**Legionella pneumophila** CsrA is a pivotal repressor of transmission traits and activator of replication

Ari B. Molofsky and Michele S. Swanson*

Department of Microbiology and Immunology, University of Michigan Medical School, Ann Arbor, MI 48109–0620, USA.

Summary

*Legionella pneumophila* can replicate inside amoebae and also alveolar macrophages to cause Legionnaires’ Disease in susceptible hosts. When nutrients become limiting, a stringent-like response coordinates the differentiation of *L. pneumophila* to a transmissive form, a process mediated by the two-component system LetA/S and the sigma factors RpoS and FliA. Here we demonstrate that the broadly conserved RNA binding protein CsrA is a global repressor of *L. pneumophila* transmission phenotypes and an essential activator of intracellular replication. By analysing *csrA* expression and the phenotypes of *csrA* single and double mutants and a strain that expresses *csrA* constitutively, we demonstrate that, during replication in broth, CsrA represses every post-exponential phase phenotype examined, including cell shape shortening, motility, pigmentation, stress resistance, sodium sensitivity, cytotoxicity and efficient macrophage infection. At the transition to the post-exponential phase, LetA/S relieves CsrA repression to induce transmission phenotypes by both FliA-dependent and -independent pathways. For *L. pneumophila* to avoid lysosomal degradation in macrophages, CsrA repression must be relieved by LetA/S before phagocytosis; conversely, before intracellular bacteria can replicate, CsrA repression must be restored. The reciprocal regulation of replication and transmission exemplified by CsrA likely enhances the fitness of microbes faced with fluctuating environments.

Introduction

To survive, microbes are adept at sensing environmental changes and altering their physiology. Concomitant with alterations in bacterial metabolism, growth rate, and stress resistance, pathogenic microbes must regulate virulence effectors that promote either replication or transmission. A strategy shared by a number of intracellular pathogens is to alternate between replicative, intracellular forms and resilient, infectious extracellular forms that avoid immune-mediated destruction. For example, the obligate intracellular bacterium *Chlamydia trachomatis* differentiates from an intracellular replicative form, RB, to a highly resistant and infectious form, EB. These two forms have distinct morphologies and properties, indicating widespread changes in gene expression (Shaw et al., 2000). By coupling developmental pathways to external cues, microbes can adapt to a variety of environments.

*Legionella pneumophila*, a Gram-negative facultative intracellular pathogen, is commonly found in aquatic environments, where protozoa are its natural hosts (Rowbotham, 1980; Fields, 1996). However, when contaminated water is inhaled by susceptible individuals, *L. pneumophila* can also replicate within human alveolar macrophages and cause a progressive pneumonia called Legionnaires’ Disease (McDade et al., 1977; Horwitz and Silverstein, 1980). Similar to *C. trachomatis*, *L. pneumophila* alternates between an infectious, non-replicating form thought to promote transmission to a new host, and an intracellular replicative form which does not express transmission traits (Rowbotham, 1986; Byrne and Swan- son, 1998). In broth cultures, as amino acids become limiting, *L. pneumophila* stops replicating and activates transmission traits, including cytotoxicity, motility, osmotic and heat resistance, sodium sensitivity, and the capability to avoid phagosomal-lysosome fusion (Byrne and Swan- son, 1998; Hammer and Swanson, 1999). Therefore, we have used exponential (E) and post-exponential (PE) phase broth cultures as a tool to model the replicative and transmissive phases, respectively, of the *L. pneumophila* lifecycle in nature.

As *L. pneumophila* coordinates regulati

© 2003 Blackwell Publishing Ltd
In Pseudomonads and Erwinia carotovora, the LetAS homologues GacA/S and ExpA/S activate synthesis of exported virulence effectors by counteracting the global regulatory protein CsrA (Blumer et al., 1999; Aarons et al., 2000; Cui et al., 2001; Heeb and Haas, 2001; Heeb et al., 2002). In particular, these two-component systems induce expression of CsrB homologues, which are non-coding regulatory RNAs that can sequester multiple copies of CsrA protein, thereby inducing CsrA-repressed virulence effectors. In general, the CsrA protein functions as a modulator of mRNA stability and translation, whereas the CsrB RNA binds and sequesters multiple copies of CsrA protein to de-repress CsrA targets.

