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Co-ordination of *Legionella pneumophila* virulence with entry into stationary phase by ppGpp

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Summary

Legionella pneumophila **survives in aquatic environments, but replicates within amoebae or the alveolar macrophages of immunocompromised individuals. Here, the signal transduction pathway that co-ordinates *L. pneumophila* virulence expression in response to amino acid depletion was investigated. To facilitate kinetic and genetic studies, a phenotypic reporter of virulence was engineered by fusing *flaA* promoter sequences to a gene encoding green fluorescent protein. When subjected to amino acid depletion, *L. pneumophila* accumulated ppGpp and converted from a replicative to a virulent state, as judged by motility and sodium sensitivity. ppGpp appeared to initiate this response, as *L. pneumophila* induced to express the *Escherichia coli* RelA ppGpp synthetase independently of nutrient depletion accumulated ppGpp, exited the exponential growth phase and expressed *flaAgfp*, motility, sodium sensitivity, cytotoxicity and infectivity, five traits correlated with virulence. Although coincident with the stationary phase, *L. pneumophila* virulence expression appeared to require an additional factor: mutant Lp120 accumulated ppGpp and acquired two stationary phase traits but none of six virulence phenotypes analysed. We propose that, when nutrients are limiting, ppGpp acts as an alarmone, triggering the expression of multiple traits that enable *L. pneumophila* to escape its spent host, to survive and disperse in the environment and to re-establish a protected intracellular replication niche.**

Introduction

Legionella pneumophila, a ubiquitous aquatic Gram-negative bacterium, can parasitize amoebae, the presumed natural reservoir for this pathogen (Fields, 1996). When immune-impaired individuals aspirate or inhale aerosolized water contaminated with *L. pneumophila*, the pathogen can invade and replicate within alveolar macrophages (Horwitz and Silverstein, 1980) and cause the severe pneumonia Legionnaires' disease (McDade *et al*., 1977). The aim of this study was to identify the regulatory pathway that enables this microbe to sense and adapt to these distinct extracellular and intracellular environments.

L. pneumophila uses amino acids as the sole source of both carbon and energy (George *et al*., 1980; Hoffman, 1984). Therefore, whether its local environment is favourable for growth could be gauged by monitoring amino acid levels. A previous study showed that, when exponential phase cells are shifted to broth depleted of amino acids, *L. pneumophila* converts to a virulent, post-exponential phase, characterized by motility, osmotic resistance, sodium sensitivity, cytotoxicity and competence to avoid phagosome–lysosome fusion (Byrne and Swanson, 1998). Accordingly, the following model of *L. pneumophila* virulence trait regulation was proposed. Plentiful nutrients and other factors within host cells support bacterial replication. When nutrients become limiting, intracellular bacteria express virulence factors that mediate escape from the spent host, survival and dispersal in the environment and the establishment of a replication niche in a new host cell.

The cellular response to starvation has been studied in detail for a variety of microorganisms. Amino acid depletion of *Escherichia coli* initiates the stringent response, a rapid inhibition of stable RNA, ribosome and protein synthesis, leading to growth arrest (for a review, see Cashel *et al*., 1996). This global response is a consequence of the binding of uncharged tRNAs to ribosomes, which activates the enzyme RelA, a guanosine 3′,5′-bispyrophosphate (ppGpp) synthetase. ppGpp accumulation triggers the adaptive response, including the synthesis and accumulation of the stationary phase sigma factor, σ^s (RpoS) (Gentry *et al*., 1993). Similarly, (p)ppGpp appears to initiate as a response to amino acid depletion fruiting body development by *Myxococcus xanthus* (Harris *et al*., 1998) and antibiotic and pigment production by *Streptomyces coelicolor* (Chakraburtty and Bibb, 1997). Thus, a variety of microbes use ppGpp as an alarmone to co-ordinate an adaptive response to amino acid starvation.

To investigate whether ppGpp controls entry into stationary phase and expression of virulence by *L. pneumophila*, the guanine nucleotide pools of replicating and starved cultures were analysed, and the effects of gratuitous *relA* expression on *L. pneumophila* growth and virulence were

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examined. To begin to define genetically the putative virulence regulon of *L. pneumophila*, the ability of a candidate regulatory mutant, Lp120, to express virulence traits in response to ppGpp was examined. A model is proposed in which ppGpp acts as a second messenger to co-ordinate *L. pneumophila* virulence expression as an adaptive response to amino acid starvation.

Results

Accumulation of ppGpp in L. pneumophila *cultures*

The hypothesis that *L. pneumophila*, like *E. coli*, accumulates ppGpp in a manner analogous to the stringent response to amino acid depletion was tested. After radiolabelling the guanosine nucleotide pools of *L. pneumophila* cultures for 6 h (two doubling times), ppGpp levels were analysed by thin-layer chromatography (Cashel, 1969). As a standard for nucleotide localization, we analysed ppGpp accumulation by *E. coli* carrying a plasmid (pMMBrelA) from which transcription of *E. coli* ppGpp synthetase *relA* could be activated by IPTG (Schreiber *et al*., 1991; Fig. 1A). Similarly, *L. pneumophila* carrying pMMBrelA also accumulated ppGpp in response to the addition of IPTG (Fig. 1A).

Two approaches were taken to test whether *L. pneumophila* accumulated ppGpp in response to amino acid starvation. Consistent with studies of growth control in other bacteria (Cashel, 1969; Harris *et al*., 1998), *L. pneumophila* ppGpp was below the limit of detection during exponential phase, but accumulated in the stationary phase (Fig. 1A). Next, we analysed ppGpp levels in exponential phase *L. pneumophila* cells transferred to broth depleted of amino acids, a treatment that induces virulence expression (Byrne and Swanson, 1998). After 30 min in amino acid-depleted medium, the amount of *L. pneumophila* ppGpp had increased significantly, and it remained elevated for 3 h (Fig. 1B), a period that corresponds to approximately one generation time. Accumulation of pppGpp, the metabolic precursor of ppGpp (Cashel, 1994), was also detected under certain conditions (Fig. 1). Thus, like *E. coli*, *L. pneumophila* accumulated ppGpp in response to amino acid depletion.

