A two-component regulator induces the transmission phenotype of stationary-phase *Legionella pneumophila*

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Summary

Pathogenic *Legionella pneumophila* evolved as a parasite of aquatic amoebae. To persist in the environment, the microbe must be proficient at both replication and transmission. In laboratory cultures, as nutrients become scarce a stringent response-like pathway coordinates exit from the exponential growth phase with induction of traits correlated with virulence, including motility. A screen for mutants that express the flagellin gene poorly identified five activators of virulence: LetA/LetS, a two-component regulator homologous to GacA/GacS of *Pseudomonas* and SirA/BarA of *Salmonella*; the stationary-phase sigma factor RpoS; the flagellar sigma factor FliA; and a new locus, letE. Unlike wild type, post-exponential-phase letA and letS mutants were not motile, cytotoxic, sodium sensitive or proficient at infecting macrophages. *L. pneumophila* also required fliA to become motile, cytotoxic and to infect macrophages efficiently and letE to express sodium sensitivity and maximal motility and cytotoxicity. When induced to express RelA, all of the strains exited the exponential phase, but only wild type converted to the fully virulent form. In contrast, intracellular replication was independent of letA, letS, letE or fliA. Together, the data indicate that, as the nutrient supply wanes, ppGpp triggers a regulatory cascade mediated by LetA/LetS, RpoS, FliA and letE that coordinates differentiation of replicating *L. pneumophila* to a transmissible form.

Introduction

Pathogens colonize hosts and promote their own transmission among the susceptible population. Pressures of economics and host defences favour organisms that elaborate traits tailored to their surroundings (Guiney, 1997). Consequently, during their life cycle, a variety of microbes convert between a vegetative form and one that is non-replicating, infectious and resistant to environmental stress. For example, dormant *Bacillus anthracis* endospores remain infectious for decades, whereas vegetative bacilli replicate profusely in blood but are not transmissible (Hanna and Ireland, 1999). Also extremely resilient and infectious is the non-replicating small cell variant of *Coxiella burnetii*; once ingested by macrophages, the microbe differentiates into a fragile large-cell variant that replicates within lysosomes (Heinzen et al., 1999). The *Leishmania* protozoan parasites also lead a biphasic life: motile promastigotes live in the gut of blood-sucking sandflies; after ingestion by macrophages at the site of an insect bite, the parasites differentiate into an amastigote form that replicates in phagolysosomes (Turco and Descoteaux, 1992). Thus, cellular differentiation enables pathogens to meet demands peculiar to their life cycle.

Bacterial differentiation is often controlled by transcription factors whose activity is regulated by local cues. The *Bordetella bronchiseptica* BvgA/S two-component regulator represses motility and induces adhesins and toxins that promote colonization of the respiratory tract; when this phosphorelay is not active, colonization factors are repressed, motility is induced and the bacteria become resistant to nutrient limitation, phenotypes likely to favour transmission (Cotter and DiRita, 2000). Similarly, the *Vibrio cholerae* ToxR regulon mediates reciprocal expression of toxin and adhesins with motility, an advantage for a pathogen that alternates between intestinal and aquatic sites (Harkey et al., 1994; Gardel and Mekalanos, 1996; Hase and Mekalanos, 1999). Within the gastrointestinal tract, *Salmonella typhimurium* HilA induces expression of SPI-1 invasion factors to promote colonization of the epithelium; once engulfed by macrophages, an active PhoP/Q two-component regulator mediates repression of hilA and SPI-1 invasion genes and induction of SPI-2 factors to allow intracellular survival and replication (Guiney et al., 1995; Cotter and DiRita, 2000). Thus, transcriptional regulators permit pathogens to adapt to their
surroundings by differentiating to forms fit for colonization, or transmission.

*Legionella pneumophila* evolved as a parasite of freshwater protozoa (Fields, 1996). Remarkably, traits that promote amoeba infection also confer virulence in the human lung (reviewed by Swanson and Hammer, 2000). Typically, ~1000 cases of Legionnaires’ disease are reported annually in the United States (Groseclose et al., 2001), yet person-to-person transmission has not been observed. Therefore, *L. pneumophila* virulence is determined by selective pressures in the environment, where survival as a planktonic cell, colonization of amoebae and transmission to a new replication niche are paramount.