Originally described as an Escherichia coli regulator of glycogen accumulation (Romeo et al., 1993), the small RNA-binding protein Carbon Storage Regulator A (CsrA) and the non-coding repressor RNA CsrB are now known to function as a global post-transcriptional regulatory system in a large number of bacterial species (reviewed in Romeo, 1998). CsrA regulates a host of E. coli PE phase traits, including central carbohydrate metabolism, motility, biofilm formation, and adherence (Sabnis et al., 1995; Yang et al., 1996; Wei et al., 2000; 2001; Jackson et al., 2002). For the E. coli glycan biosynthesis gene glgC, CsrA binds near the ribosomal binding site of the mRNA, preventing ribosomal binding and destabilizing the transcript (Baker et al., 2002). CsrA can also stabilize RNA transcripts, such as the E. coli master flagellar regulator flhDC, but the mechanism for this stabilization is not thoroughly understood (Wei et al., 2001). In the plant pathogen Erwinia carotovora, the CsrA homologue RsmA affects production of several lytic enzymes, motility and quorum sensing, whereas CsrA of Salmonella typhimurium regulates the Salmonella pathogenicity island 1, including genes important for cell invasion (Chatterjee et al., 1995; Cui et al., 1995; Altier et al., 2000a,b). Pseudomonas aeruginosa RsmA represses extracellular virulence effectors and production of quorum sensing genes during exponential growth (Pessi et al., 2001). In L. pneumophila, constitutive expression of wild-type csrA inhibits the PE phase traits motility and pigment production (Fettes et al., 2001). In sum, the CsrA/B global regulatory system is widely conserved and functions to repress PE phase virulence traits in many pathogenic bacteria.

In this work, we extend our stringent response model for L. pneumophila differentiation by proposing that CsrA functions as a pivotal regulator of the L. pneumophila lifecycle. By analogy to homologous regulatory circuits, we postulated that during the replicative phase CsrA represses transmission traits and coordinately promotes replication. When L. pneumophila sense that amino acid levels are low, activated LetAS would relieve CsrA repression of PE traits, including several required for virulence. To test this model, we constructed both csrA constitutive expression and csrA conditional mutant strains in wild type and a panel of regulatory mutant backgrounds and analysed their expression of transmissive phase traits.

**Results**

**Identification of csrA**

Fettes et al. (2001) recently identified the L. pneumophila csrA gene and demonstrated that, in high copy, the wild-type locus inhibits expression of the PE phase traits motility, pigment production and cell shortening. To extend this analysis by examining its regulation of L. pneumophila differentiation and virulence, we first cloned L. pneumophila csrA and verified its ability to complement the glycogen storage phenotype of E. coli csrA mutants as described previously (data not shown, Fettes et al., 2001).

Legionella pneumophila express csrA during the replication phase

Because in other bacteria CsrA inhibits post-exponential (PE) phase genes, including virulence factors, we predicted L. pneumophila csrA would be active during the replication period to repress virulence-associated phenotypes. To monitor csrA expression during the L. pneumophila lifecycle, broth cultures of wild-type microbes transformed with a plasmid containing a csrA:gfpr transcriptional fusion (WT pcsrAgfp) were analysed by fluorometry (Fig. 1A). The csrA promoter was active throughout the exponential (E) phase, then its activity declined slightly as the microbes progressed into the PE phase. In comparison, expression of the known PE phase flagellar subunit gene flaA (Heuner et al., 1995; 1999; Hammer and Swanson, 1999) was undetectable during exponential growth, but was strongly activated as the microbes entered PE phase, and green fluorescent protein (GFP) continued to accumulate (Fig. 1B). Although the stability of GFP precludes strict interpretations of promoter activity, maintenance of fluorescence by replicating
bacteria requires constant pcsrAgfp expression. Therefore, the csrA promoter is active preferentially during the bacterial replication period in vitro, a conclusion consistent with northern analysis monitoring csrA transcript levels during broth growth (Fettes et al., 2001).

