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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.

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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 *Bacillis 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 amoebae 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 factors that block phagosome maturation to establish a protected niche within the next phagocyte.

As the stationary-phase sigma factor RpoS induces expression of only some of the *L. pneumophila* virulence traits (Bachman and Swanson, 2001), additional regulators were sought. Our classical genetic strategy exploited the *mariner* transposon, the *L. pneumophila* genome sequence, the microbe's natural competence, and a *flaA–gfp* reporter of virulence (Hammer and Swanson, 1999). This approach identified two sigma factors, a new locus and a widespread two-component regulator that induces the transmission phenotype in stationary phase or in response to induction of the RelA ppGpp synthetase but is dispensable for replication.

Results

Identification of regulatory mutants

Exploiting its coordinate expression with flagellar synthesis (Pruckler *et al*., 1995; Merriam *et al*., 1997; Byrne and Swanson, 1998; Hammer and Swanson, 1999), positive activators of *L. pneumophila* virulence were sought by screening *mariner* mutants for defective expression of a *flaAgfp* reporter (Hammer and Swanson, 1999). From two independent pools of ~10^4 Kan^R mutants, 69 isolates were identified on solid medium that expressed *flaAgfp* poorly relative to the wild-type (WT) parent (Fig. 1). Post-exponential phase (PE) broth cultures of each mutant were also less fluorescent and motile than wild type (data not shown). Next, the 69 mutants were classified by screening three other phenotypes correlated to virulence: cytotoxicity (Husmann and Johnson, 1994; Byrne and Swanson, 1998; Kirby *et al*., 1998; Hammer and Swanson, 1999; Alli *et al*., 2000), sodium sensitivity (Sadosky *et al*., 1993; Vogel *et al*., 1996; Byrne and Swanson, 1998; Hammer and Swanson, 1999) and growth in macrophages (Cianciotto *et al*., 1989).

Two classes of false-positive mutants were discarded. Five strains carried transposons on the reporter plasmid, as detected by linkage of Kan^R to Amp^R. Another 27 isolates harboured second-site mutations that affected virulence, 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 (σ^s), the flagellar sigma factor FliA (σ^{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 are 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).

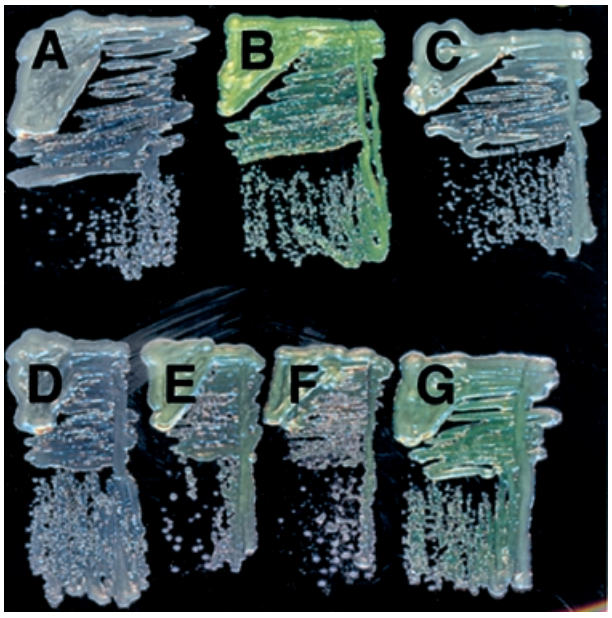

Fig. 1. Identification of mutants defective for *flaAgfp* expression.Colony colour in ambient light after 4 days at 37°C of WT Lp02 (A) compared with flaAgfp transformants: (B) wild-type strain MB355, (C) *rpoS-52* strain MB422, (D) *fliA-35* strain MB411, (E) *letA-22* strain MB414, (F) *letS-36* strain MB417 and (G) *letE-121* strain MB420.