Rowbotham’s pioneering microscopic studies (Rowbotham, 1980) together with recent experimental research indicate that *L. pneumophila* converts from a replicative to a transmissible form when nutrients are limiting (Swanson and Hammer, 2000). By a pathway analogous to the *E. coli* stringent response, amino acid depletion stimulates production of ppGpp, a second messenger that coordinates entry into the stationary phase with induction of traits thought to promote transmission (Byrne and Swanson, 1998; Hammer and Swanson, 1999; Bachman and Swanson, 2001). According to this model, as intracellular conditions deteriorate, progeny express a cytotoxin to escape the host, osmotic resistance to tolerate fresh water, motility to disperse and express a cytotoxin to escape the host, osmotic resistance to tolerate fresh water, motility to disperse and therefore, as judged by failure of the back-crossed insertion alleles to confer a mutant phenotype. Surprisingly, 26 of these mariner insertions mapped to a homologue of the *E. coli* periplasmic stress response regulator CpxR (32% identical, 51% similar; Danese et al., 1995). However, any relationship between CpxRA and virulence expression appeared complex and indirect: typically, the Kan\(^R\) locus was in the same transcriptional orientation as cpxR; no mutations mapped to the 3’ cpxA sensor kinase homologue (30% identity, 49% similarity); although at least 11 different insertion alleles were isolated (data not shown), all of the cpxR mutants originated from one pool; the phenotype of the original isolates was not stable; and neither back-crossed alleles nor a cpxRA null mutation affected virulence (data not shown). Presumably, when combined with a second-site mutation and/or under particular growth conditions, mariner insertions in cpxR reduce *L. pneumophila* virulence.

To gain insight to the mechanism of *L. pneumophila* virulence expression, the loci identified by the remaining 37 pleiotropic mutants were analysed (Experimental procedures). Identified were four transcription factors that induce the transmission phenotype of PE *L. pneumophila*: the stationary-phase sigma factor RpoS (\(\sigma^V\)), the flagellar sigma factor Flia (\(\sigma^{28}\)) and an ancient two-component regulator known as GacA/GacS of *Pseudomonas*, SirA/BarA of *Salmonella* and UvrY/BarA of *E. coli*. A fifth locus that enhanced the expression of transmission traits is postulated to encode a regulatory RNA. Molecular and phenotypic analysis of these five loci is described below. The location of nine other insertion mutations was not determined.

RpoS, RelA and SpoT

We have postulated that ppGpp positively activates RpoS to promote transmission of stationary-phase *L. pneumophila* (Bachman and Swanson, 2001). Therefore, we screened the pale *flaAgfp* mutants for *rpoS*, *relA* and

spoT insertion mutations. The *L. pneumophila* rpoS sequence has been described (Hales and Shuman, 1999); two genes predicted to encode ppGpp synthetases were identified in the *L. pneumophila* genome database as homologous to *E. coli* RelA (44% identity, 63% similarity) and SpoT (53% identity, 71% similarity). After amplification, the sizes of the rpoS, relA and spoT loci of the mutants were compared with the wild type. As expected for genes of redundant function (Cashel et al., 1996), relA and spoT were intact in all of the mutants. As predicted by the phenotype of an *L. pneumophila* rpoS deletion mutant (Bachman and Swanson, 2001), one isolate harboured an insertion in rpoS that conferred sodium resistance and poor flaAgfp expression, motility, and intracellular growth, whereas cytotoxicity was similar to WT (Figs 1C and 2A and data not shown).

FliA

*Legionella pneumophila* flagellum expression correlates with efficient host cell infection (Pruckler et al., 1995; Byrne and Swanson, 1998; Hammer and Swanson, 1999; Dietrich et al., 2001). Therefore, we investigated by a similar approach whether any mutations mapped to fliA, which encodes the flagellar sigma factor that activates flaA (Heuner et al., 1997). One mutant carried an insertion at a position corresponding to codon 201 of the 238 residue FliA protein (Fig. 2B). Upstream of fliA are additional flagellar genes; immediately downstream is a putative operon predicted to encode homologues of MotA of *Rhodobacter sphaeroides* (32% identity, 32% similarity; Shah and Sockett, 1995) and MotB of *Bacillus subtilis* (33% identity, 49% similarity; Mirel et al., 1992). As MotA and MotB regulate flagellar rotation (Yamaguchi et al., 1986), polar effects of the fliA insertion are not likely to be manifested in mutants that do not express flaA, the gene for the major flagellar subunit (Heuner et al., 1995; Fig. 1D). Consistent with the phenotype of *L. pneumophila* flaA mutants (Dietrich et al., 2001), the fliA mutant was non-motile and it infected macrophages poorly, yet its sodium sensitivity and intracellular replication resembled PE wild-type cells (Table 2, Fig. 3). Unlike wild type, the fliA mutant was not cytotoxic (Fig. 3A).
To gain insight into \textit{L. pneumophila} virulence regulation, we sought to identify the loci affected in the 35 remaining pleiotropic mutants. In broth, the mutants grew similarly to wild type (data not shown). Southern hybridization assays demonstrated that the majority (62\%) contained one transposon, but 19\% had two and 16\% had more than two. The nucleotide sequence disrupted in representative single mutants was determined and compared with the \textit{L. pneumophila} genome database. To determine whether other mutants belonged to the same linkage group, the genomic locus was amplified from each of the remaining candidates and sized. To map each insertion, the polymerase chain reaction (PCR) products obtained using a fixed chromosomal primer and either of two transposon primers were sized. Finally, to verify linkage of a transposon to the mutant phenotypes, representative alleles were back-crossed via natural transformation before quantifying their effects on virulence.


