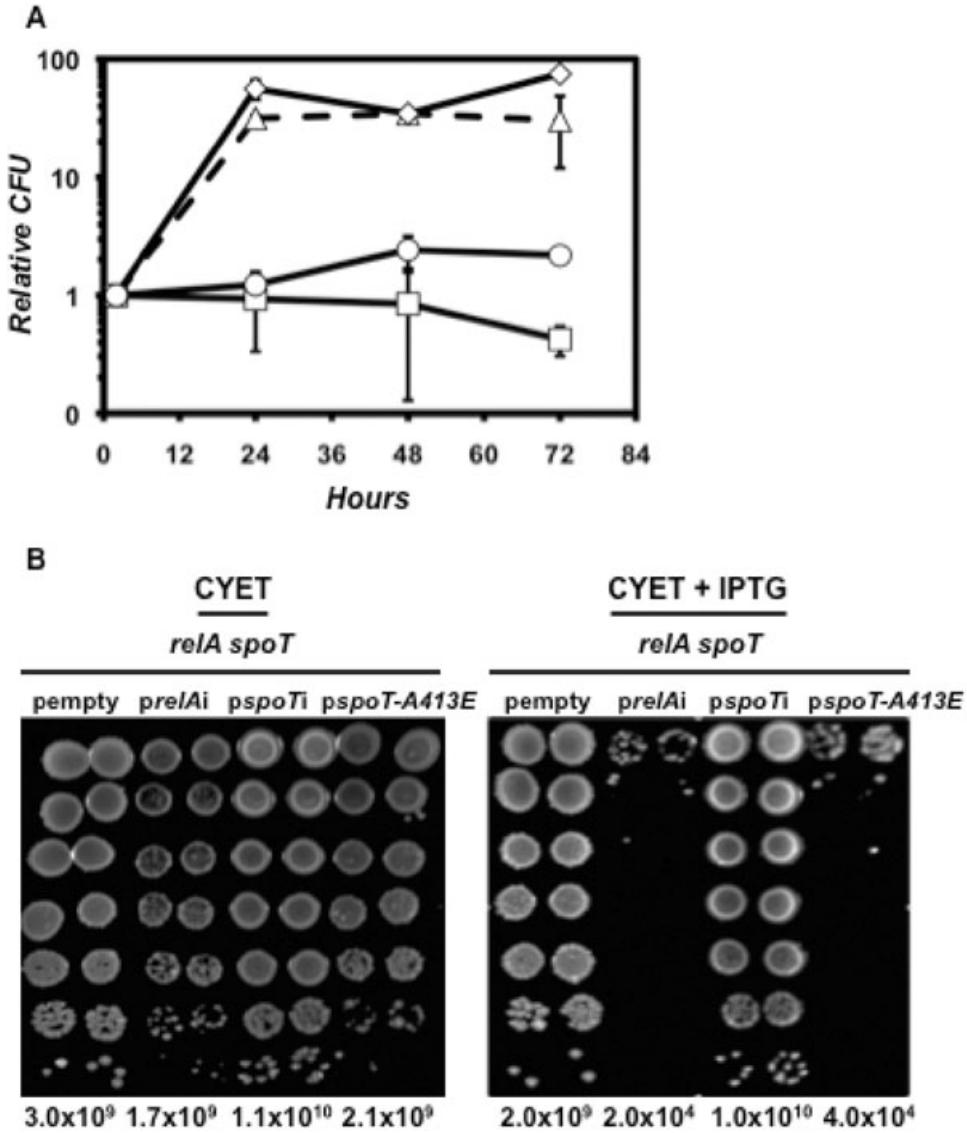

Fig. 8. Transmissive *L. pneumophila* requires SpoT to enter the replicative phase.
A. To determine if SpoT hydrolase activity contributes to the transmissive to replicative phase transition within host cells, *relA spoT* mutant *L. pneumophila* carrying either p*relA*i (squares), p*spoT*i (diamonds) or p*spoT-A413E* (circles) were induced with 200 μM IPTG in mid-E phase, cultured into PE phase (4–5 h), and then used to infect macrophages at an moi of ~1. During the 72 h infection, 200 μM IPTG was maintained. WT carrying empty vector cultured into PE phase (triangles, dashed line) served as a positive control in this assay. Relative cfu was as described above. Shown are mean fold increase in cfu ± SE from duplicate wells in one of three independent experiments.
B. As in (A), *relA spoT* mutant derivatives of *L. pneumophila* were cultured in mid-E phase to PE phase (4–5 h) in the presence of 200 μM IPTG. Then, to mimic the induction conditions used in the macrophage experiment, bacteria were plated onto CYET with or without 200 μM IPTG. Colony-forming units (displayed beneath image) were calculated as the number of bacteria from the induction in broth capable of initiating replication to form a colony in the presence or absence of IPTG. Shown are images of CYET agar plates of one of three independent experiments.

ated by SpoT in this medium is sufficient to induce with timing similar to WT *L. pneumophila* not only the *flaA* promoter, but also motility, sodium sensitivity and evasion of lysosomes (*relA* mutant; Figs 1B and 4, and data not shown).

In addition to triggering its panel of transmission traits, ppGpp from either synthetase is also critical for *L. pneumophila* to survive the PE phase in broth cultures (Fig. 1C). In *E. coli*, ppGpp-dependent control of fatty acid and phospholipid metabolism allows the bacterium to selectively modify its membranes during stress. For example, ppGpp inhibits fatty acid and phospholipid biosynthesis through repression of *plsB* (sn-glycerol-3-phosphate acyltransferase), resulting in accumulation of long-chain acyl-ACPs and cell shortening (Heath *et al.*, 1994). Indeed, the *E. coli* stringent response both acti-