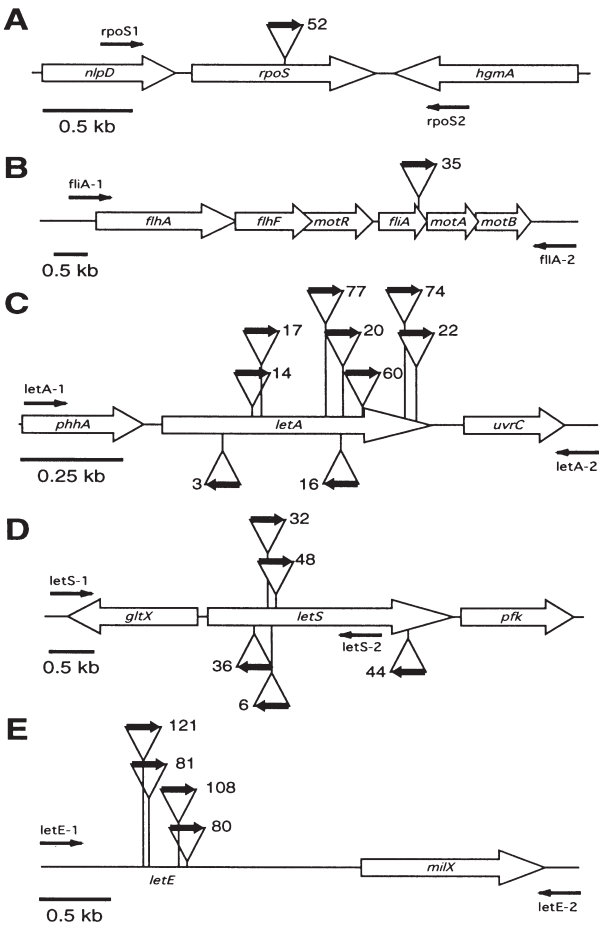

Fig. 2. Maps of loci required by *Legionella* to express transmission phenotypes. Indicated are the orientation, location and allele designation of mariner insertions (thick black arrows), relevant primers (thin black arrows) and the chromosomal loci (thick white arrows) encoding (A) *rpoS*, (B) *fliA*, (C) *letA*, (D), *letS* and (E) *letE*. Not shown are eight other *letA* insertion mutations whose independence from the alleles shown was ambiguous.