To extend the results obtained from our in vitro model, L. pneumophila expression of pcsrAgfp and pflaAgfp was next compared during its lifecycle in macrophages. Ten minutes after infection with PE phase bacteria, the pflaAgfp microbes were strongly fluorescent, whereas the pcsrAgfp bacteria were only faintly so (Fig. 1C), consistent with the broth culture fluorometry data (Fig. 1A and B). By 16 h after infection, intracellular replicating L. pneumophila containing pcsrAgfp were bright green (Fig. 1C) and...
most replication vacuoles (86% ± 5.7%) contained a majority of GFP-positive microbes (Fig. 1D). At the same time, replicating flaAgfp microbes displayed little fluorescence (Fig. 1C), and few vacuoles (9.8% ± 7.9%) contained a majority of GFP-positive bacteria (Fig. 1D). The reciprocal pattern of expression observed for csrA and the flaA transmission phase gene during the L. pneumophila lifecycle is consistent with the observation that constitutive csrA expression decreases mRNA levels of the flagellar sigma factor flaA (Fettes et al., 2001) and with the hypothesis that CsrA is a vital regulator during the replicative phase.

Growth and morphology of L. pneumophila that either lack or constitutively express csrA

To test the prediction that CsrA is a replication phase repressor of PE traits, we created strains that lack or constitutively express csrA, then determined their transmission phenotypes in both E and PE phases. We and others were unable to recover csrA null mutant strains using standard procedures (see Experimental procedures, Fettes et al., 2001), suggesting that L. pneumophila requires csrA to grow on bacteriological agar. Instead, we created csrA conditional null mutants in which the chromosomal csrA locus was deleted and the expression of a plasmid-encoded wild-type csrA could be induced with IPTG (p206-csrA), hereafter referred to as csrA mutants (MB464, MB465). As expected, in the absence of IPTG, csrA mutants formed colonies poorly. When cultured in broth that contained IPTG to induce csrA, the bacteria replicated at a wild-type rate (Supplementary material, Fig. S1). However, when transferred to broth without IPTG, the yield of csrA mutants increased slowly, and the cells were short and coccoid-shaped, resembling PE phase wild-type microbes (Supplementary material, Figs S1 and S2). Furthermore, unlike wild-type Lp02 L. pneumophila, which become motile at culture densities of OD600nm > 3.0 (Byrne and Swanson, 1998; Hammer and Swanson, 1999), some csrA mutant cells swam at densities as low as 0.1; by an OD600nm of 2.0, a majority of csrA mutant cells were motile (Table 1).

As a complementary approach to study CsrA function, a wild-type strain in which the csrA gene could be constitutively expressed with addition of IPTG (wild-type pcsrA) was constructed and examined for morphology and motility. Unlike p206-csrA, used in the conditional csrA mutants, pcsrA has an IPTG-responsive P lac promoter that drives high-level expression of csrA, and was therefore appropriate for studies of constitutive csrA expression. Even in the absence of IPTG induction, the pcsrA microbes displayed a leaky csrA expression, based on their elongated cell shapes and partially repressed transmission phase phenotypes, including motility, infectivity, sodium-sensitivity, and cytotoxicity (data not shown). All subsequent experiments with constitutive csrA expressing microbes (wild-type pcsrA) maintained the cultures with IPTG throughout broth growth. When induced with IPTG, L. pneumophila wild-type pcsrA cells grew as well as wild-type bacteria in broth (data not shown). However, when compared to wild-type L. pneumophila, the cells constitutively expressing csrA due to IPTG induction became even more elongated, similar to bacteria lacking the two-component response regulator letS, and their motility was reduced and flaA expression was <30% of wild-type levels (Table 1, Fig. S2, Fettes et al., 2001). Observations of cells that lack or that constitutively express csrA are consistent with a model whereby L. pneumophila produce CsrA to repress a host of PE traits during the replicative period; during the transmissive phase, CsrA repression must be relieved to activate motility and cell shortening.

CsrA inhibits pigment production and stress resistance of PE phase L. pneumophila

As L. pneumophila progresses through the PE phase, it produces a melanin-like, soluble pigment that protects these aquatic microbes from UV damage but is not required for intracellular growth (Warren and Miller, 1979; Wiater et al., 1994; Wintermeyer et al., 1994; Steinert et al., 1995). Our model predicts that when RelA senses amino acid starvation, it produces ppGpp to activate the two-component system LetA/S, which in turn represses CsrA activity to induce expression of PE phase traits. Legionella pneumophila that lack relA or constitutively express csrA are defective for pigment accumulation (Fettes et al., 2001; Zusman et al., 2002). Therefore, we predicted that letA and letS mutants produced little pigment.