To address whether ppGpp might initiate conversion of replicating *L. pneumophila* to the virulent form, the kinetics of ppGpp accumulation and virulence trait expression by *L. pneumophila* in response to amino acid depletion were compared. In particular, we quantified the efficiency of colony formation on medium containing sodium, which decreases ≈1000-fold when virulent *L. pneumophila* enter the stationary phase (Sadosky *et al*., 1993; Vogel *et al*., 1996; Byrne and Swanson, 1998). As expected, *L. pneumophila* cultures maintained in the exponential phase were relatively sodium resistant. However, after 2 h in amino

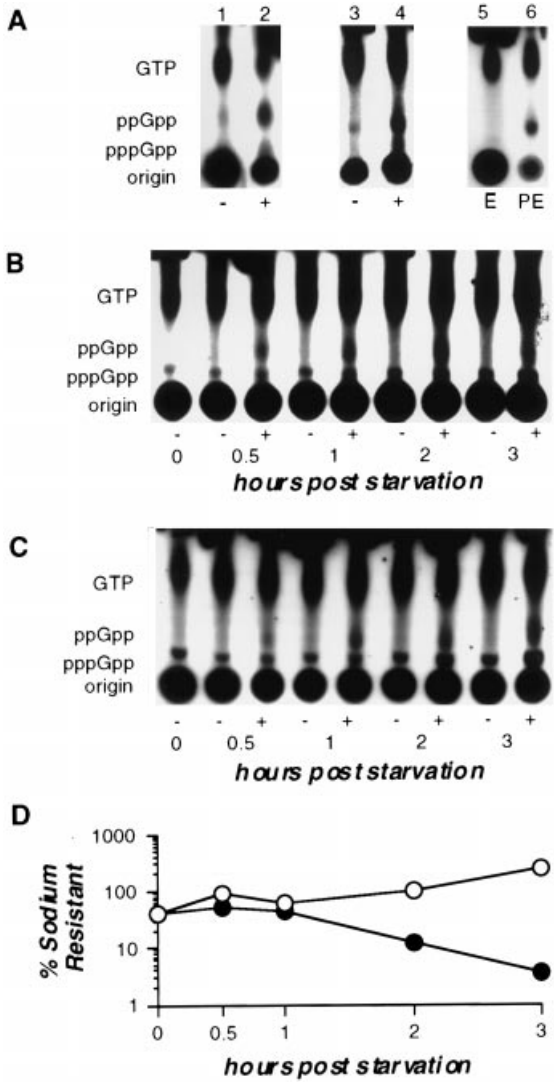

Fig. 1. ppGpp accumulation in *L. pneumophila*. After ^{32}P labelling of the nucleotide pools, cell extracts were prepared and separated by PEI-TLC as described in *Experimental procedures*.
A. Nucleotide pools of *E. coli* strain DH5α/pMMBrelA (lanes 1 and 2) and *L. pneumophila* strain Lp02/pMMBrelA (lanes 3 and 4) incubated during exponential phase without (−) or with (+) IPTG for 1 h and 4.5 h, respectively, and *L. pneumophila* strain Lp02 (lanes 5 and 6) grown to the exponential phase (E) and post-exponential phase (PE).
Wild-type Lp02 (B) and mutant Lp120 (C) exponential phase cells were transferred to either exponential phase supernatant (− starvation) or post-exponential phase Lp02 supernatant (+ starvation) for the period shown. Then, nucleotide pools were analysed. Representative chromatograms from one of two independent experiments are shown for each *L. pneumophila* strain.
D. Sodium sensitivity of non-radioactive wild-type Lp02 cultures prepared in parallel with those analysed in (B) (open circles, − starvation; closed circles, + starvation). The percentage sodium resistant was determined by plating duplicate samples on CYET and on CYET containing 100 mM NaCl. The mean values obtained from one representative experiment of two performed are shown.

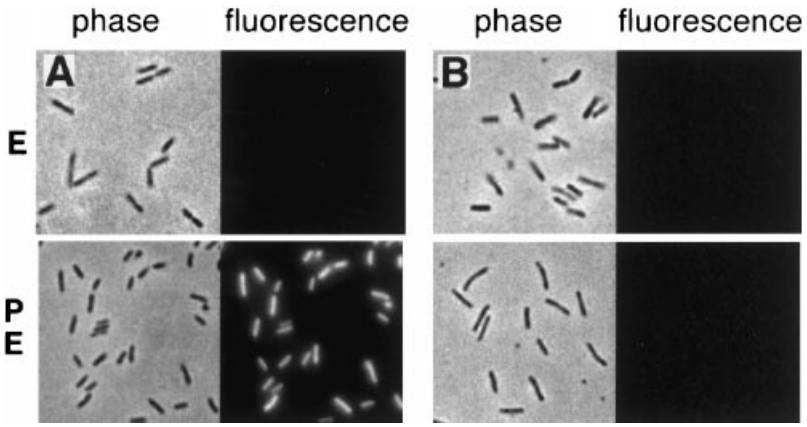

Fig. 2. *flaAgfp* is expressed by post-exponential phase *L. pneumophila*. Wet mounts of exponential phase (E) and post-exponential phase (PE) *L. pneumophila* Lp02/pflaG (A) and Lp120/pflaG (B) broth cultures were examined by phase and fluorescence microscopy. For wild type, cultures of OD_{600} 0.4 (E) and 2.2 (PE) were analysed; for mutant Lp120, cultures of OD_{600} 0.8 (E) and 2.3 (PE) were analysed. An identical exposure time was used for each of the fluorescence micrographs shown. Similar patterns of expression were observed for several hundred cells examined during each of at least three independent experiments.

acid-depleted medium, sodium sensitivity was apparent; after 3 h, it had increased ≈100-fold (Fig. 1D). Likewise, maximal cytotoxicity and motility, two other activities correlated with *L. pneumophila* virulence, occur more than 3 h after exponential phase cells are transferred to spent medium (B. Byrne and M. S. Swanson, unpublished; Byrne and Swanson, 1998). That ppGpp accumulates before expression of *L. pneumophila* virulence traits is consistent with its postulated role as an inducer in this regulatory pathway.

flaAgfp, *a reporter of* L. pneumophila *virulence*

To facilitate more detailed genetic and kinetic studies of *L. pneumophila* virulence regulation, we designed a visual and quantifiable marker of the *L. pneumophila* virulent phenotype. A number of observations suggested that the *L. pneumophila flaA* promoter could be used to construct such a genetic reporter. First, *L. pneumophila* becomes motile as it exits the exponential phase of growth in broth and in cells (Rowbotham, 1986; Byrne and Swanson, 1998). Secondly, flagellar synthesis appears to be regulated co-ordinately with virulence (Pruckler *et al*., 1995; Merriam *et al*., 1997). Thirdly, flagella production is regulated during growth in macrophages (Byrne and Swanson, 1998). Fourthly, transcription of the *L. pneumophila* flagellin gene *flaA* is regulated by temperature (Heuner *et al*., 1997). Reasoning that *flaA* expression was also likely to be regulated by nutrient levels, we constructed a gene fusion in which the *L. pneumophila flaA* promoter controlled the expression of green fluorescent protein (GFP).