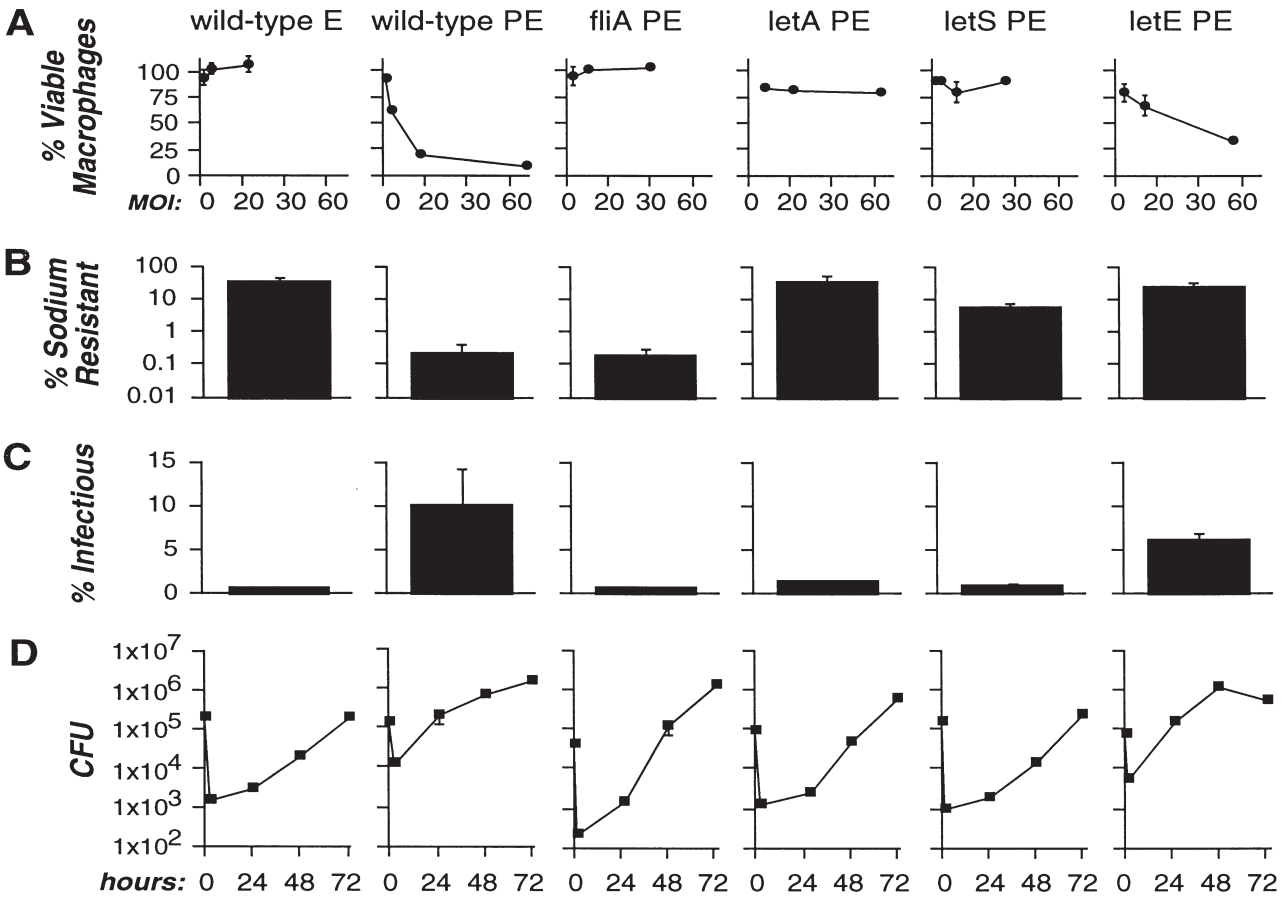

Fig. 3. *Legionella* mutants do not express transmission phenotypes, but do replicate in macrophages. PE cultures of *fliA-35* (MB410), *letA-22* (MB414), *letS-36* (MB417) and *letE-121* (MB420) mutants were compared with exponential-phase and PE cultures of wild-type Lp02 in four assays of virulence.

A. Contact-dependent cytotoxicity requires *fliA*, *letA* and *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 *fliA* at a MOI of 156; 81% for PE *letA* at a MOI of 190; 83% for PE *letS* at a MOI of 40; and 14% for PE *letE* at a MOI of 220.

B. Sodium sensitivity requires *letA*, *letE* and *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 *fliA*, *letA* and *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 *fliA*, *letA*, *letS* and *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'.

To gain insight into *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 *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.

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; Reimmann *et al*., 1997; 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

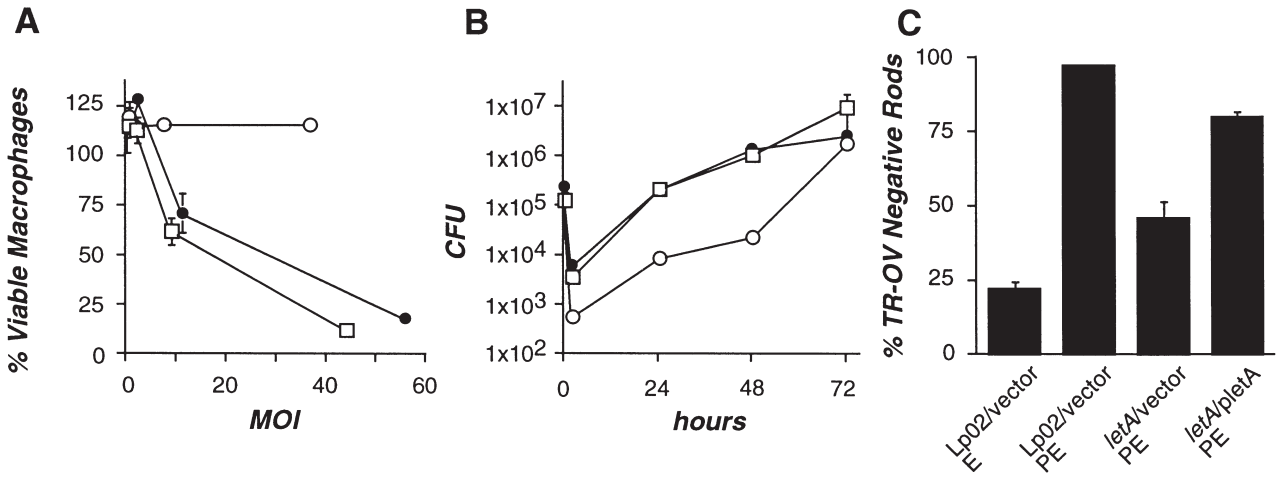

Fig. 4. The *letA-22* mutation is complemented by plasmid-borne *letA*.
A**.** Cytotoxicity was measured as in Fig. 3A. The *letA-22* mutant transformed with the vector is not cytotoxic (open circles; MB434), whereas the *letA-22* mutant carrying pLetA (filled circles; MB435) is as cytotoxic as the wild-type 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* (Reimmann *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 data base 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 alarmone, 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 p_{Tac}-*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 *L. pneumophila* in response to nutrient limitation is mediated by LetA/S, RpoS, FliA and *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 *Vibrio cholerae* (Wong *et al*., 1998), siderophores that enable uropathogenic *E. coli* to grow in urine (Zhang and Normark, 1996), catalases that protect *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 *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 *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 *Pseudomonas fluorescens*, *gacA* expression is induced in the stationary phase (Laville *et al*., 1992), and for *P. fluorescens* and *E. coli,* full induction of RpoS requires GacA/S (Whistler *et al*., 1998; Mukhopadhyay *et al*., 2000). In *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 *L. pneumophila*. While replicating in macrophages, *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 *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, *L. pneumophila* alternates between a replicative and a transmissible form, and these can be separated genetically by *letA/S* mutations.