Wild-type L. pneumophila and rpoS mutants accumulated substantial pigment, as demonstrated previously (Supplementary material, Fig. S3A; Table 1; Hales and Shuman, 1999; Bachman and Swanson, 2001). As predicted, letA or letS mutants accumulated little pigment, a phenotype similar to that observed for the constitutively active csrA strain (Supplementary material, Fig. S3B; Table 1, Fettes et al., 2001). In addition, compared to wild-type microbes, bacteria that either lacked the LetA or LetS activators or constitutively expressed the CsrA repressor exhibited a significant loss of absorbance at OD600nm as cultures progressed into late PE, yet a corresponding loss in CFU was not observed during the same period (data not shown). More detailed studies are required to determine whether the decline in OD600nm is due to further changes in bacterial shape or small amounts of cell lysis difficult to detect by CFU assays.
Table 1. Effects of CsrA on general PE phase phenotypes in L. pneumophila.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Growth phase</th>
<th>Motility</th>
<th>Coccolid shape</th>
<th>Pigment</th>
<th>Heat-resistance</th>
<th>Osmotic-Resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type Lp02/vector (MB473)</td>
<td>E</td>
<td>–</td>
<td>+/-</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Wild-type Lp02/vector (MB473)</td>
<td>PE</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>(17% ± 7.8%)</td>
</tr>
<tr>
<td>csrA (MB464, MB465)</td>
<td>E</td>
<td>+</td>
<td>++</td>
<td>–</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>csrA (MB464, MB465)</td>
<td>PE</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>Lp02 pcsrA + IPTG (MB472)</td>
<td>PE</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>letAVector (MB434)</td>
<td>PE</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>csrA letA (MB466)</td>
<td>PE</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>letA pcsrA + IPTG (MB476)</td>
<td>PE</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>ND</td>
</tr>
<tr>
<td>fliA Vector (MB462)</td>
<td>PE</td>
<td>–</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>ND</td>
</tr>
<tr>
<td>csrA fliA (MB467)</td>
<td>PE</td>
<td>–</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>ND</td>
</tr>
<tr>
<td>fliA pcsrA + IPTG (MB475)</td>
<td>PE</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>dotA (MB460)</td>
<td>PE</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>csrA dotA (MB468)</td>
<td>PE</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>rpoS Vector (MB478)</td>
<td>PE</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>(67% ± 45%)</td>
</tr>
<tr>
<td>rpoS pcsrA + IPTG (MB474)</td>
<td>PE</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

a. Motility was gauged qualitatively by microscopy of wet-mounts and is based upon numerous independent observations. The motility of csrA mutants (MB464 465) increased as cultures progressed from early to late E phase. Wild-type Lp02 constitutively expressing csrA and carrying the pTLp6-flaAgfp reporter plasmid (MB470, +IPTG) expressed flaA at <30% of the level of wild-type Lp02 pTLP6-flaAgfp (MB471), as measured by quantifying GFP fluorescence of PE phase bacteria by fluorometry.

b. PE phase L. pneumophila adopt a more coccolid, compact shape than E phase, replicating microbes. Shape was assessed qualitatively by microscopy of wet-mounts of numerous independent cultures. (+) indicates compact, wild-type PE shape (+/-) indicates longer, replicating shape, and (–) indicates extremely long shape (see Supplementary Fig S2).

c. L. pneumophila secrete a melanin-like pigment in the PE phase. Representative graphs demonstrating pigment accumulation over time are shown in Supplementary Fig S3. As actual absolute pigment values of wild-type strains varied between experiments and were affected by carriage of empty vectors, results are represented here as ++ (exceeding wild-type pigment), + (wild-type pigment levels), and – (very low pigment accumulation). For all experiments, a wild-type strain with appropriate empty vector was included as a reference. Data shown represent 2–6 independent experiments.

d. In the PE phase, L. pneumophila become resistant to a variety of environmental stresses, including heat and osmotic shock. (+) indicates >10% survival (+) indicates 1–10% survival, and (–) indicates <1% survival. Shown are the means of 2–5 independent experiments ± SEM. Data shown represent wild-type microbes carrying the empty pMMBGent-mob-invcsrA control vector (MB463).

As L. pneumophila alter their physiology to accommodate nutrient limitation, resistance to various environmental stresses is activated (Bandyopadhyay and Steinman, 1998; Hales and Shuman, 1999; Hammer and Swanson, 1999; Bachman and Swanson, 2001). Legionella pneumophila letA mutants are deficient in stress resistance, as are E. coli that lack the LetA orthologue UvrY (Pernestig et al., 2001; Lynch et al., 2003). As LetA activates and CsrA represses a number of PE phase traits of L. pneumophila, we tested the prediction that CsrA also represses resistance to environmental stress.