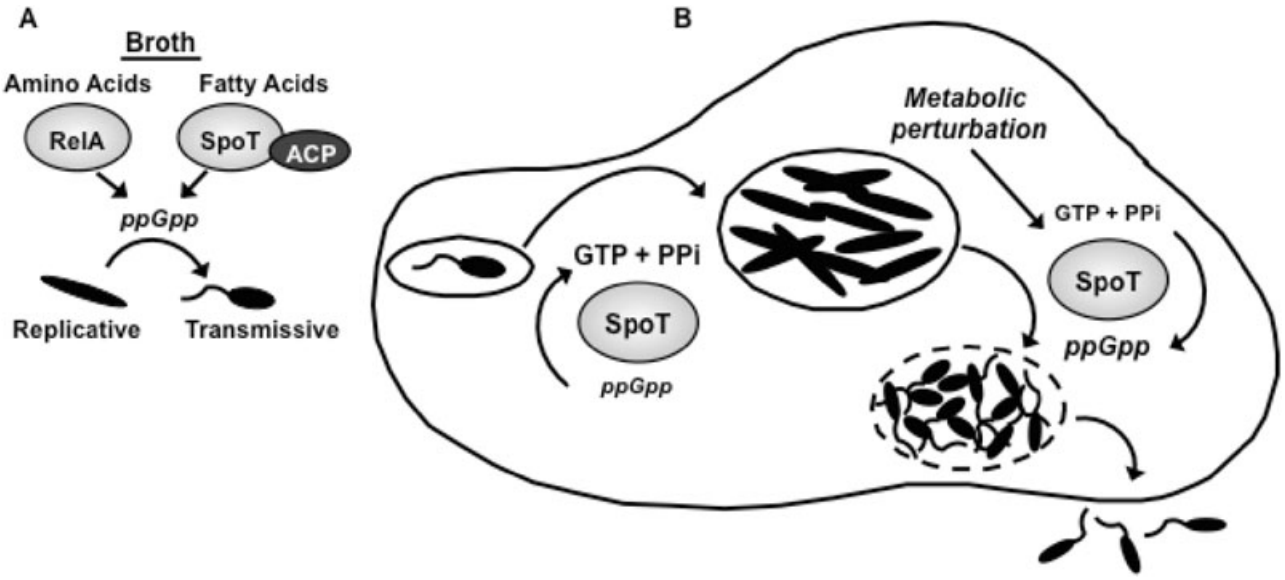

Fig. 9. SpoT governs *L. pneumophila* differentiation in host macrophages.
A. To co-ordinate exit from the replicative state and entry into the transmissive state in broth, *L. pneumophila* utilizes its two ppGpp synthetases, RelA and SpoT. Each enzyme responds to distinct metabolic cues and SpoT monitors fatty acid biosynthesis likely through an interaction with acyl-carrier protein (ACP).
B. During the intracellular life cycle of *L. pneumophila*, the bifunctional enzyme SpoT plays a dual role. When transmissive bacteria have evaded initial host cell defences and gain access to nutrients, SpoT-dependent ppGpp hydrolysis enables transformation into the replicative form. Following robust replication, nutrients become exhausted and SpoT synthetase activity is elicited. Accumulation of SpoT-dependent ppGpp initiates global metabolic change as replicative traits are repressed and the bacterium converts back to the transmissive form. Transmissive bacteria are equipped to exit the depleted host cell and prepared to infect a naïve one.

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

To alternate between transmissive and replicative forms in macrophages, *L. pneumophila* uses the SpoT enzyme to tightly regulate ppGpp synthesis and degradation. As reported previously (Zusman *et al*., 2002; Abu-Zant *et al*., 2006), the RelA enzyme itself is dispensable for *L. pneumophila* to survive phagocytosis by macrophages and to replicate intracellularly (Fig. 6A). On the other hand, the alarmone ppGpp is critical to induce the virulence factors that equip *L. pneumophila* to establish its protected intracellular niche, since mutants that lack both *relA* and *spoT* survive poorly within macrophages (Fig. 6). For the replicative to transmissive transition in broth, the source of ppGpp is inconsequential, since expression of either RelA or SpoT largely bypasses the transmissive trait defects of *relA spoT* double mutant bacteria (Fig. 5A and B). Once replication ensues in the