Broth cultures of the *letA, letS,* and *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 *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 *letA/S* defect. Acidity may contribute to this putative signal transduction pathway, as mature replication vacuoles are ~pH 5.5 (Sturgill-Koszycki and Swanson, 2000), and incubation of exponential-phase bacteria in 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 *L. pneumophila* in settings that more closely mimic its natural reservoir.

A precedent for the cellular differentiation mechanism we propose for *L. pneumophila* is the regulated development of *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; Ellehauge *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.

Legionella pneumophila requires *fliA* for both motility and contact-dependent cytotoxicity (Fig. 3). By analogy to *Salmonella* and *Yersinia* (Young *et al*., 1999; Eichelberg and Galan, 2000; Schmiel *et al*., 2000), 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 3 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 phagosome–lysosome 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 (OD_{660} 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 pGEMT-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 (Promega) to create pGEMletA; to generate pLetA, the pGEMletA *Eco*RI fragment encoding *letA* was ligated to the *Eco*RI site of the pMMB-GentΔmob derivative of pMMB-Gent (Hammer and Swanson, 1999), which lacks the *Age*I 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.

Table 1. Primers.

marout2	5′-GCT CTT GAA GGG AAC TAT GTT G-3′
marout4	5′-GCA TTT AAT ACT AGC GAC GCC-3′
fliA1	5′-GAT GAA TAC AAA GAG ACG GAA GG-3′
fliA2	5′-GAA GTC AAA GAT ACC CCC TAA GC-3′
letA1	5′-CGC CGG GGG AAT TTT ATC TT-3′
letA2	5′-GGA GGG CGG CTT CTT TTT ACT TC-3′
letS1	5′-ATT GGC CAT TTT AAC CTG ATA GA-3′
letS2	5′-GCC ATT TCG CCA TCG TCA ACC-3′
letE1	5′-ATG GAA GGT TGG TTA ATG GTT GAA-3′
letE2	5′-TTC CCA TGC CAT AAT ATC CAC CTA-3′
spoT1	5′-TGA GTT GCA CCC TAA ATA CAT ACG-3′
spoT2	5′-TGA GCG TTG AAT TTG TCC ACC ATC-3′
relA1	5′-GAT GGA AGG GGG ATA GCA CGA G-3′
relA2	5′-CAG GAT TCC CCA TGC CAA AGT C-3′

Mutagenesis and phenotypic screens

The suicide delivery plasmid pFD1 (*ori*R6K, Str^S), 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 *flaAgfp* fluorescence; exponential-phase and PE Lp02/pflaG cultures served as controls. For phenotypic

screens, master microtitre plates containing 10-fold serial dilutions of 1×10^8 to 1×10^2 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 qualitatively by phase microscopy and quantitatively after incubating ~4 h with Alamar Blue. To determine whether motile mutants that failed to express *flaAgfp* carried a Kan^R transposon on the reporter plasmid, DH5αλpir was electroporated directly with the suspect colony, then the number of Kan^R–Amp^R transformants was scored; MB355 (WT/pflaG) was the negative control.

Analysis of candidates

Southern analysis of *Bgl*II chromosomal digests used a digoxigenin-labelled *Mlu*I internal Kan^R fragment from pFD1 (Roche). To identify the locus disrupted by each transposon, the *Bgl*II cleavage products were also ligated into the *Bam*HI site of pBluescript KS+, plasmids were electroporated into DH5α, then transformants were selected on LB-Kan (25 µg ml^{-1}). Nucleotide sequences of insertion sites were generated by The University of Michigan Sequencing Core Facility using either marout2 or marout4 and compared with the *L. pneumophila* genome database. To determine whether other mutations mapped to the same locus, its chromosomal region was amplified and sized relative to wild type. To determine the orientation and location of each transposon, the product generated by PCR using a chromosomal primer of known position and either marout2 or marout4 was sized. To map the *fliA* mutation, the PCR product generated with fliA1 and fliA2 was cloned into pGEMT-Easy, then sequenced. To verify the posted *letE* nucleotide sequence, the locus was amplified using primers letE-1 and letE-2 (Fig. 2), then a 0.5 kb region defined by the four insertions mutations was sequenced on both strands by our Core Facility using letE-1 and two custom *letE* primers (data not shown). To back-cross 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).

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Table 2. Effects of RelA induction on transmission phenotypes of defined mutants.