Whether expression of the *flaAgfp* reporter was growth phase dependent in broth cultures of *L. pneumophila* was evaluated first. Wild-type strain Lp02 carrying plasmid pflaG expressed *flaA* specifically in the stationary phase as judged by fluorescence microscopy. Exponential phase cells were neither motile nor fluorescent, whereas at least 95% of post-exponential phase cells were motile and intensely fluorescent (Fig. 2A).

To determine the kinetics of *flaA* induction, GFP-dependent fluorescence of Lp02/pflaG broth cultures growing at 37°C was measured by spectrofluorometry. Bacterial fluorescence increased sharply and more than 30-fold as cultures reached the post-exponential phase (Fig. 3A). Similar results were obtained with cultures expressing *flaAgfp* on ColE1 replicon pTLPflaG (data not shown). Interestingly, growth phase regulation of GFP-dependent fluorescence was also observed when cells were cultured at 30°C (data not shown), indicating that the promoter sequences used in this study were not sufficient to confer the temperature-regulated *flaA* expression reported previously (Heuner *et al*., 1997). Thus, *flaAgfp* expression coincided with the conversion of *L. pneumophila* from the replicative to the virulent form.

Next, we tested whether *flaAgfp* was also regulated by *L. pneumophila* growing intracellularly. Macrophages were infected with post-exponential phase Lp02/pflaG at a low multiplicity to ensure that only one bacterium entered each macrophage. Then, expression of *flaAgfp* was assessed by fluorescence microscopy. Macrophages infected for 1 h

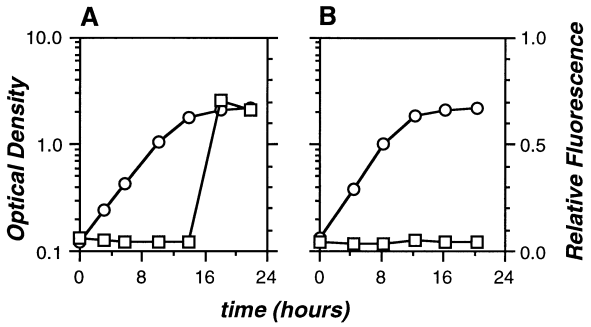

Fig. 3. *flaAgfp* expression is regulated by growth phase in broth cultures of *L. pneumophila*. Lp02/pflaG (A) and Lp120/pflaG (B) cultures were sampled at ≈3 h intervals for spectrophotometric determination of optical density (circles) and spectrofluorometric analysis of fluorescence intensity (squares), as described in *Experimental procedures*. Similar results were obtained in two additional independent experiments.

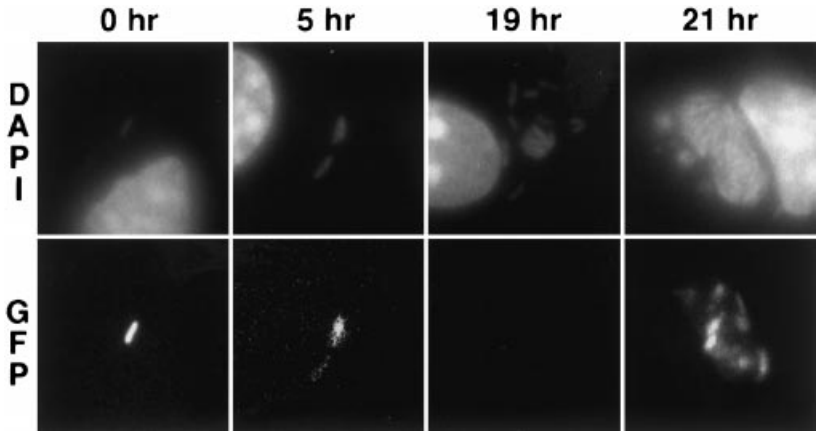

Fig. 4. *flaAgfp* expression by *L. pneumophila* growing intracellularly. Mouse macrophages infected with stationary phase Lp02/pflaG for the times shown were fixed and stained with DAPI to localize macrophage nuclei and intracellular bacteria. GFP-mediated fluorescence was detected immediately after infection (0 h), but diminished during the intracellular replication period (5 and 19 h). *flaAgfp* expression was detected again at the end of the primary infection period when cell lysis began to occur (21 h). Representative results from one of two independent experiments are shown.

typically harboured a single fluorescent bacterium (Fig. 4). After 5 h, bacterial replication was evident, but GFP-mediated fluorescence of the bacteria was markedly diminished (Fig. 4). By 19 h after infection, macrophages contained large numbers of non-fluorescent bacteria (Fig. 4). After 21 h, when macrophage lysis was first apparent by phase microscopy, *flaAgfp* fluorescence was visible within the crowded phagosome (Fig. 4). Likewise, intracellular *L. pneumophila* become sodium sensitive and flagellated during this late stage of infection (Byrne and Swanson, 1998). That *flaAgfp* expression was coincident with the virulent phase of *L. pneumophila* cultured in broth or in macrophages indicates that it is a valid phenotypic marker of the virulent state.

Effects of E. coli relA *induction on exponential phase* L. pneumophila *cultures*

In the virulent, post-exponential phase, *L. pneumophila* contained elevated levels of ppGpp, whereas replicating bacteria did not (Fig. 1). Whether ppGpp can trigger conversion of replicating *L. pneumophila* to the virulent state was examined using a molecular genetic approach previously applied by others (Schreiber *et al*., 1991; Harris *et al*., 1998). To study the effect of ppGpp accumulation independent of nutrient levels or growth state, we used plasmid pMMBrelA from which transcription of *E. coli relA* by *L. pneumophila* could be activated by IPTG (Fig. 1A). After inducing exponential cultures to express ppGpp synthetase, we measured their growth and the expression of four traits previously correlated with virulence: *flaAgfp*-dependent fluorescence, sodium sensitivity, cytotoxicity and infectivity.

To learn whether gratuitous ppGpp synthetase expression triggered a switch to the virulent state, *flaAgfp*-dependent fluorescence of cells induced to express *relA* was analysed. For this purpose, a *L. pneumophila* strain carrying both pMMBrelA and a compatible replicon encoding *flaAgfp* (pTLPflaG) was constructed. As expected, the fluorescence of wild-type cells carrying both plasmids increased upon entry into stationary phase (Fig. 5A). However, exponential phase cells induced to express *relA* also became fluorescent, concomitant with an apparent decrease in the growth rate (Fig. 5A). When wet mounts were observed by phase-contrast and fluorescence microscopy, at least 90% of the cells were fluorescent and 10% were motile (data not shown). Thus, an accumulation of ppGpp appeared to trigger the transition into stationary phase and initiate flagellar biosynthesis.