macrophage, ppGpp is no longer required, since the few *relA spoT* mutant bacteria that do survive the initial infection replicate efficiently during the subsequent 24 h primary infection period (Fig. 6A). When vacuolar conditions deteriorate and *L. pneumophila* transit to a new host, SpoT is required, since induction of SpoT but not RelA bypasses the growth defect of *relA spoT* mutant bacteria beyond the 24 h time point (Fig. 7). During the transmission period, the synthetase activity of SpoT is sufficient to promote escape from and entry into a new host (*relA* mutant bacteria; Fig. 6A), whereas its hydrolase is absolutely required for newly engulfed bacteria to initiate replication (Fig. 8A). Thus, SpoT governs differentiation of *L. pneumophila* at two critical nodes of its life cycle in macrophage cultures.

Legionella pneumophila has evolved two ppGpp synthetases capable of responding to distinct metabolic inputs during growth *in vitro* (Fig. 2); however, during intercellular transmission only SpoT is essential (Figs 6A and 7). Therefore, the stress signal that promotes replicative bacteria to convert to the transmissive form in macrophages must be sensed by SpoT. In *E. coli*, RelA responds to amino acid limitation, whereas SpoT activates the stringent response pathway during all other types of stresses and starvation (Potrykus and Cashel, 2008). Amino acid depletion also stimulates *L. pneumophila* to produce ppGpp and induce

differentiation by a RelA-dependent pathway (Fig. 2A and B). On the other hand, to respond to perturbations in FAB, *L. pneumophila* relies on SpoT (Fig. 2C and D). Since SpoT alone is sufficient to induce transmission in macrophages (Figs 6A and 7), we postulate that the ability of SpoT to sense variations in fatty acid metabolism equips *L. pneumophila* to respond to fluctuations in the lipid supply during its intracellular life cycle. Whether SpoT also equips *L. pneumophila* to respond to other metabolic stimuli that are critical within host cells remains to be explored experimentally.