Strain	Relevant genotype	Growth phase	IPTG	Motility[a]	Toxicity	Na^{Sb}
MB359	Wild-type/	E	–	–	–	–
	pMMBrelA	E	+	+	++	+
		PE	–	++	++	++
MB412	fliA/	E	–	–	–	–
	pMMBrelA	E	+	–	–	ND
		PE	–	–	–	++
MB415	letA/	E	–	–	–	–
	pMMBrelA	E	+	–	–	+
		PE	–	–	–	+
MB418	letS/	E	–	–	–	–
	pMMBrelA	E	+	–	–	+
		PE	–	–	–	+
MB412	letE/	E	–	–	–	–
	pMMBrelA	E	+	–	+	+
		PE	–	+	+	–

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 +.

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 Universite 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

Aarons, S., Abbas, A., Adams, C., Fenton, A., and O'Gara, F. (2000) A regulatory RNA (PrrB RNA) modulates expression of secondary metabolite genes in *Pseudomonas fluorescens* F113. *J Bacteriol* **182:** 3913–3919.

Ahmer, B.M., van Reeuwijk, J., Watson, P.R., Wallis, T.S., and Heffron, F. (1999) *Salmonella* SirA is a global regulator of genes mediating enteropathogenesis. *Mol Microbiol* **31:** 971–982.

Alli, O.A.T., Gao, L.-Y., Pedersen, L.L., Zink, S., Radulic, M., Doric, M., and Abu Kwaik, Y. (2000) Temporal pore formation-mediated egress from macrophages and alveolar epithelial cells by *Legionella pneumophila. Infect Immun* **68:** 6431–6440.

Altier, C., Suyemoto, M., Ruiz, A.I., Burnham, K.D., and Maurer, R. (2000a) Characterization of two novel regulatory genes affecting *Salmonella* invasion gene expression. *Mol Microbiol* **35:** 635–646.

Altier, C., Suyemoto, M., and Lawhon, S.D. (2000b) Regulation of *Salmonella enterica* serovar Typhimurium invasion genes by *csrA. Infect Immun* **68:** 6790–6797.

Bachman, M.A., and Swanson, M.S. (2001) RpoS co-operates with other factors to induce *Legionella pneumophila* virulence in the stationary phase. *Mol Microbiol* **40:** 1201–1214.

Berger, K.H., and Isberg, R.R. (1993) Two distinct defects in intracellular growth complemented by a single genetic locus in *Legionella pneumophila. Mol Microbiol* **7:** 7–19.

Blumer, C., Heeb, S., Pessi, G., and Haas, D. (1999) Global GacA-steered control of cyanide and exoprotease production in *Pseudomonas fluorescens* involves specific ribosome binding sites. *Proc Natl Acad Sci USA* **96:** 14073–14078.

Brinkman, F.S.L., MacFarlane, E.L.A., Warrener, P., and Hancock, R.E.W. (2001) Evolutionary relationships among virulence-associated histidine kinases. *Infect Immun* **69:** 5207–5211.

Brown, M.R., and Barker, J. (1999) Unexplored reservoirs of pathogenic bacteria: protozoa and biofilms. *Trends Microbiol* **7:** 46–50.

Byrne, B., and Swanson, M.S. (1998) Expression of *Legionella pneumophila* virulence traits in response to growth conditions. *Infect Immun* **66:** 3029–3034.

Cashel, M., Gentry, D.R., Hernandez, V.J., and Vinella, D. (1996) The stringent response. In: Escherichia coli *and* Salmonella*: Cellular and Molecular Biology.* Neidhardt, F.C. (ed. in chief). Washington, DC: American Society for Microbiology Press, pp. 1458–1496.

Cianciotto, N., Eisenstein, B.I., Engleberg, N.C., and Shuman, H. (1989) Genetics and molecular pathogenesis of *Legionella pneumophila*, an intracellular parasite of macrophages. *Mol Biol Med* **6:** 409–424.

Coers, J., Monahan, C.N., and Roy, C.R. (1999) Modulation of phagosome biogenesis by *Legionella pneumophila* creates an organelle permissive for intracellular growth. *Nature Cell Biol* **1:** 451–453.

Cotter, P.A., and DiRita, V.J. (2000) Bacterial virulence gene regulation: an evolutionary perspective. *Annu Rev Microbiol* **54:** 519–565.

Danese, P.N., Snyder, W.B., Cosma, C.L., Davis, L.J., and Silhavy, T.J. (1995) The Cpx two-component signal transduction pathway of *Escherichia coli* regulates transcription of the gene specifying the stress-inducible periplasmic protease, DegP. *Genes Dev* **9:** 387–398.

Dietrich, C., Heuner, K., Brand, B.C., Hacker, J., and Steinert, M. (2001) Flagellum of *Legionella pneumophila* positively affects the early phase of infection of eukaryotic host cells. *Infect Immun* **69:** 2116–2122.