In response to amino acid depletion, *L. pneumophila* accumulated ppGpp and converted to a sodium-sensitive form (Fig. 1). Therefore, we used a culture of *L. pneumophila* carrying pMMBrelA to investigate whether ppGpp was sufficient to trigger sodium sensitivity independent of culture conditions. Whereas uninduced exponential phase cultures were relatively sodium resistant, cultures

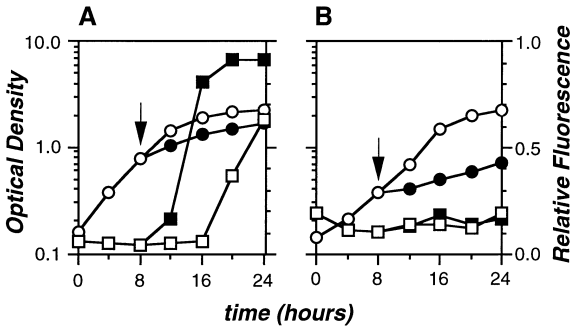

Fig. 5. Growth and GFP-mediated fluorescence of exponential phase *L. pneumophila* induced to express *relA*. Cultures of *L. pneumophila* wild-type Lp02 (A) or mutant Lp120 (B) strains containing both pTLPflaG and pMMBrelA were divided at the time indicated (arrows); one was treated with IPTG to induce *relA* (closed symbols) and the other was not (open symbols). At the times shown, samples were collected for spectrophotometric determination of optical density (circles) and spectrofluorometric determination of relative fluorescence (squares). Similar results were obtained in two other independent experiments.

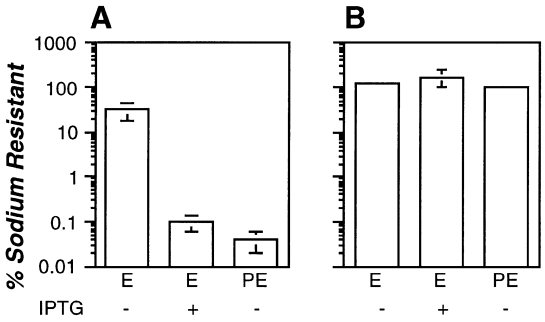

Fig. 6. Sodium sensitivity of exponential phase *L. pneumophila* induced to express *relA*. Sodium sensitivity of cultures of wild-type Lp02 (A) and mutant Lp120 (B) strains containing pMMBrelA grown to the exponential (E) or the post-exponential (PE) phase that either were induced for 4.5 h with IPTG (+) or were not (−) was determined by plating duplicate samples on CYET and CYET supplemented with 100 mM NaCl. The values plotted represent the mean ± standard error determined in three independent experiments.

induced to express *relA* were ≈1000-fold more sensitive to sodium (Fig. 6A), similar to the effect measured for amino acid-starved cultures (Fig. 1D) and post-exponential phase cultures (Fig. 6A and Table 1).

We next examined whether gratuitous RelA activity induced contact-dependent cytotoxicity, a stationary phase activity thought to be important for *L. pneumophila* to establish and/or escape from its replication vacuole within amoebae and macrophages (Husmann and Johnson, 1994; Byrne and Swanson, 1998; Kirby and Isberg, 1998; Kirby *et al*., 1998). To determine whether *relA* induction was sufficient to convert exponential phase *L. pneumophila* to the cytotoxic form, exponential phase cultures of Lp02/pMMBrelA were treated with IPTG for 4.5 h; then, their cytotoxicity was compared with that of two control cultures. As expected, stationary phase cultures killed macrophages efficiently, while exponential phase cultures of Lp02/pMMBrelA did not (Fig. 7A). However, exponential phase cultures induced to express *relA* killed macrophages with an efficiency similar to that of stationary phase cultures (Fig. 7A). Therefore, ppGpp accumulation triggered *L. pneumophila* to express cytotoxicity, a trait correlated with virulence.

Finally, we analysed whether *relA* induction enhanced *L. pneumophila* entry and survival in macrophages. Consistent with results published previously, within 2 h of infection, ≈5% of stationary phase *L. pneumophila* cells carrying pMMBrelA associated with macrophages (Fig. 8), whereas less than 1.0% of exponential phase Lp02/pMMBrelA cells did so (Fig. 8). However, exponential phase Lp02/pMMBrelA cells expressing *relA* entered and survived in macrophages at least as efficiently as stationary phase cells (11%; Fig. 8). Together, the results of this series of experiments indicate that, when *L. pneumophila* is starved of amino acids, ppGpp accumulates and initiates both entry into stationary phase and expression of multiple virulence traits, including motility, sodium sensitivity, cytotoxicity and enhanced infectivity.

Avirulent mutant Lp120 may identify a virulence regulator

Although co-ordinate expression of several *L. pneumophila* virulence traits has been observed in the stationary phase (Byrne and Swanson, 1998), the regulatory proteins that presumably control the timing of virulence expression have not yet been identified. To begin to identify such regulators genetically, we considered the phenotypes of a collection of intracellular growth mutants characterized previously (Swanson and Isberg, 1996a). When mutant Lp120 is cultured within macrophages, the majority of bacteria in the inoculum are degraded in the lysosomes, while a minority replicates as efficiently as wild type (Swanson and Isberg, 1996a). Based on the supposition that the partial loss of function of a virulence activator could cause the observed heterogeneity, mutant Lp120 was chosen for further study.

Reasoning that a mutation in a global regulator would probably have a pleiotropic effect on virulence, we compared mutant Lp120 and wild-type cultures in four assays

Table 1. Comparison of *L. pneumophila* avirulent mutant Lp120 with wild-type Lp02 during exponential and post-exponential growth.

Strain	Growth phase	% Na^+ resistant [a]	% Osmotic resistant [b]	% Heat resistant [c]
Lp02	Exponential	50 ± 14	11 ± 5.8	0.002 ± 0.003
	Post-exponential	0.002 ± 0.0005	62 ± 9.1	20 ± 10
Lp120	Exponential	110 ± 18	10 ± 5	0.0007 ± 0.0006
	Post-exponential	100 ± 30	10 ± 5.2	20 ± 20

a. The percentage sodium resistant was measured as described in Fig. 6. Values represent the mean ± standard error for three experiments performed in duplicate.
b. The percentage osmotic resistant was measured by incubating broth cultures for 1 h in AYET that contained 0.3 M KCl (AYET + KCl) or did not, diluting in water or AYET + KCl, then plating duplicate samples on CYET to quantify cfu. The values represent the mean ± standard error for three experiments performed in duplicate.
c. Percentage heat resistant was determined by diluting broth cultures in H_2O to $\approx 1 \times 10^7$ cfu, incubation at room temperature or 57°C for 20 min, then plating on CYET to quantify cfu. The values represent the mean ± standard error for three experiments performed in duplicate.