Our genetic data suggest that, as in *E. coli*, the *L. pneumophila* response to perturbations in FAB requires SpoT interaction with ACP; by some mechanism, this interaction regulates SpoT enzyme activity (Battesti and Bouveret, 2006). In particular, Battesti and Bouveret identified a single amino acid substitution in SpoT that abrogates a physical interaction with ACP and renders the mutant bacteria blind to fatty acid starvation (Battesti and Bouveret, 2006). Likewise, the analogous *spoT-A413E* mutant *L. pneumophila* fail to trigger cytotoxicity in response to excess SCFA (Fig. 3A). In addition, interaction between SpoT and ACP affects the ability of SpoT to reduce the ppGpp pool, as judged by three phenotypic assays. *L. pneumophila* treated to express strongly the *spoT-A413E* allele apparently contain excessive ppGpp, as the cells are more cytotoxic than bacteria expressing WT *spoT* (Fig. 3B), and they survive yet fail to replicate when cultured either in macrophages or on rich medium (Fig. 8A and B). Based on the capacity of the analogous *E. coli spoT-A404E* mutant to adapt to histidine starvation, Battesti and Bouveret (2006) also deduced that excess ppGpp accumulates when the SpoT–ACP interaction is disrupted. We note two caveats to our experimental approaches. The contribution of a physical interaction between SpoT and ACP to the *L. pneumophila* fatty acid response is based on a precedent established in *E. coli* and our analogous genetic analysis, which remains to be verified by direct biochemical assays. Second, the simple interpretation that increased concentrations of IPTG used to induce either the *spoT* or the *spoT-A413E* allele increases the amount of the respective proteins (Fig. 3) has not been verified. Our attempts to monitor SpoT protein levels by Western analysis using a previously described polyclonal antibody specific to the *E. coli* enzyme (Gentry and Cashel, 1996) were not successful.

Given our genetic data and the published genetics and biochemistry (Battesti and Bouveret, 2006), several working models remain in play. When FAB is perturbed, an unidentified signalling ligand either accumulates or is depleted, thereby altering ACP in a manner that is sensed by SpoT. Consequently, ACP could either repress SpoT-dependent ppGpp hydrolysis, or activate SpoT-dependent ppGpp synthesis. Biochemical studies can distinguish between these possibilities. In any case, when FAB is perturbed, SpoT-dependent ppGpp accumulates and replicative *L. pneumophila* transition to the transmissive form.

In addition to monitoring FAB, SpoT activity is also critical for transmissive bacteria to re-enter the replicative state when nutrients are abundant, likely due to the need to hydrolyse the alarmone (Fig. 8A and B). The intracellular phenotype of *L. pneumophila* that generate excess ppGpp (i.e. *relA spoT* mutant bacteria that constitutively express either *relA* or the mutant allele *spoT-A413E*) is similar to that of *phtA* mutant *L. pneumophila*, which lack the high-affinity transporter for threonine (Sauer *et al.*, 2005). In each case, the mutant bacteria persist as single, intact, rods within their vacuoles, while WT microbes replicate robustly (Sauer *et al.*, 2005). Without the capacity to acquire threonine, as essential amino acid, *L. pneumophila* may become locked in a state of ppGpp synthesis. Thus, the local nutrient supply dictates when transmissive bacteria activate replicative functions. To resume multiplying, *L. pneumophila* must activate the SpoT hydrolase to reduce its ppGpp pool (Fig. 9B).

The intracellular pathogen *M. tuberculosis* encodes a single-bifunctional Rel–Spo homologue (RSH), Rel, required for long-term survival *in vitro* (Primm *et al.*, 2000) and long-term persistence in mice (Dahl *et al.*, 2003; Karakousis *et al.*, 2004). The ability of this pathogen to differentiate to a persister state may contribute to the failure of front-line antimicrobial agents (Warner and Mizrahi, 2006). Thus, understanding the biochemistry of the stringent response enzymes provides an avenue to analyse the life cycle of a variety of intracellular pathogens and, more importantly, to develop new strategies and therapeutics to eradicate them.