Wild-type PE phase L. pneumophila tolerated both heat and osmotic stress well, whereas E phase microbes were sensitive (Table 1, Hales and Shuman, 1999; Hammer and Swanson, 1999). As predicted, bacteria that constitutively expressed csrA remained sensitive to heat and osmotic stress in the PE phase, resembling both E phase wild-type microbes and PE phase letA mutants. Conversely, when E phase csrA mutants were subjected to heat, they were partially heat resistant; as they entered the PE phase, the csrA mutants became fully heat resistant (Table 1). Thus, csrA represses heat resistance during replication, but other factors may also activate heat resistance of PE phase L. pneumophila. When subjected to pH or oxidative stress, both letA mutants and csrA constitutively expressing microbes survived as efficiently as PE phase wild-type and rpoS mutant cultures, whereas E phase wild-type microbes were
sensitive (data not shown, Hales and Shuman, 1999; Bachman and Swanson, 2001). However, Lynch et al. (2003) report that letA mutants are sensitive to pH and oxidative stress; the most likely explanation for this discrepancy is strain differences. In sum, CsrA represses several physiological changes characteristic of the PE phase, but other factors may also regulate some PE phase traits, including tolerance of heat, acid and oxidative stress (Table 1). Alternately, it is possible that our csrA conditional null mutant retains low levels of IPTG-independent csrA expression, and these traits may be repressed by low levels of CsrA that do not repress the majority of transmission traits.

**CsrA represses cytotoxicity and sodium-sensitivity, two L. pneumophila virulence traits**

As *L. pneumophila* coordinates expression of virulence with general PE phase traits, we postulated that CsrA also represses virulence-associated traits during the replication period. In the PE phase, wild-type *L. pneumophila* are cytotoxic to bone marrow-derived macrophages, whereas E phase bacteria are not (Byrne and Swanson, 1998). This contact-dependent cytotoxicity depends upon the Dot/Icm type IV secretion system and the PE phase activator proteins LetA, LetS, and FliA (Kirby et al., 1998; Hammer et al., 2002) and may aid in bacterial escape from spent hosts (Ali et al., 2000). Consistent with our model, wild-type PE phase cultures were highly cytotoxic, whereas PE phase cells that constitutively express csrA were not, comparable to E phase wild-type bacteria (Fig. 2A). Conversely, csrA mutants in the E phase were prematurely cytotoxic, comparable to PE phase wild type (Fig. 2B).

Virulent *L. pneumophila* are sensitive to sodium, as judged by their poor plating efficiency on agar containing 100 mM NaCl (Catrenich and Johnson, 1989). Although the mechanism is not understood, several of the dot/icm type IV secretion mutants were originally identified on the basis of their growth in high NaCl concentrations, and LetA/S activates sensitivity to sodium in the PE phase (Sadosky et al., 1993; Vogel et al., 1996; Byrne and Swanson, 1998; Hammer et al., 2002). As predicted, PE phase cells that constitutively express csrA remained resistant to sodium (Fig. 3A), comparable to E phase wild-type control cultures and PE phase letA mutants (Fig. 3B, Hammer et al., 2002). Conversely, E phase csrA mutants were partially sodium sensitive, becoming fully sodium sensitive in the PE phase (Fig. 3B). Therefore, the virulence-associated traits of cytotoxicity and sodium-sensitivity are under CsrA-mediated repression during replication; upon transition into the PE phase, this repression must be relieved to allow expression of virulence traits.