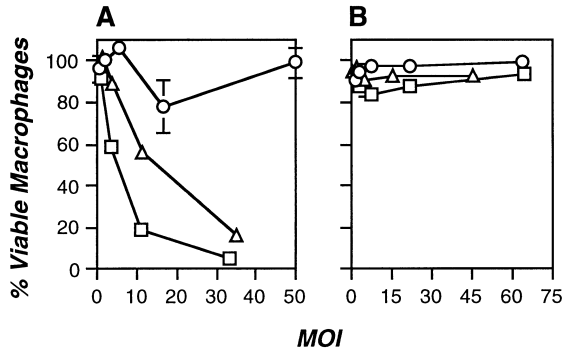

Fig. 7. Cytotoxicity to macrophages of exponential phase *L. pneumophila* induced to express *relA*. Broth cultures of Lp02/pMMBrelA (A) and Lp120/pMMBrelA (B) grown to the exponential phase and treated for 4.5 h with IPTG (triangles) or not (circles) or to the stationary phase (squares) were added to triplicate wells of macrophage cultures at the multiplicities of infection (MOIs) shown. After a 1 h incubation, cytotoxicity was measured as the ability of viable macrophages to reduce the colorimetric dye Alamar blue as described in *Experimental procedures*. The values plotted represent the mean ± standard error for triplicate samples determined in one of three similar experiments.

of virulence. First, although virulent *L. pneumophila* converts from a sodium-resistant to a sodium-sensitive form upon exiting exponential phase, mutant Lp120 remained sodium resistant in all stages of growth (Table 1). Secondly, unlike stationary phase cultures of wild-type *L. pneumophila*, broth cultures of Lp120 did not exhibit contact-dependent cytotoxicity at any growth phase or bacterial density measured (Fig. 7B, data not shown). Finally, post-exponential phase cultures of Lp120/pflaG did not induce *flaAgfp* expression, as judged by microscopic examination (Fig. 2B) and spectrofluorometry (Fig. 3B), nor did cells become motile (data not shown). Because it was completely defective for the expression of all four virulence-associated phenotypes assessed, including cytotoxicity, sodium sensitivity, motility and expression of *flaAgfp*, and is partially defective for growth in macrophages, evasion of phagosome–lysosome fusion and association with the endoplasmic reticulum (Swanson and Isberg, 1996a,b), mutant Lp120 is likely to harbour a mutation affecting a global activator of *L. pneumophila* virulence.

Because the expression of *L. pneumophila* virulence is co-ordinately regulated with entry into stationary phase, a common signal transduction pathway may control both. To investigate whether the putative Lp120 activator was also required for the expression of stationary phase phenotypes, we compared the expression by wild-type and mutant Lp120 cells of two hallmark stationary phase traits described for *E. coli*: resistance to osmotic shock (McCann *et al*., 1991) and heat shock (Jenkins *et al*., 1990).

Unlike wild-type *E. coli* and *L. pneumophila*, which become resistant to osmotic shock upon entering post-exponential phase (McCann *et al*., 1991; Byrne and Swanson, 1998), mutant Lp120 was osmotically sensitive during both exponential and post-exponential phases (Table 1). Like *E. coli*, wild-type *L. pneumophila* acquired heat resistance in the stationary phase: after a 20 min incubation at 57°C, less than 0.01% of exponential phase cells were recovered, whereas 20% of post-exponential cells survived the heat shock (Table 1). Interestingly, in this assay, mutant Lp120 resembled wild-type Lp02: cells were heat sensitive in the exponential phase, but heat resistant in the post-exponential phase (Table 1). The phenotype of mutant Lp120 predicts that *L. pneumophila* encodes a factor that co-ordinately regulates a number of virulence genes, but is dispensable for ppGpp-dependent growth arrest and the expression of at least one stationary phase trait, heat resistance.

Gratuitous relA *expression does not restore virulence gene expression by mutant Lp120*

Its pleiotropic phenotype suggested that mutant Lp120 either fails to synthesize ppGpp or lacks a regulatory factor that responds to ppGpp accumulation. To investigate where in the signal transduction pathway the Lp120 gene product acts, we subjected exponential phase cultures of the mutant to amino acid depletion, then analysed ppGpp synthesis. Similar to wild type, mutant cells accumulated ppGpp within 0.5 h of transfer to supernatant obtained from wild-type stationary phase cultures, and the levels remained elevated for at least 3 h (Fig. 1C). Therefore, mutant Lp120 appeared to be competent to respond to amino acid depletion by synthesizing ppGpp.

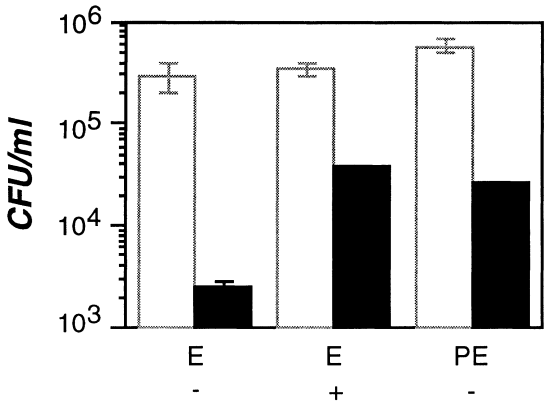

Fig. 8. Infection efficiency of exponential phase *L. pneumophila* induced to express *relA*. Wild-type strain Lp02 containing pMMBrelA was grown to the post-exponential phase (PE) or to the exponential phase (E) and treated with IPTG for 4.5 h (+) or not (−) before infection of macrophages. The number of cfu added to macrophage monolayers at 0 h (white bars) and the number associated with monolayers after a 2 h infection period (black bars) was determined by plating onto CYE triplicate samples of infection medium and macrophage lysates respectively. The mean cfu ± standard error determined in one of two similar experiments are shown.

As an independent approach to place the putative Lp120 activator in the virulence regulation pathway, we investigated whether gratuitous *relA* expression suppressed the virulence defects of the mutant. In particular, we examined the effect of gratuitous *relA* expression on mutant Lp120 growth and three virulence-associated traits: *flaAgfp* expression, sodium sensitivity and cytotoxicity. Lp120/pMMBrelA, like Lp02/pMMBrelA, responded to *relA* induction by reducing its growth rate (Fig. 5B). Therefore, the Lp120 mutation did not interfere with the accumulation of ppGpp or the subsequent growth response. In contrast to its effects on wild-type *L. pneumophila*, replicating mutant cells induced to express *relA* did not convert to the virulent form. Broth cultures of mutant Lp120 expressing the *E. coli relA* gene were amotile and did not express *flaAgfp* (Fig. 5B), sodium sensitivity (Fig. 6B) or cytotoxicity (Fig. 7B). Together, these data support the hypothesis that mutant Lp120 lacks a factor that co-ordinates *L. pneumophila* virulence expression in response to ppGpp, the intracellular signal of amino acid starvation.