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

Experimental procedures

Bacterial strains, culture conditions and reagents

Legionella pneumophila strain Lp02 (*thyA hsdR rpsL*; MB110), a virulent thymine auxotroph derived from Philadel-

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

relA *and* relA spoT *mutant strain construction*

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

To construct the *relA spoT* double mutant, we generated pGEM-*spoT* by amplifying the *spoT* locus (lpg2009) plus flanking sequence from Lp02 genomic DNA using the primers spoT1 and spoT2. pGEM-*spoT* was digested with HindIII, which cuts at sites ~98 and ~588 bp 3′ to the translational start, releasing an ~490 bp fragment encoding the consensus HD motif required for hydrolase activity, as well as a significant portion of the predicted synthetase coding regions at the N-terminus of SpoT. The deletion leaves intact 32 amino acids at the extreme N-terminus, as well as amino acids 195–715 at the C-terminus. By analogy to deletion analysis conducted with the *E. coli spoT* gene (Gentry and Cashel, 1996), our deletion should have eliminated both enzymatic activities of SpoT. The linear fragment containing the *spoT* deletion was blunted, and a kanamycin cassette was ligated to the blunted fragment, generating pGEM-*spoT::kan*. After verification by PCR and restriction enzyme digest, the deletion/insertion alleles were used to transform *relA::gent* mutant *L. pneumophila* MB695 by natural competence, and transformants were selected using the appropriate antibiotic (Stone and Abu Kwaik, 1999). Recombination of the desired *spoT* deletion/insertion allele into the *relA::gent* mutant chromosome was confirmed by PCR. The resulting *relA::gent spoT::kan* double mutant *L. pneumophila* was designated MB697. MB355 contains the p*flaA*gfp plasmid that encodes thymidylate synthetase as a selectable marker and a transcriptional fusion of the *flaA* promoter to *gfp* (Hammer and Swanson, 1999; Hammer *et al*., 2002). MB696 and MB697 were transformed with the p*flaA*gfp reporter plasmid to create MB684 and MB685 respectively.

Inducible relA *and* spoT *expression*

To generate strains in which expression of either *relA* or *spoT* could be induced, we cloned promoterless fragments of *relA* and *spoT* into pMMB206-Δmob, a plasmid containing a $P_{taclacUV5}$ IPTG-inducible promoter and a chloramphenicol resistance cassette. To construct pGEM-*relA*i, the *relA* locus was amplified from Lp02 genomic DNA using the primers relAi1 and relAi2, each containing a SalI restriction site. The fragment was excised from pGEM-*relA*i using SalI and ligated into pMMB206-Δmob at the SalI site within the MCS immediately 3′ of the $P_{taclacUV5}$ promoter, generating p*relA*i. Insertion and orientation were confirmed by both PCR and restriction enzyme digest. p*relA*i was used to transform MB697, and transformants were selected on chloramphenicol, creating MB687 for inducible *relA* expression. To construct pGEM-*spoT*i, the *spoT* locus was amplified from Lp02 genomic DNA using the primers spoTi1, which contains a SalI restriction site, and spoTi2, which contains a HindIII site. pGEM-*spoT*i and pMMB206-Δmob were each digested with SalI and HindIII and then ligated, generating p*spoT*i. Insertion and orientation of *spoT* in the MCS of pMMB206-Δmob were confirmed by PCR and restriction enzyme digest. p*spoT*i was used to transform MB697, generating MB688 for inducible *spoT* expression.

Inducible spoT-A413E *expression*

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

Detection of ppGpp

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

Fluorometry

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

Sodium sensitivity

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

Macrophage cultures

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

Lysosomal degradation

The percentage of intracellular *L. pneumophila* that remain intact after a 2 h macrophage infection was quantified by fluorescence microscopy (Bachman and Swanson, 2001). Briefly, macrophages were plated at 2.5×10^5 onto coverslips in 24-well plates. Then, E- or PE-phase microbes were added to macrophage monolayers at a multiplicity of infection (moi) ~1. The cells were centrifuged at 400 *g* for 10 min at 4°C and then incubated for 2 h at 37°C. Extracellular bacteria were removed by washing the monolayers three times with RPMI/FBS, and the macrophages were fixed, permeabilized and stained for *L. pneumophila* as described (Molofsky *et al.*, 2005). Except for longer incubation times (18 and 24 h), identical procedures were used to image and score infected macrophages at later time points of the primary infection period (Fig. 6B and C).