\begin{figure}[h]
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\includegraphics[width=\textwidth]{fig3}
\caption{\textit{Legionella} mutants do not express transmission phenotypes, but do replicate in macrophages. PE cultures of \textit{fliA-35} (MB410), \textit{letA-22} (MB414), \textit{letS-36} (MB417) and \textit{letE-121} (MB420) mutants were compared with exponential-phase and PE cultures of wild-type Lp02 in four assays of virulence.}
\end{figure}

A. Contact-dependent cytotoxicity requires \textit{fliA}, \textit{letA} and \textit{letS}. Macrophage viability was assessed after a 1 h incubation with bacteria at a range of MOI. Shown are means ± standard error (SE) for duplicate samples from a representative experiment of the 2–6 performed. The trends depicted were consistent with results obtained when macrophages were infected with more bacteria. In the experiment shown, macrophage viability was 107\% for exponential-phase wild-type cells at a MOI of 86; 121\% for PE \textit{fliA} at a MOI of 156; 81\% for PE \textit{letA} at a MOI of 190; 83\% for PE \textit{letS} at a MOI of 40; and 14\% for PE \textit{letE} at a MOI of 220.

B. Sodium sensitivity requires \textit{letA}, \textit{letE} and \textit{letS}. The percentage of colony-forming units (CFU) that were sodium resistant was determined by plating duplicate aliquots of broth cultures onto CYE lacking or containing 100 mM NaCl. Shown are the means ± SE determined for 4–8 samples from six experiments.

C. Efficient macrophage infection requires \textit{fliA}, \textit{letA} and \textit{letS}. Infectivity, a measure of bacterial binding, entry and survival in macrophages, was calculated as: CFU associated with monolayers at 2 h/CFU added to macrophages at 0 h × 100. Shown are the means for 2–4 duplicate samples from four experiments.

D. Intracellular replication is independent of \textit{fliA}, \textit{letA}, \textit{letS} and \textit{letE}. Macrophages were infected at an MOI of 0.3 ± 0.2, then, at the times shown, the number of viable bacteria per well was determined. Shown are mean CFU ± SE from duplicate samples. Similar results were obtained in at least one other experiment. Note that the 0 and 2 h data are displayed in C as ‘% Infectious’.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig3}
\caption{Fig. 3. Legionella mutants do not express transmission phenotypes, but do replicate in macrophages. PE cultures of \textit{fliA-35} (MB410), \textit{letA-22} (MB414), \textit{letS-36} (MB417) and \textit{letE-121} (MB420) mutants were compared with exponential-phase and PE cultures of wild-type Lp02 in four assays of virulence.}
\end{figure}
LetA and LetS

The mariner insertions of 17 mutants mapped to a gene predicted to encode a 220 residue homologue of GacA, a two-component response regulator of *P. aeruginosa* (55% identity, 72% similarity; Reimmann *et al.*, 1997; Fig. 2C, data not shown). Two additional conserved features substantiated its designation as a gacA homologue: a predicted TTG start codon (Moolenaar *et al.*, 1987) and its 3' linkage to a homologue of *P. fluorescens* UvrC (47% identity, 64% similarity; Laville *et al.*, 1992; Fig. 2C). In many pathogens, GacA activity is coupled to a sensor kinase, GacS; together the proteins regulate motility and other virulence traits, often in the stationary phase (Willis *et al.*, 1990; Laville *et al.*, 1992; Eriksson *et al.*, 1998; Wong *et al.*, 1998; Kinscherf and Willis, 1999; Tan *et al.*, 1999; Brinkman *et al.*, 2001; Goodier and Ahmer, 2001).

As gacA and gacS homologues of Gram-negative bacteria are not genetically linked, we searched the *L. pneumophila* database for homologues of *Pseudomonas fluorescens* GacS (GenBank accession no. AAG13658.1). An open reading frame (ORF) was identified that is predicted to encode a 910 residue protein homologous to *P. syringae* GacS (34% identity, 56% similarity; Hrabak and Willis, 1992), including the conserved His-Asp-His phosphotransfer domains characteristic of the subfamily of tripartite sensor kinases to which GacS and BvgS belong (Cotter and DiRita, 2000). As predicted by the isolation of 17 gacA alleles, five independent insertions mapped to the gacS homologue (Fig. 2D). Based on their molecular features and their mutant phenotypes, described below, we designate the gacA homologue as letA and the gacS homologue as letS, for *Legionella* transmission activator and sensor, respectively.