Eichelberg, K., and Galan, J.E. (2000) The flagellar sigma factor FliA (sigma (28) regulates the expression of *Salmonella* genes associated with the centisome 63 type III secretion system. *Infect Immun* **68:** 2735–2743.

Ellehauge, E., Nørregaard-Madsen, M., and Søgaard-Andersen, L. (1998) The FruA signal transduction protein provides a checkpoint for the temporal co-ordination of intercellular signals in *Myxococcus xanthus* development. *Mol Microbiol* **30:** 807–817.

Engleberg, N.C., Cianciotto, N., Smith, J., and Eisenstein, B.I. (1988) Transfer and maintenance of small, mobilizable plasmids with ColE1 replication origins in *Legionella pneumophila. Plasmid* **20:** 83–91.

Eriksson, A.R., Andersson, R.A., Pirhonen, M., and Palva, E.T. (1998) Two-component regulators involved in the global control of virulence in *Erwinia carotovora* subsp. *carotovora. Mol Plant–Microbe Interact* **11:** 743–752.

Fields, B.S. (1996) The molecular ecology of legionellae. *Trends Microbiol* **4:** 286–290.

Gardel, C.L., and Mekalanos, J.J. (1996) Alterations in *Vibrio cholerae* motility phenotypes correlate with changes in virulence factor expression. *Infect Immun* **64:** 2246–2255.

Goodier, R.I., and Ahmer, B.M. (2001) SirA orthologs affect both motility and virulence. *J Bacteriol* **183:** 2249–2258.

Groseclose, S.L., Hall, P.A., Knowles, C.M., Adams, D.A., Park., S., Perry, F., *et al.* (2001) Summary of notifiable diseases, United States, 1999. *Morbid Mortal Wkly Rep* **48:** 1–104.

Guiney, D.G. (1997) Regulation of bacterial virulence gene expression by the host environment. *J Clin Invest* **99:** 565–569.

Guiney, D.G., Libby, S., Fang, F.C., Krause, M., and Fierer, J. (1995) Growth-phase regulation of plasmid virulence genes in *Salmonella. Trends Microbiol* **3:** 275–279.

Hales, L.M., and Shuman, H.A. (1999) The *Legionella pneumophila rpoS* gene is required for growth within *Acanthamoeba castellanii. J Bacteriol* **181:** 4879–4889.

Hammer, B.K., and Swanson, M.S. (1999) Co-ordination of *Legionella pneumophila* virulence with entry into stationary phase by ppGpp. *Mol Microbiol* **33:** 721–731.

Hanna, P.C., and Ireland, J.A. (1999) Understanding *Bacillus anthracis* pathogenesis. *Trends Microbiol* **7:** 180–182.

Harb, O.S., and Abu Kwaik, Y. (2000) Characterization of a macrophage-specific infectivity locus (*milA*) of *Legionella pneumophila. Infect Immun* **68:** 368–376.

Harkey, C.W., Everiss, K.D., and Peterson, K.M. (1994) The *Vibrio cholerae* toxin-coregulated-pilus gene *tcpI* encodes a homolog of methyl-accepting chemotaxis proteins. *Infect Immun* **62:** 2669–2678.

Harris, B.Z., Kaiser, D., and Singer, M. (1998) The guanosine nucleotide (p)ppGpp initiates development and A-factor production in *Myxococcus xanthus. Genes Dev* **12:** 1022–1035.

Hase, C.C., and Mekalanos, J.J. (1999) Effects of changes in membrane sodium flux on virulence gene expression in *Vibrio cholerae*. *Proc Natl Acad Sci USA* **96:** 3183–3187.

Hayashi, F., Smith, K., Ozinsky, A., Hawn, T., Yi, E., Goodlett, D., *et al*. (2001) The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. *Nature* **410:** 1099–1103.

Heinzen, R.A., Hackstadt, T., and Samuel, J.E. (1999) Developmental biology of *Coxiella burnetii. Trends Microbiol* **7:** 149–154.

Heuner, K., Bender-Beck, L., Brand, B.C., Luck, P.C., Mann, K.-H., Marre, R., *et al*. (1995) Cloning and genetic characterization of the flagellum subunit gene (*flaA*) of *Legionella pneumophila* serogroup 1. *Infect Immun* **63:** 2499–2507.

Heuner, K., Hacker, J., and Brand, B.C. (1997) The alternative sigma factor σ^{28} *Legionella pneumophila* restores flagellation and motility to an *Escherichia coli fliA* mutant. *J Bacteriol* **179:** 17–23.