Infectivity and intracellular growth

Infectivity is a gauge of the ability of *L. pneumophila* to bind, enter and survive inside macrophages during a 2 h incubation, as previously described (Byrne and Swanson, 1998; Molofsky and Swanson, 2003). In brief, macrophages were infected with strains at an moi of ~1, with centrifugation at 400 *g* for 10 min. Cells were incubated for 2 h at 37°C, washed three times with fresh RPMI, and lysed in a 1:1 solution of RPMI and saponin, then intracellular cfu were enumerated. Results are expressed as the percentage of the inoculated cfu recovered from lysed macrophages at 2 h post infection. To gauge intracellular growth, the pooled macrophage supernatant and lysate were plated for cfu at various times post infection, as described elsewhere (Molofsky and Swanson, 2003). Results for intracellular growth are expressed as either total cfu recovered or relative cfu. In the latter, cfu recovered at 24, 48 and 72 h are divided by the number of cfu at 2 h. Culturing conditions for WT and mutant *L. pneumophila* varied depending on the experimental question addressed. For experiments assaying infectivity and intracellular survival of WT, *relA* and *relA spoT* bacteria lacking expression vectors, cultures were grown to E or PE phase prior to infection. To test the contribution of *relA* and *spoT* to the replicative to transmissive phase transition in

broth (Fig. 5), overnight cultures in mid-E-phase OD_{600} of 1.0–1.75 were back diluted to early E-phase OD_{600} of 0.50–0.85. Cultures were incubated for approximately 3 h at 37°C to an OD_{600} of 1.0–1.75; then, 200 μM IPTG was added and maintained until PE phase. To assess the role of *relA* and *spoT* in the transmissive to replicative phase transition within host cells (Fig. 8A), bacteria were cultured in broth with 200 μM IPTG to PE phase, and 200 μM IPTG was maintained throughout macrophage infection. A similar protocol was used to test the transmissive to replicative transition in bacteriological medium by plating bacteria that had been induced with 200 μM IPTG and cultured into PE phase onto CYET with 200 μM IPTG (Fig. 8B).

Cytotoxicity

To determine contact-dependent cytotoxicity of *L. pneumophila* following macrophage infection, bacteria were added to macrophage monolayers at the indicated moi. After centrifugation at 400 *g* for 10 min at 4°C (Molofsky *et al*., 2005), the cells were incubated for 1 h at 37°C. For quantification of macrophage viability, RPMI/FBS containing 10% alamarBlue™ (Trek Diagnostic Systems) was added to the monolayers for 6–12 h, and the reduction of the colorimetric dye was measured spectrophoretically as described (Molofsky *et al*., 2005). Protocols for culturing WT and mutant microbes varied depending on the experimental question. To determine the effect *relA* and *spoT* induction on cytoxicity of *relA spoT* mutant bacteria (Fig. 5A), microbes were cultured with 200 μM IPTG to PE phase in broth prior to the assay, as described in *Infectivity and intracellular growth*. To determine if expression of WT and mutant *spoT* was sufficient to rescue the cytoxicity defect of *relA spoT* mutant bacteria in which FAB had been perturbed (Fig. 3), overnight cultures in mid-E-phase OD_{600} of 1.0–1.75 were back diluted to early E-phase OD_{600} of 0.45–0.60 and treated with either 25 or 50 μM IPTG for 1 h in the case of the 25 μM induction and 2 h for the 50 μM induction. After incubation on a roller at 37°C, mutant cultures were split and either mock-treated or treated with propionic acid, acetic acid or cerulenin. Cultures were incubated at 37°C for an additional 3 h prior to assaying cytotoxicity.

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