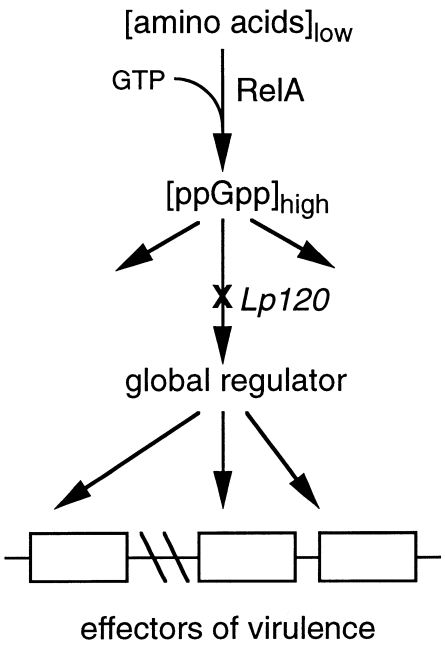

Fig. 9. Model for co-ordination of *L. pneumophila* virulence by ppGpp as described in the text. The 'effectors of virulence' refer to the class of genes presumably required for traits correlated with virulence, including motility, sodium sensitivity, cytotoxicity and evasion of phagosome–lysosome fusion.

Discussion

In its natural habitat, the opportunistic pathogen *L. pneumophila* survives in fresh water but replicates within amoebae. Its ability to sense and respond to these alternative conditions is presumably critical for survival and must require co-ordinate regulation of particular traits required in each environment. In broth cultures, *L. pneumophila* responds to amino acid depletion by switching from a replicative to a virulent form, a state characterized by cytotoxicity, osmotic resistance, motility, sodium sensitivity and the capacity to evade lysosomal degradation (Byrne and Swanson, 1998). This report demonstrates that, in response to amino acid depletion, *L. pneumophila* accumulated ppGpp. Furthermore, gratuitous expression of the RelA ppGpp synthetase induced ppGpp accumulation and triggered *L. pneumophila* virulence expression, independent of nutrient levels or cell density. Finally, in response to amino acid depletion, mutant Lp120 produced ppGpp, exited the exponential phase and became heat resistant, yet failed to convert to the virulent phenotype. Accordingly, we propose that ppGpp acts as an alarmone, which requires the Lp120 factor to co-ordinate expression of *L. pneumophila* virulence traits as an adaptive response to amino acid starvation (Fig. 9). Virulent progeny can then efficiently escape from the spent host, survive and disperse in the aquatic environment and re-establish a protected intracellular replication niche.

ppGpp has been shown to initiate in a variety of environmental microbes developmental programs that are critical for surviving periods of starvation (Chakraburtty and Bibb, 1997; Wendrich and Marahiel, 1997; Harris *et al*., 1998). In response to amino acid depletion, RelA synthesis of ppGpp by *Myxococcus xanthus* initiates the formation of a multicellular fruiting body, which subsequently differentiates into environmentally resistant myxospores (Harris *et al*., 1998). ppGpp accumulation by *Streptomyces coelicolor* plays a role in the production of antibiotic and the pigmentation characteristic of mature spores (Chakraburtty and Bibb, 1997). When the soil bacterium *Bacillus subtilis* is starved of amino acids, ppGpp accumulates and activates the expression of stress response proteins that may contribute to sporulation (Wendrich and Marahiel, 1997). Similarly, the aquatic bacterium *L. pneumophila* appears to use ppGpp to trigger the expression of virulence traits, which may facilitate its escape from a nutrient-poor environment and subsequent transmission to a new host.

In *E. coli*, accumulation of ppGpp alters cell physiology dramatically. In addition to a rapid block in the synthesis of proteins and stable RNAs, known as the stringent response, effects of ppGpp on metabolism, DNA replication, cell cycle control and accumulation of stationary phase sigma factor σ^S (RpoS) have been described (for a review, see Cashel *et al*., 1996). However, the molecular mechanism by which ppGpp arrests bacterial growth and induces stationary phase processes is not completely understood. Indirect evidence indicates that inhibition of RNA synthesis during the stringent response may result from direct binding of ppGpp to RNA polymerase (Reddy *et al*., 1995). Numerous studies have described the effects of ppGpp on transcription at

either the level of initiation (Baracchini and Bremer, 1988), pausing (Hernandez and Bremer, 1993) or readthrough (Faxén and Isaksson, 1994), as well as on translation (Svitil *et al*., 1993), underscoring the complexity and lack of consensus regarding the mechanism of ppGpp-mediated growth arrest.

E. coli strains that lack RelA and SpoT, two ppGpp synthetases, have a pleiotropic phenotype similar to that of *rpoS* mutants, suggesting that much of the stringent response is a consequence of RpoS induction by ppGpp (Cashel *et al*., 1996). By analogy with *E. coli*, RpoS may activate expression of *L. pneumophila* virulence genes in the stationary phase. Indeed, RpoS is required for maximal virulence of several pathogens, including Salmonella species (Fang *et al*., 1992), *Shigella flexneri* (Small *et al*., 1994), toxigenic *E. coli* (Waterman and Small, 1996) and the plant pathogen *Erwinia carotovora* (Mukherjee *et al*., 1998). However, it has been reported that an *L. pneumophila rpoS* mutant strain resembles wild type in its capacity to a kill a macrophage cell line (L. M. Hales and H. A. Shuman, unpublished). Whether RpoS is required for expression of the panel of virulence traits examined here remains to be determined.

Based on its pleiotropic phenotype and its ability to synthesize ppGpp, we predict that the Lp120 mutation will identify an activator of the *L. pneumophila* virulence regulatory pathway (Fig. 9). Whether Lp120 lacks an RpoS activity has not been tested directly. However, like *E. coli rpoS* mutants, stationary phase Lp120 cells remain elongated (Fig. 2B) and sensitive to osmotic shock (Table 1), and they lose viability when subjected to prolonged incubation in nutrient-limiting conditions (B. K. Hammer and M. S. Swanson, unpublished; Lange and Hengge-Aronis, 1991a,b; McCann *et al*., 1991). On the other hand, mutant Lp120 cells acquire heat resistance in the post-exponential phase (Table 1), a trait that is RpoS dependent in *E. coli* (Lange and Hengge-Aronis, 1991b). Interpretation of the phenotype of mutant Lp120 is complicated: because this strain was generated by chemical mutagenesis, it may carry a partial loss-of-function allele or multiple mutations. Therefore, isolation and characterization of the respective null mutant strain is required to define the role of the Lp120 gene product in the co-ordinate expression of virulence and stationary traits by *L. pneumophila*.