**Fig. 2.** CsrA represses *L. pneumophila* contact-dependent cytotoxicity. A. Bacteria were incubated with macrophages at the multiplicity of infection shown for 1 h, then macrophage viability was quantified by the reduction of the colorimetric dye Alamar Blue. Wild-type Lp02 (MB473) cultured to the PE phase (solid circles) was cytotoxic, but wild-type Lp02 pcsrA cultured with IPTG to the PE phase (MB472; solid diamonds) resembled the non-cytotoxic control, E phase wild-type Lp02 (MB473; open circles). B. E phase csrA::kan mutant microbes are cytotoxic (MB464, solid triangles), resembling PE phase wild type (MB463, solid circles), and the negative control E phase wild-type strain is not (MB463, open circles). C. Both PE phase csrA::kan mutant microbes (MB464, solid triangles) and PE phase csrA letA double mutants (MB466, open squares) are cytotoxic, but PE phase letA single mutants are not (MB413, solid squares). PE and E phase wild type controls are labelled as in A and B. Shown are representative graphs from three or more independent experiments performed in duplicate or triplicate.
LetA induces PE phase transmission traits by relieving CsrA repression

By analogy to homologous regulatory circuits in other Gram-negative bacteria, CsrA repression of *L. pneumophila* transmission traits is predicted to be relieved in the PE phase by activation of the two-component system LetA/S. If so, genetic inactivation of CsrA should bypass the requirement for LetA/S in transmission phenotype expression. To test this aspect of our model, we mutated the *letA* locus in the *csrA* conditional null background, then analysed the phenotype of *csrA letA* double mutants by culturing the cells in broth that lacked IPTG.

When CsrA repression was relieved by mutation, the LetA activator was dispensable for *L. pneumophila* to express every transmission phenotype examined. After reaching the PE phase, both the *csrA* single mutant and the *csrA letA* double mutant became coccoid, fully motile, and pigmented, comparable to wild-type *L. pneumophila* (Table 1). Loss of *csrA* also restored to PE phase *letA* mutants heat resistance (Table 1), cytotoxicity (Fig. 2C), and sodium-sensitivity (Fig. 3B). Finally, the *csrA letA* double mutants were as infectious for macrophages as PE phase wild-type *L. pneumophila* (Fig. 4B), as they efficiently avoided lysosomal degradation (Fig. 4D), traits discussed in detail below. The observation that loss of CsrA activity bypassed all of the *letA* mutant transmission defects indicates that, for the phenotypes assayed, LetA solely functions to repress CsrA activity, thereby inducing transmission traits.

As a test for specificity of the genetic suppression observed, we asked whether loss of the CsrA repressor also compensated for another pleiotropic mutation, *dotA*. As a putative integral component of the type IV secretion complex, DotA is thought to be required for delivery of virulence factors to the host cell (Berger *et al*., 1994; Roy and Isberg, 1997; Roy *et al*., 1998; Vogel *et al*., 1998). Accordingly, loss of the CsrA repressor should not bypass the virulence defects of *dotA* secretion mutants. As predicted, loss of *csrA* did not restore cytotoxicity to *dotA* mutant microbes: After 1 h at an MOI of 25, only ~5% of macrophages incubated with PE wild-type microbes were viable, whereas ~95% of macrophages incubated with PE *dotA* single mutants or *csrA dotA* double mutants were viable. However, PE phase *csrA dotA* double mutants were 10-fold more sodium-sensitive than PE phase *dotA* single mutants (Fig. 3B), indicating either that loss of csrA non-specifically causes partial restoration of sodium-sensitivity or that a genetic link exists between *csrA* and *dotA* that remains to be understood. Both PE phase *dotA* single mutants and *csrA dotA* double mutants were resistant to heat and secreted the melamin-like pigment (Table 1), demonstrating that the type IV secretion apparatus is not required for stress resistance or pigment production. Even in the absence of a functional type IV secretion system, *L. pneumophila* required *csrA* to repress motility and to replicate at wild-type rates (data not shown), suggesting that CsrA may also activate the replication phenotype.
CsrA repression is mediated by both FliA-dependent and -independent pathways

CsrA is postulated to repress the flagellar sigma factor FliA, thereby inhibiting motility and certain virulence traits, based on the loss of fliA mRNA when csrA is expressed constitutively (Fettes et al., 2001). To test genetically whether other CsrA-repressed transmission traits are dependent upon FliA activation, we constructed and analysed csrA fliA double mutants in a csrA conditional null background.

As observed for the csrA, csrA dotA and csrA letA mutant strains, the csrA fliA double mutant strain also had an apparent slow growth rate, illustrating that CsrA function is critical for L. pneumophila replication in broth (data not shown). However, when it eventually reached high culture densities, the csrA fliA double mutant strain was not motile, infectious for macrophages, or cytotoxic (Table 1; Fig. 4B; data not shown). Therefore, even when CsrA repression is relieved by mutation, L. pneumophila requires the flagellar sigma factor FliA to express three of its transmission traits.