When cultured to the PE phase, letA and letS mutants retained the characteristics of exponential-phase wild-type *L. pneumophila*. The mutants expressed flaAgfp poorly (Fig. 1E and F), and they were impaired for motility (Table 2), cytotoxicity (Fig. 3A) and sodium sensitivity (Fig. 3B). In macrophage infections, PE letA and letS mutants also had a fate similar to exponential-phase wild-type cells: compared to PE wild-type cells, 10-fold fewer bacteria were viable and cell associated after 2 h (Fig. 3C), and by 2.5 h 50% of the PE letA mutants were degraded in a lysosomal compartment (Fig. 4C). Nevertheless, those PE letA and letS bacteria that survived macrophage infection did replicate, as shown by a >100-fold increase in CFU from 24 to 72 h (Fig. 3C and D). Although not ruled out experimentally, selection of spontaneous unlinked mutations that mask a replication defect is unlikely to account for the intracellular growth observed, for the following reasons. The apparent growth rate of the letA and letS mutant strains, which resembled wild-type exponential-phase cultures, was reproducible for duplicate samples in each of three experiments; each infection

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**Fig. 4.** The letA-22 mutation is complemented by plasmid-borne letA.

A. Cytotoxicity was measured as in Fig. 3A. The letA-22 mutant transformed with the vector is not cytotoxic (open circles; MB434), whereas the letA-22 mutant carrying pLetA (filled circles; MB435) is as cytotoxic as the wild-type strain transformed with the vector (open squares; MB433).

B. Bacterial infection of and replication in macrophages was quantified as in Figs 3C and D. The letA-22 mutant transformed with the vector infects macrophages poorly (open circles; MB434), whereas the letA-22 mutant carrying pLetA (filled circles; MB435) infects macrophages as well as the wild-type strain transformed with the vector (open squares; MB433).

C. Evasion of phagosome–lysosome fusion was quantified microscopically after a 2.5 h infection of macrophages whose lysosomal compartments contained Texas red–ovalbumin. Shown for the exponential-phase and PE wild-type strain MB 433, the PE letA-22 strain MB434, and the PE complemented letA-22 strain MB435 are the mean fraction of cell-associated bacteria ± SE that were intact in a non-fluorescent compartment calculated by scoring 50 bacteria in each of three experiments.
was initiated by ~10^5 CFU, an insufficient pool to supply the number of spontaneous mutants needed to generate the number of CFU observed at each time point; and 18 independent Kan^R letA-22 clones isolated 48 h after infection of macrophages retained the defect in cytotoxicity and motility (data not shown).

Genetic complementation tests verified that expression of the transmission phenotype required letA. A plasmid-borne letA locus fully complemented a letA mutant for cytotoxicity and macrophage infectivity, and partially restored its capacity to evade phagosome–lysosome fusion (Fig. 4). Therefore, in the PE phase, LetA/S positively activates motility, sodium sensitivity, cytotoxicity and lysosome evasion, traits likely to promote transmission of *L. pneumophila* to a new host. In contrast, intracellular replication appeared to be independent of the putative LetA/S regulon.

To test whether the homologous regulators of *L. pneumophila* and *P. aeruginosa* function similarly, interspecies complementation tests were performed. Although pyocyanin synthesis by a *P. aeruginosa* gacA mutant is restored by plasmid-encoded *P. aeruginosa* gacA (Reimann et al., 1997), the defect was not complemented by pLetA. Likewise, none of the *L. pneumophila* letA mutant phenotypes was complemented by *P. aeruginosa* gacA (data not shown). Hence, although the LetA/LetS and the GacA/GacS regulators share several features, their expression and/or function differs.

**LetE**

Four independent mutants carried an insertion in a ∼0.4 kb chromosomal region that does not appear to encode a protein (Fig. 2E). The nucleotide sequence of the letE region determined by the Columbia Genome Center and verified by us revealed a single ORF which extends just 372 basepairs and lacks significant homology to any other NCBI database proteins (i.e. tblastx E-values from a search of the NCBI database were >1). Approximately 1 kb downstream is a locus similar to the *L. pneumophila* infectivity gene milA (Harb and Abu Kwaik, 2000), but no insertions mapped there. Although not ruled out, simple models in which the *mariner* mutations alter *milX* expression are inconsistent with the genetic data: letE-121 conferred a stronger phenotype than did letE-80, an insertion located downstream (M. Bachman and M. Swanson, unpublished). The letE mutants replicated in macrophages as well as the wild-type parent (Fig. 3D), but they exhibited a severe decrease in sodium sensitivity (Fig. 3B) and more modest reductions in *flaAgfp* expression (Fig. 1G), motility (Table 2), and cytotoxicity (Fig. 3A). To emphasize its intermediate effect on traits thought to promote transmission, the locus was named *letE* for *Legionella* transmission enhancer.