We have presented evidence that an extracellular signal, amino acid starvation (Byrne and Swanson, 1998), stimulates the production of an intracellular second messenger, ppGpp, which co-ordinates the expression of traits critical for *L. pneumophila* virulence. It remains possible that additional environmental signals act through this or another signal transduction pathway to regulate *L. pneumophila* virulence expression. For example, environmental regulation of *S. typhimurium* virulence requires numerous transcriptional regulators (Guiney *et al*., 1995; Slauch *et al*., 1997). Nonetheless, the stringent response paradigm will probably facilitate identification of the presumptive transcriptional regulators and their target effector genes, which together form the *L. pneumophila* virulence regulon.

Experimental procedures

Bacterial strains and culture conditions

L. pneumophila strain Lp02, a virulent thymine auxotroph (Berger and Isberg, 1993), and Lp120, an intracellular growth mutant isolated by chemical mutagenesis of Lp02 (Swanson and Isberg, 1996a), were grown in *N*-(2-acetamino)-2-aminoethanesulphonic acid (ACES; Sigma)-buffered yeast extract broth supplemented when necessary with 100 μg ml^{-1} thymidine (AYET) at 37°C with agitation. Bacteria were plated on ACES-buffered charcoal yeast extract agar supplemented with 100 μg ml^{-1} thymidine (CYET) at 37°C. Where indicated, chloramphenicol was added to a final concentration of 5 μg ml^{-1} and gentamicin to 10 μg ml^{-1}.

Macrophage cultures

Bone marrow-derived macrophages were prepared from female A/J mice (Jackson Laboratory) as described previously (Swanson and Isberg, 1995). After a 7 day culture period in L-cell conditioned medium, macrophages were collected by centrifugation, suspended in RPMI-1640 containing 10% fetal bovine serum (RPMI-FBS; Gibco BRL), and plated as described below for the analysis of intracellular *flaAgfp* expression and infectivity.

Plasmids

To exploit the observed correlation between motility and expression of virulence traits, a genetic reporter was constructed in which transcription of a gene encoding green fluorescent protein (GFP) was controlled by the *L. pneumophila flaA* promoter. Plasmid pflaG was generated by subcloning the 0.8 kb *Xba*I/*Pst*I fragment encoding GFP and the phage T7 *gene 10* ribosome binding site obtained from plasmid pGFPmut3 (Cormack *et al*., 1996) into the multiple cloning site (MCS) of the IncQ plasmid pJB98, which encodes thymidylate synthetase as a selectable marker. Plasmid pJB98 was generated from pKB5 (Berger and Isberg, 1993) by deletion of an ≈1.5 kb *Hpa*I/*Eco*RI fragment containing the P_{tac} promoter and the *lacIq* gene (J. P. Vogel and R. R. Isberg, unpublished). The *flaA* promoter sequences, including its −35, −10 and transcriptional start sites, were amplified from wild-type Lp02 chromosomal DNA by the polymerase chain reaction (PCR). The forward primer was 5′-AAA AAG GAT CCT TTG CGA CTT TTC AAA AAA GC-3′, which introduced a *Bam*HI restriction site, and the reverse primer was 5′-AAA AAT CTA GAC CTG AAT CCC TGG TAT GGG AC-3′, which introduced an *Xba*I restriction site. The *Bam*HI/*Xba*I flagellar promoter product (P_{flaA}) generated from Lp02 chromosomal DNA was ligated to the *Bam*HI and *Xba*I sites located directly upstream of the *gfp* gene (Heuner *et al*., 1997). The plasmid pTLPflaG was constructed by subcloning an ≈1 kb *Bam*HI/*Sph*I *flaAgfp*

fragment from pflaG into the ≈4.6 kb *Bam*HI/*Sph*I fragment of the ColE1 replicon pTLP6 (McClain *et al*., 1996), which encodes chloramphenicol resistance as a selectable marker.

To analyse the effects of expression of the *E. coli relA* gene in *E. coli* and *L. pneumophila*, plasmid pMMBrelA was constructed by subcloning the *Eco*RI/PstI *relA* fragment of pALS13 (Svitil *et al*., 1993), which encodes a truncated, metabolically active form of RelA (Schreiber *et al*., 1991), into the MCS of the broad-host-range IncQ vector pMMB67EH (Frey *et al*., 1983). The location of the MCS directly downstream of the P_{tac} promoter and the presence of *lacIq* on pMMB67EH enabled IPTG induction of the *E. coli relA* gene. Preliminary experiments determined that 200 μM IPTG was sufficient for maximum growth arrest (data not shown). To permit selection in *L. pneumophila*, an *Eco*RI fragment encoding gentamicin resistance was treated with Klenow enzyme, then ligated to the unique *Dra*I site of pMMB67EH.

Detection of ^{32}P-labelled ppGpp in L. pneumophila

Analysis of ppGpp accumulation by thin-layer chromatography was performed using an established protocol (Cashel, 1969). To provide a standard for ppGpp localization, we analysed the nucleotide pools of *E. coli* strain DH5α carrying pMMBrelA, which were or were not induced with 200 μM IPTG for 1 h, as described previously (Schreiber *et al*., 1991). To ensure uniform labelling of nucleotide pools, *L. pneumophila* wild-type Lp02 and mutant Lp120 cultures grown in AYET were labelled with carrier-free [^{32}P]-phosphoric acid at 100 μCi ml^{-1} (ICN Pharmaceuticals) for ≈6 h (two generation times) before sampling.

To analyse the effect of *relA* induction on ppGpp accumulation in *L. pneumophila*, aliquots were removed after incubation of exponential phase Lp02/pMMBrelA (OD_{600} ≈0.2) cultures without or with 200 μM IPTG for 4.5 h. For experiments determining the growth phase-dependent accumulation of ppGpp, analysis was performed with aliquots collected from wild-type *L. pneumophila* strain Lp02 grown to the exponential phase (OD_{600} ≈0.4) and stationary phase (OD_{600} ≈2.1). To subject *L. pneumophila* to amino acid depletion, aliquots of *L. pneumophila* wild-type Lp02 and mutant Lp120 cultures grown to exponential phase (OD_{600} ≈0.4) were collected by centrifugation and resuspended with supernatants obtained by filter sterilization of either corresponding exponential phase non-radioactive cultures (OD_{600} ≈0.4) or post-exponential phase Lp02 supernatant (OD_{600} = 2.1–2.3). Aliquots were removed for analysis before dividing the culture and at designated times after starvation. The starvation protocol was essentially as described previously (Byrne and Swanson, 1998), except the supernatants were supplemented with thymidine (100 μg ml^{-1}), cysteine (400 μg ml^{-1}) and ferric nitrate (135 μg ml^{-1}), and ^{32}P (100 μCi ml^{-1}) was added to labelled culture supernatants.