Hrabak, E.M., and Willis, D.K. (1992) The *lemA* gene required for pathogenicity of *Pseudomonas syringae* pv. *syringae* on bean is a member of a family of two-component regulators. *J Bacteriol* **174:** 3011–3020.

Husmann, L.K., and Johnson, W. (1994) Cytotoxicity of extracellular *Legionella pneumophila. Infect Immun* **62:** 2111–2114.

Johnston, C., Pegues, D.A., Hueck, C.J., Lee, A., and Miller, S.I. (1996) Transcriptional activation of *Salmonella typhimurium* invasion genes by a member of the phosphorylated response-regulator superfamily. *Mol Microbiol* **22:** 715–727.

Kim, S.K., and Kaiser, D. (1990) Cell alignment required in differentiation of *Myxococcus xanthus. Science* **249:** 926–928.

Kinscherf, T.G., and Willis, D.K. (1999) Swarming by *Pseudomonas syringae* B728a requires *gacS* (*lemA*) and *gacA* but not the acyl-homoserine lactone biosynthetic gene *ahlI. J Bacteriol* **181:** 4133–4136.

Kirby, J.E., Vogel, J.P., Andrews, H.L., and Isberg, R.R. (1998) Evidence of pore-forming ability by *Legionella pneumophila. Mol Microbiol* **27:** 323–336.

Laville, J., Voisard, C., Keel, C., Maurhofer, M., Defago, G., and Haas, D. (1992) Global control in *Pseudomonas fluorescens* mediating antibiotic synthesis and suppression of black root rot of tobacco. *Proc Natl Acad Sci USA* **89:** 1562–1566.

Liu, Y., Cui, Y., Mukherjee, A., and Chatterjee, A.K. (1998) Characterization of a novel RNA regulator of *Erwinia carotovora* ssp. *carotovora* that controls production of extracellular enzymes and secondary metabolites. *Mol Microbiol* **29:** 219–234.

Merriam, J.J., Mathur, R., Maxfield-Boumil, R., and Isberg, R.R. (1997) Analysis of the *Legionella pneumophila fliI* gene: intracellular growth of a defined mutant defective for flagellum biosynthesis. *Infect Immun* **65:** 2497–2501.

Mirel, D.B., Lustre, V.M., and Chamberlin, M.J. (1992) An operon of *Bacillus subtilis* motility genes transcribed by the sigma D form of RNA polymerase. *J Bacteriol* **174:** 4197–4204.

Moolenaar, G.F., van Sluis, C.A., Backendorf, C., and van de Putte, P. (1987) Regulation of the *Escherichia coli* excision repair gene *uvrC*. Overlap between the *uvrC* structural gene and the region coding for a 24 kD protein. *Nucleic Acids Res* **15:** 4273–4289.

Mukhopadhyay, S., Audia, J.P., Roy, R.N., and Schellhorn, H.E. (2000) Transcriptional induction of the conserved alternative sigma factor RpoS in *Escherichia coli* is dependent on BarA, a probable two-component regulator. *Mol Microbiol* **37:** 371–381.

Ogawa, M., Fujitani, S., Mao, X., Inouye, S., and Komano, T. (1996) FruA, a putative transcription factor essential for the development of *Myxococcus xanthus. Mol Microbiol* **22:** 757–767.

Parkins, M.D., Ceri, H., and Storey, D.G. (2001) *Pseudomonas aeruginosa* GacA, a factor in multihost virulence, is also essential for biofilm formation. *Mol Microbiol* **40:** 1215–1226.

Pruckler, J.M., Benson, R.F., Moyenuddin, M., Martin, W.T., and Fields, B.S. (1995) Association of flagellum expression and intracellular growth of *Legionella pneumophila. Infect Immun* **63:** 4928–4932.

Rahme, L.G., Stevens, E.J., Wolfort, S.F., Shao, J., Tompkins, R.G., and Ausubel, F.M. (1995) Common virulence factors for bacterial pathogenicity in plants and animals. *Science* **268:** 1899–1902.

Reimmann, C., Beyeler, M., Latifi, A., Winteler, H., Foglino, M., Lazdunski, A., and Haas, D. (1997) The global activator GacA of *Pseudomonas aeruginosa* PAO positively controls the production of the autoinducer *N*-butyryl-homoserine lactone and the formation of the virulence factors pyocyanin, cyanide and lipase. *Mol Microbiol* **24:** 309–319.

Rich, J.J., Kinscherf, T.G., Kitten, T., and Willis, D.K. (1994) Genetic evidence that the *gacA* gene encodes the cognate response regulator for the *lemA* sensor in *Pseudomonas syringae. J Bacteriol* **176:** 7468–7475.