**Test of the stringent response model**

In *L. pneumophila* broth cultures, ppGpp coordinates expression of virulence traits with entry into stationary phase by RpoS-dependent and -independent mechanisms (Hammer and Swanson, 1999; Bachman and Swanson, 2001). To determine whether *L. pneumophila* requires LetA/LetS, *letE* or FliA to respond to the alar-mone, we tested whether the mutants converted to the transmissible form when induced to express the ppGpp synthetase RelA. When exponential-phase cultures of the wild-type strain carrying a pREL*relA* plasmid (pMMBrelA) were treated with isopropyl β-D-thiogalactopyranoside (IPTG), the cells differentiated to the non-replicating virulent form, as judged by assays of the culture optical density and bacterial motility, cytotoxicity and sodium resistance (Table 2; Hammer and Swanson, 1999). In contrast, although the apparent growth rate of the regulatory mutants did slow in response to *relA* induction, indicating ppGpp accumulation (data not shown), the strains did not become motile or cytotoxic (Table 2). We did observe a modest increase in sodium sensitivity when the *letA*, *letS* and *letE* cells expressed *relA*, a phenomenon reported previously for *rpoS* mutants (Bachman and Swanson, 2001). The most striking *relA*-dependent suppression was observed for the *letE* mutants: whereas both exponential-phase and PE *letE* mutants were sodium resistant, exponential-phase phase *letE* cultures induced to express RelA yielded 10-fold fewer CFU in the presence of 100 mM NaCl (Fig. 3B and Table 2). Carriage of the pMMBrelA plasmid also enhanced the sodium sensitivity of PE-phase wild-type and mutant strains cultured without IPTG (Fig. 3A and Table 2). Apparently, colony formation on medium containing 100 mM sodium is affected by multiple mechanisms. Nevertheless, it was apparent that *L. pneumophila* requires *letE*, *letA/S*, FliA and RpoS (Bachman and Swanson, 2001) for complete expression of the transmission phenotype in response to *relA* induction.

**Discussion**

In nature, *L. pneumophila* encounters freshwater, biofilms and phagocytic amoebae (Rogers and Keevil, 1992; Rogers et al., 1994; Fields, 1996). To thrive, the parasite must tailor its phenotype to its surroundings. When inhaled into the lung, a similar strategy apparently promotes colonization of alveolar macrophages and the tissue damage characteristic of Legionnaires’ disease (reviewed by Swanson and Hammer, 2000). In both ponds and humans, the pathogen travels among professional phagocytes to establish an intracellular replication...
niche. To do so, the microbe alternates between a replicative and transmissible form (Byrne and Swanson, 1998). Our genetic data argue that differentiation of \textit{L. pneumophila} in response to nutrient limitation is mediated by LetA/S, RpoS, FliA and \textit{letE} (Byrne and Swanson, 1998; Hammer and Swanson, 1999; Bachman and Swanson, 2001).

Virulence expression by a variety of plant and animal pathogens is controlled by homologues of the same highly conserved two-component regulator. Among extracellular pathogens, GacA/S homologues induce production of cholera toxin and the toxin co-regulated pilus by \textit{Vibrio cholerae} (Wong et al., 1998), siderophores that enable uropathogenic \textit{E. coli} to grow in urine (Zhang and Normark, 1996), catalases that protect \textit{E. coli} from oxidative stress (Mukhopadhyay et al., 2000) and secretion of cyanide, proteases, lipases and also biofilm formation by the pseudomonads (Rich et al., 1994; Rahme et al., 1995; Reimmann et al., 1997; Parkins et al., 2001). In the case of \textit{Salmonella typhimurium}, an enteric facultative intracellular pathogen, the GacA/S homologue SirA/BarA represses the flagellar regulon (Johnston et al., 1996; Ahmer et al., 1999; Altier et al., 2000a; Goodier and Ahmer, 2001) and induces epithelial cell invasion and the inflammatory response characteristic of gastroenteritis (Johnston et al., 1996; Ahmer et al., 1999; Altier et al., 2000a; Goodier and Ahmer, 2001). In the case of \textit{L. pneumophila}, another intracellular pathogen, LetA/S induces the transmission phenotype (Figs 1, 3 and 4). In general, virulence factors regulated by GacA/S are either known or predicted to be exported, often in conditions suboptimal for growth.

Although the signal(s) that activates this conserved phosphorelay is not known, linkage of GacA/S to stationary phase has been reported. In \textit{Pseudomonas fluorescens}, gacA expression is induced in the stationary phase (Laville et al., 1992), and for \textit{P. fluorescens} and \textit{E. coli}, full induction of RpoS requires GacA/S (Whistler et al., 1998; Mukhopadhyay et al., 2000). In \textit{L. pneumophila}, amino acid depletion or ppGpp accumulation triggers a LetA/S- and RpoS-dependent cellular differentiation (Fig. 3 and Table 2; Hammer and Swanson, 1999; Bachman and Swanson, 2001). An intriguing possibility is that ppGpp directly activates the GacA/S phosphorelay (Mukhopadhyay et al., 2000).