Formic acid extracts of each 50 μl culture aliquot were prepared, applied to a PEI-cellulose thin-layer chromatography (TLC) sheet (20×20 cm) and developed with 1.5 M KH_2PO_4 (pH 3.4) as described previously (Cashel, 1969; Cashel, 1994). Equal volumes (40 μl) of extracts were chromatographed in each experiment, except for the volume of post-exponential phase extract in the growth phase experiment, which was reduced to compensate for the calculated increase in cfu ml^{-1} with growth phase. Autoradiographs of each chromatogram were converted to digital images with a Hewlett Packer ScanJet IIcx scanner, and the image quality was optimized using Adobe Photoshop software (Adobe Systems). Optical density and cfu ml^{-1} were measured for non-radioactive cultures grown under identical conditions.

Osmotic sensitivity

Osmotic sensitivity was measured by diluting aliquots of exponential and stationary phase cultures to ≈1×10^7 cfu ml^{-1} AYET that did not or did contain 0.3 M KCl (AYET + KCl). After incubation for 1 h at 37°C with agitation, the cultures were diluted with either H_2O or AYET + KCl, then plated onto CYET. The percentage of bacteria that were osmotically resistant was determined for samples incubated in AYET + KCl according to the following formula: (cfu ml^{-1} 'H_2O-diluted' samples)/(cfu ml^{-1} 'AYET + KCl-diluted' samples)×100. As expected, the plating efficiencies of control broth samples treated only with H_2O or only with AYET + KCl were identical.

Heat resistance

Heat resistance was quantified by diluting cultures to ≈1×10^7 cfu ml^{-1} H_2O, transferring a 300 μl aliquot of each sample to a 1.5 ml culture tube, then incubating for 20 min at room temperature or in a 57°C water bath. Next, cultures were diluted in H_2O and plated on CYET to enumerate surviving cfu. The percentage of bacteria that were heat resistant was determined by the following calculation: (cfu ml^{-1} heated sample)/(cfu ml^{-1} unheated sample)×100.

Sodium sensitivity

The sodium sensitivity of wild-type Lp02 and mutant Lp120 broth cultures was quantified by diluting aliquots in H_2O and plating on CYET containing or lacking 100 mM NaCl. The percentage of bacteria that were sodium resistant was determined by the following calculation: (cfu ml^{-1} on CYET + 100 mM NaCl)/(cfu ml^{-1} on CYET)×100 as described previously (Byrne and Swanson, 1998).

Cytotoxicity

Cytotoxicity of wild-type Lp02 and mutant Lp120 carrying plasmid pMMBrelA was determined essentially as described previously (Byrne and Swanson, 1998). Exponential phase cultures at OD_{600} < 1.0 were divided into two samples: one sample was induced with 200 μM IPTG and the other was not. After an additional 4.5 h incubation, both exponential phase cultures and an untreated stationary phase culture were each resuspended in RPMI-FBS and added at the indicated ratios to macrophage monolayers in 96-well tissue culture plates. The multiplicity of infection (MOI) was calculated by plating the broth cultures on CYET in duplicate. After incubation at 37°C for 1 h, extracellular bacteria were washed away, and the macrophages were incubated with RPMI-FBS containing 10% Alamar blue (Acumed) for ≈4 h to allow viable cells to reduce the colorimetric dye. To generate a standard curve, twofold dilutions of uninfected macrophages cultures in the range of ≈5×10^4 (100%) to 1.6×10^3 (3.1%) per well

were included in each assay. The absorbance of samples was determined at 570 nm and 600 nm using a Microtek plate reader. The percentage of macrophages that were viable was calculated from the average A570/600 of duplicate or triplicate samples, and the slope of the plot of A570/600 versus uninfected macrophages was obtained from the standard curve.

Infectivity

The infectivity assay was performed essentially as described previously (Byrne and Swanson, 1998). Because strains carrying plasmid pMMBrelA were gentamicin resistant, extracellular bacteria were removed at 2 h by washing monolayers three times with RPMI rather than by gentamicin treatment. Colony-forming units added at time 0 h and intracellular cfu at time 2 h were calculated from plating triplicate aliquots on CYET.

flaAgfp *expression in broth cultures and in infected macrophages*

Expression of *flaAgfp* by *L. pneumophila* broth cultures was analysed by phase-contrast and fluorescence microscopy. Wet mounts of exponential and post-exponential phase cultures were photographed with Kodak T-Max ASA 400 film. Negatives were converted to digital images with an Archiboldi Leafscan-45 film scanner, and the image quality was optimized using Adobe Photoshop software (Adobe Systems).

Expression of *flaAgfp* by intracellular *L. pneumophila* was analysed by fluorescence microscopy. Macrophages cultured on 12 mm glass coverslips were infected at an MOI of ≈1 for 1 h, washed to remove the majority of extracellular bacteria and then incubated in fresh medium. At that time (0 h), and after specific time periods (5, 19 and 21 h), a set of coverslips was fixed with periodate–lysine–paraformaldehyde (McLean and Nakane, 1974) prewarmed to 37°C, washed three times with PBS, methanol extracted and again washed three times with PBS before staining the bacterial DNA fluorescently with 0.1 μg of 4′,6-diamidino-2-phenylindole (DAPI) per ml of PBS. Photographs were processed as above.

Quantification of fluorescence

Expression of *flaAgfp* was quantified by fluorometry for *L. pneumophila* wild-type Lp02 and mutant Lp120 cultured in AYET containing appropriate antibiotics. For experiments measuring the effect of *relA* expression on GFP-mediated fluorescence, 200 μM IPTG was added to one-half of the culture at the indicated time. At the times indicated, one aliquot of each culture was collected to determine optical density. To ensure that similar bacterial concentrations were analysed by fluorometry, one aliquot of cells was then collected by centrifugation and resuspended in water to an OD_{600} of ≈0.10. The maximum signal was established using a stationary phase culture of wild-type Lp02 expressing *flaAgfp*, and all fluorescence values for a given experiment were determined relative to the positive control. Relative fluorescence intensity was measured on a SPF-500C spectrophotometer (SLM Instruments) using an excitation of 488 nm with a bandpass width of 2 nm, and emission of 510 nm with bandpass width of 1 nm.

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