Rogers, J., and Keevil, C.W. (1992) Immunogold and fluorescein immunolabelling of *Legionella pneumophila* within an aquatic biofilm visualized by using episcopic differential interference contrast microscopy. *Appl Environ Microbiol* **58:** 2326–2330.

Rogers, J., Dowsett, A.B., Dennis, P.J., Lee, J.V., and Keevil, C.W. (1994) Influence of temperature and plumbing material selection on biofilm formation and growth of *Legionella pneumophila* in a model potable water system containing complex microbial flora. *Appl Environ Microbiol* **60:** 1585–1592.

Rowbotham, T.J. (1980) Preliminary report on the pathogenicity of *Legionella pneumophila* for freshwater and soil amoebae. *J Clin Pathol* **33:** 1179–1183.

Rubin, E.J., Akerley, B.J., Novik, V.N., Lampe, D.J., Husson, R.N., and Mekalanos, J.J. (1999) *In vivo* transposition of mariner-based elements in enteric bacteria and mycobacteria. *Proc Natl Acad Sci USA* **96:** 1645–1650.

Sadosky, A.B., Wiater, L.A., and Shuman, H.A. (1993) Identification of *Legionella pneumophila* genes required for growth within and killing of human macrophages. *Infect Immun* **61:** 5361–5373.

Schmiel, D.H., Young, G.M., and Miller, V.L. (2000) The *Yersinia enterocolitica* phospholipase gene *yplA* is part of the flagellar regulon. *J Bacteriol* **182:** 2314–2320.

Shah, D.S., and Sockett, R.E. (1995) Analysis of the *motA* flagellar motor gene from *Rhodobacter sphaeroides*, a bacterium with a unidirectional, stop-start flagellum. *Mol Microbiol* **17:** 961–969.

Stone, B.J., and Abu Kwaik, Y. (1999) Natural competence for DNA transformation by *Legionella pneumophila and* its association with expression of type IV pili. *J Bacteriol* **181:** 1395–1402.

Sturgill-Koszycki, S., and Swanson, M.S. (2000) *Legionella pneumophila* replication vacuoles mature into acidic, endocytic organelles. *J Exp Med* **192:** 1261–1272.

Swanson, M.S., and Hammer, B.K. (2000) *Legionella pneumophila* pathogenesis: a fateful journey from amoebae to macrophages. *Annu Rev Microbiol* **54:** 567–613.

Tan, M.W., Rahme, L.G., Sternberg, J.A., Tompkins, R.G., and Ausubel, F.M. (1999) *Pseudomonas aeruginosa* killing of *Caenorhabditis elegans* used to identify *P. aeruginosa* virulence factors. *Proc Natl Acad Sci USA* **96:** 2408–2413.

Turco, S.J., and Descoteaux, A. (1992) The lipophosphoglycan of *Leishmania* parasites. *Annu Rev Microbiol* **46:** 65–94.

Vogel, J.P., Roy, C., and Isberg, R.R. (1996) Use of salt to isolate *Legionella pneumophila* mutants unable to replicate in macrophages. *Ann NY Acad Sci* **797:** 271–272.

Whistler, C.A., Corbell, N.A., Sarniguet, A., Ream, W., and Loper, J.E. (1998) The two-component regulators GacS and GacA influence accumulation of the stationary-phase sigma factor sigma S and the stress response in *Pseudomonas fluorescens* Pf-5. *J Bacteriol* **180:** 6635–6641.

Willis, D.K., Hrabak, E.M., Rich, J.J., Barta, T.M., Lindow, S.E., and Panopoulos, N.J. (1990) Isolation and characterization of a *Pseudomonas syringae* pv. *syringae* mutant deficient in lesion formation on bean. *Mol Plant–Microbe Interact* **3:** 149–156.

Wong, S.M., Carroll, P.A., Rahme, L.G., Ausubel, F.M., and Calderwood, S.B. (1998) Modulation of expression of the ToxR regulon in *Vibrio cholerae* by a member of the two-component family of response regulators. *Infect Immun* **66:** 5854–5861.

Yamaguchi, S., Fujita, H., Ishihara, A., Aizawa, S.-I., and Macnab, R.M. (1986) Subdivision of flagellar genes of *Salmonella typhimurium* into regions responsible for assembly, rotation and switching. *J Bacteriol* **166:** 187–193.

Young, G.M., Schmiel, D.H., and Miller, V.L. (1999) A new pathway for the secretion of virulence factors by bacteria: the flagellar export apparatus functions as a protein-secretion system. *Proc Natl Acad Sci USA* **96:** 6456–6461.

Zhang, J.P., and Normark, S. (1996) Induction of gene expression in *Escherichia coli* afer pilus-mediated adherence. *Science* **273:** 1234–1236.