Phenotypic analysis indicated that, whereas LetA/LetS is required to induce the transmission phenotype in stationary phase, it does not contribute to intracellular replication (Figs 3 and 4). The fact that conditions suboptimal for growth activate the GacA/S regulon is consistent with the previously documented pattern of virulence expression by \textit{L. pneumophila}. While replicating in macrophages, \textit{L. pneumophila} downregulates flagella production, sodium sensitivity, cytotoxicity and the capacity to block phagosome maturation (Byrne and Swanson, 1998; Hammer and Swanson, 1999; Alli et al., 2000; Sturgill-Koszycki and Swanson, 2000). Conversely, as the replication period ends, intracellular \textit{L. pneumophila} express flagella, sodium sensitivity and cytotoxicity (Byrne and Swanson, 1998; Hammer and Swanson, 1999; Alli et al., 2000), traits that are LetA/LetS dependent (Fig. 3). In addition, although establishment of a replication vacuole requires a Dot/Icm secretion system, intracellular growth does not (Coers et al., 1999). Thus, \textit{L. pneumophila} alternates between a replicative and a transmissible form, and these can be separated genetically by \textit{letA/S} mutations.

Broth cultures of the \textit{letA}, \textit{letS}, and \textit{fliA} mutants exhibited marked defects in macrophage infection (Fig. 3C and D), yet macrophage-grown bacteria appeared to initiate secondary and tertiary infections as efficiently as wild type, as indicated by the similarity of the slopes of intracellular growth curve assays from 24 to 72 h (Fig. 3D). It is possible that cultured macrophages become less stringent with age. Alternatively, LetA/S-independent pathways may induce the transmission phenotype of intracellular \textit{L. pneumophila} (Bachman and Swanson, 2001). In this model, some environmental stress that is absent from PE broth but present in mature replication vacuoles triggers a regulatory pathway that bypasses the \textit{letA/S} defect. Acidity may contribute to this putative signal transduction pathway, as mature replication vacuoles are \textit{pH} 5.5 (Sturgill-Koszycki and Swanson, 2000), and incubation of exponential-phase bacteria in \textit{pH} 5.5 broth for 4 h induces partial motility and cytotoxicity (S. Sturgill-Koszycki and M. S. Swanson, unpublished). More detailed studies are needed to address how the LetA/S regulator contributes to transmission of \textit{L. pneumophila} in settings that more closely mimic its natural reservoir.

A precedent for the cellular differentiation mechanism we propose for \textit{L. pneumophila} is the regulated development of \textit{Myxococcus xanthus}. For this soil microbe, ppGpp triggers a regulatory cascade that controls formation of a multicellular fruiting body which disperses stress-resistant spores (Harris et al., 1998). Differentiation requires coordination of multiple signalling pathways: ppGpp-dependent (A-factor) and -independent (C-factor) signals regulate production and activity of FruA, a FixJ family response regulator that activates the independent motility and sporulation pathways (Ogawa et al., 1996; Ellehaeug et al., 1998). Thus, a two-component regulator integrates ppGpp signalling with other cues to orchestrate differentiation of vegetative cells to a motile, transmissible form.

\textit{Legionella pneumophila} requires \textit{fliA} for both motility and contact-dependent cytotoxicity (Fig. 3). By analogy to \textit{Salmonella} and \textit{Yersinia} (Young et al., 1999; Eichelberg and Galan, 2000; Schmiel et al., 2000), \textit{FliA} may regulate more than flagellar genes. Alternatively, flagella may act

as an adhesin, or as a ligand that activates a macrophage signal transduction cascade that contributes directly or indirectly to contact-dependent cytotoxicity (Hayashi et al., 2001). More complex models hold that cytotoxin delivery requires either the electrochemical gradient that propels flagella or an oriented movement to generate a contact-dependent signal, as for M. xanthus C-signalling (Kim and Kaiser, 1990). Although correlated, motility is not sufficient for cytotoxicity, as dotB mutants are motile but not cytotoxic (B. Byrne and M. S. Swanson, unpublished). Understanding how the L. pneumophila flagellar regulon contributes to contact-dependent cytotoxicity requires further investigation.

Four independent mutants identified letE, a locus required for maximal expression of the L. pneumophila transmission phenotype (Figs 1, 2 and Table 2). The letE region does not appear to encode a protein; therefore, by analogy to homologous regulatory systems, we favour a model in which letE encodes a regulatory RNA. For example, P. fluorescens produces a regulatory RNA, PrrB, that sequesters RsmA, a negative regulator of GacA/S-dependent expression of secreted enzymes and secondary metabolites (Blumer et al., 1999; Aarons et al., 2000). Similar ribonucleoprotein complexes regulate the GacA/S-dependent production of exoenzymes and secondary metabolites by stationary-phase E. carotovora ssp. carotovora (Liu et al., 1998) and also the type III secretion system-dependent invasion of epithelial cells by S. typhimurium (Altier et al., 2000a, b). Consistent with this model, preliminary Northern analysis has indicated that the letE locus encodes an RNA whose expression is aberrant in rpoS mutants and absent in letE-121 cells (M. Bachman and M. Swanson, unpublished). More detailed molecular genetic studies of the letE–milX locus can determine how the letE mariner mutations affect L. pneumophila virulence.

In nature, L. pneumophila probably persists in multispecies biofilms as an occasional resident of amoebae (Brown and Barker, 1999). Cultured phagocytes facilitate studies of some virulence traits, including evasion of phagosomal–lysosomal fusion, cytotoxicity and intracellular replication. To evaluate how particular traits contribute to L. pneumophila fitness in the environment, biofilm models will be valuable tools (Rogers et al., 1994). In both experimental systems, the stringent response model for L. pneumophila cellular differentiation provides a conceptual framework for experiments to identify virulence factors that allow microbes to grow in macrophages.

Experimental procedures

Bacteria, plasmids and macrophages

The L. pneumophila virulent parent, MB355, is the thymine auxotroph Lp02 (Berger and Isberg, 1993) carrying pflaG, a plasmid bearing a transcriptional fusion of the flaA promoter to gfp (Hammer and Swanson, 1999). L. pneumophila was cultured on charcoal yeast extract (CYE) agar or in ACES-buffered yeast extract (AYE) broth to the exponential (OD660 0.3–1.6) or post-exponential (PE; OD 600 3.6–4.0) phase. Molecular cloning utilized E. coli strains DH5α, DH5αλpir and JM109 and plasmids pBluescript KS+ (Stratagene) and pGEM-Easy (Promega). pMMBrElA was described previously (Hammer and Swanson, 1999). Primers are listed in Table 1, except rpoS1 and rpoS2 (Bachman and Swanson, 2001); marout2 and marout4 (a gift from Dr David Hendrixson, University of Michigan, Ann Arbor, MI, USA) anneal adjacent to the mariner 5′-and 3′-terminal inverted repeats respectively. The flia, relA, spoT and gacS loci were identified in the L. pneumophila database (Columbia Genome Center Legionella Genome Project; http://genome3.cpmc.columbia.edu/~legion/) using L. pneumophila DNA sequence (flia; Heuner et al., 1995) or E. coli (RelA and SpoT) or Pseudomonas fluorescens (GacS) protein sequences and the tblastn algorithm. For complementation tests, a 1466 bp fragment containing letA and 396 bp of 5′ DNA amplified from Lp02 using letA1 and letA2 was cloned into pGEMT-Easy to create pGEMletA; to generate pLetA, the letA1 and letA2 were ligated to the EcoRI site of the pMGM-Gent λmarob derivative of pMGM-Gent (Hammer and Swanson, 1999), which lacks the Agel fragment encoding mobAB, such that p_tac can induce letA (data not shown). Macrophages derived from A/J mouse bone marrow were cultured in RPMI containing 10% fetal bovine serum (Byrne and Swanson, 1998). All incubations were at 37°C, except where noted, and media were supplemented with thymidine when necessary.

Mutagenesis and phenotypic screens

The suicide delivery plasmid pFD1 (orR6K, Strβ), encoding the Himar1 transposase and the Himar1-based magellin3 minitransposon conferring resistance to kanamycin (Kan; Rubin et al., 1999), was transferred from DH5αλpir to L. pneumophila carrying pflaG by a triparental mating (Engleberg et al., 1988), then transconjugants were selected on CYE-Kan (25 μg·ml⁻¹) and streptomycin (50 μg·ml⁻¹). After colony purification, PE cultures were scored microscopically for motility and flaGfp fluorescence; exponential-phase and PE Lp02/pflaG cultures served as controls. For phenotypic

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### Table 1. Primers.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5′-3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>marout2</td>
<td>5′-GCT CTT GAA GGG AAC TAT GTT G-3′</td>
</tr>
<tr>
<td>marout4</td>
<td>5′-GCA TTT ACT AGC GAC GCC-3′</td>
</tr>
<tr>
<td>flia1</td>
<td>5′-GAT GAA TAC AAA GAG ACG GAA GG-3′</td>
</tr>
<tr>
<td>flia2</td>
<td>5′-GAA GGA TAA AAT GAC ACC CCC TAA CAT ACG-3′</td>
</tr>
<tr>
<td>letA1</td>
<td>5′-CGG ATT CTC TCA TCG TCA ACC-3′</td>
</tr>
<tr>
<td>letA2</td>
<td>5′-GTA GGC TTT AAT TCG TGC ACC-3′</td>
</tr>
<tr>
<td>letS1</td>
<td>5′-ATT GGC CAT TTT AAC CGT ATA GA-3′</td>
</tr>
<tr>
<td>letS2</td>
<td>5′-GCC ATT TCG CCA TCG TCA ACC-3′</td>
</tr>
<tr>
<td>letE1</td>
<td>5′-ATG GAG GGT TGG TTA ATG GTT GAA-3′</td>
</tr>
<tr>
<td>letE2</td>
<td>5′-TTC CCA TGC CAT ATO CAC CAT CTA-3′</td>
</tr>
<tr>
<td>spoT1</td>
<td>5′-TGA GTT GCA CCC TAA ATC CAT ACC-3′</td>
</tr>
<tr>
<td>spoT2</td>
<td>5′-TGA GGC TTG AAT TGC TGG ACC ATC-3′</td>
</tr>
<tr>
<td>relA1</td>
<td>5′-GAT GGA AGG GGA AGT GCA CGA G-3′</td>
</tr>
<tr>
<td>relA2</td>
<td>5′-CAG GAT TCC CCA TGC CAA AGT C-3′</td>
</tr>
</tbody>
</table>
screens, master microtitre plates containing 10-fold serial dilutions of $1 \times 10^6$ to $1 \times 10^7$ colony-forming units (CFU) of PE culture per millilitre of phosphate-buffered saline were prepared. Sodium sensitivity was calculated by spotting 10 µl aliquots from each master plate onto CYE with and without 100 mM NaCl and enumerating CFU (Byrne and Swanson, 1998). Contact-dependent cytotoxicity was quantified after incubating macrophages for 1 h at a multiplicity of infection (MOI) of ~100 by the Alamar Blue colorimetric assay (Hammer and Swanson, 1999). Replication-dependent killing of macrophages was judged 24 and 48 h after infecting duplicate microtitre wells of macrophages at MOI of ~0.1 and 1 representative alleles, insertion mutations were amplified and transferred by natural transformation (Stone and Abu Kwaik, 1999) to Lp02 and Lp02/pflaG by the method of Dr Joseph Vogel (Washington University, St. Louis, MO, USA). L. pneumophila was patched onto CYE, mixed with ~1 µg of PCR product, then incubated at 30°C for 2 days before selecting transformants on CYE-Kan. Disruption of the corresponding chromosomal allele was verified by PCR analysis using primers complementary to its 5’ and 3’ regions; wild type served as the reference. Represented in Figs 1, 3 and 4 and Table 2 are the transmission phenotypes of two or more different (where available) back-crossed alleles determined using a series of quantitative assays for cytotoxicity, infectivity, sodium sensitivity, intracellular growth and phagosome–lysosome fusion described previously (Byrne and Swanson, 1998; Hammer and Swanson, 1999).

Analysis of candidates

Southern analysis of BglII chromosomal digests used a digoxigenin-labelled MluI internal KanR fragment from pFD1 (Roche). To identify the locus disrupted by each transposon, the BglII cleavage products were also ligated into the BarnHI site of pBluescript KS+, plasmids were electroporated directly with the suspect colony, then the number of KanR–AmpR transformants was scored; MB355 (WT/pflaG) was the negative control.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>Growth phase</th>
<th>IPTG</th>
<th>Motility</th>
<th>Toxicity</th>
<th>NaS</th>
<th>Table 2. Effects of RelA induction on transmission phenotypes of defined mutants.</th>
</tr>
</thead>
<tbody>
<tr>
<td>MB359</td>
<td>Wild-type/ pMMBrelA</td>
<td>E</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>Wild-type and mutant strains containing pMMBrelA were cultured to the PE phase or to the exponential phase and treated with 200 µM IPTG for 4.5 h (+) or not (−)</td>
</tr>
<tr>
<td>MB412</td>
<td>fliA/ pMMBrelA</td>
<td>E</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>MB415</td>
<td>letA/ pMMBrelA</td>
<td>E</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>MB418</td>
<td>letS/ pMMBrelA</td>
<td>E</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>MB412</td>
<td>letE/ pMMBrelA</td>
<td>E</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
</tbody>
</table>

Wild-type and mutant strains containing pMMBrelA were cultured to the PE phase or to the exponential phase and treated with 200 µM IPTG for 4.5 h (+) or not (−), then assayed as described previously (Hammer and Swanson, 1999).

a. Phenotypes were scored relative to a wild-type exponential-phase negative control (−) and wild-type PE-phase positive control (++) as in Fig. 3; + indicates an intermediate phenotype.
b. To reflect the phenotypes of the positive and negative control cultures, plating efficiencies of <0.2% are designated +; values >10% are −; and intermediate values are depicted as +.

Acknowledgements

We gratefully acknowledge the contributions of several colleagues. Mike Bachman identified in the database the L. pneumophila gacS homologue, analysed a pqaB mutation, directed the letE DNA sequencing, and made numerous insightful comments throughout the project and on the
manuscript; Brian Hefferman cloned the letE mutant allele; Jonathon Low analysed a mutation linked to fleSR; Dr Dave Hendrixson provided valuable advice and reagents related to mariner technology and critical comments on the manuscript; Dr Dieter Haas of the Université de Lausanne supplied the P. fluorescens gacA mutant and plasmid; Dr James Russo of the Columbia Genome Center provided timely information and advice. This project was funded by NIH grant AI 44212–01 and a University of Michigan Rackham Predoctoral Fellowship to B.H.

References