Other transmission traits are expressed by L. pneumophila independently of FliA. In the PE phase, both fliA single and csrA fliA double mutants were cocccoid-shaped and sodium sensitive (Hammer et al., 2002; data not shown). Likewise, when subjected to a variety of environmental stresses, both the fliA and csrA fliA mutants resembled PE phase wild-type and rpoS cultures, unless they constitutively expressed csrA (Table 1; Hales and...
Shuman, 1999; Bachman and Swanson, 2001). Therefore, neither the FliA nor the RpoS sigma factors are required for the general resilience of PE phase L. pneumophila.

Unexpected effects of FliA and LetA activity on pigment production were observed. When compared to PE phase wild-type L. pneumophila, fliA mutants were hyper-pigmented (data not shown), suggesting FliA either directly or indirectly represses pigment production. Although constitutive expression of csrA by wild type, rpoS, or fliA mutants repressed pigment production, hyper-accumulation of pigment was observed when letA mutants were induced to express csrA constitutively (Table 1), even though this strain is predicted to have high levels of CsrA activity unopposed by LetA-dependent inhibition. Additional experiments are required to understand the excess pigmentation in supernatants of strains that lack FliA or have deregulated CsrA activity.

**CsrA is necessary for intracellular growth, but dispensable for initial infection**

Studies of broth cultures indicate that during the replication period, CsrA represses a range of PE traits, including those likely to promote transmission to a new host. To assess the validity of our interpretations generated from in vitro studies, macrophages were infected with L. pneumophila csrA mutant or constitutively expressing microbes, then the efficiency of infection and intracellular replication were quantified.

When ingested by macrophages, many bacteria and inert particles are swiftly delivered to lysosomal compartments and degraded. However, as L. pneumophila sense amino acid starvation and transit into the PE state, they become competent to evade delivery to bactericidal lysosomes for several hours (Horwitz, 1983; Byrne and Swanson, 1998; Sturgill-Koszycki and Swanson, 2000). Unlike highly infectious PE phase L. pneumophila (30% ± 12%), PE phase bacteria that constitutively express csrA exhibited low infectivity (0.56% ± 0.31%), similar to the E phase wild-type cultures (Fig. 4A). In contrast, in the E phase, csrA mutants were highly infectious (22% ± 10%), similar to wild-type PE phase microbes (Fig. 4B). To verify that efficient macrophage infectivity reflects their capacity to evade lysosomal degradation, we assayed by fluorescence microscopy the fate of L. pneumophila 2 h after infection of macrophages. As expected, <20% of E phase wild-type bacteria avoided degradation (Fig. 4C and D) and >80% of PE phase wild-type bacteria remained intact (Fig. 4D). In contrast, whether cultured to either the E or PE phase, >80% of csrA mutants retained their rod shape 2 h after ingestion (Fig. 4C and D). Thus, in the absence of CsrA activity, L. pneumophila become highly infectious and avoid immediate lysosomal degradation, as judged by both the CFU-dependent infectivity assays and direct microscopic inspection (Fig. 4).

Although PE phase L. pneumophila that constitutively express csrA are poorly infectious (Figs 4A and 5A), those bacteria that survived the initial infection then multiplied at wild-type rates, as judged by the similar slopes of the respective growth curves from 2 to 24 h (Fig. 5A). As predicted, letA mutants behaved identically to csrA constitutively expressing cells in assays of infectivity and intracellular growth (Figs 4B and 5A; Hammer et al., 2002).

---

**Fig. 5.** CsrA is essential for L. pneumophila growth in macrophages, but its repression must be relieved for efficient transmission.

A. Constitutive csrA expression, due either to induction of pcsrA or deletion of letA, inhibits initial infection of macrophages but not intracellular growth. Macrophages were infected for 2 h at an MOI of ~1.0 with either wild-type Lp02 (MB473) cultured to the E (hollow circles) or PE phase (solid circles), wild-type Lp02 transformed with pcsrA and cultured with IPTG to PE phase (MB472; diamonds), or PE mutant letA carrying the vector (MB434, squares). At each time indicated, the total CFU per well was quantified. Shown are the mean CFU ± SD calculated from duplicate wells in one of three independent experiments. B. csrA mutants cannot replicate in macrophages until csrA expression is restored. CFU were quantified as described in A after infection of macrophages with the PE phase wild-type control strain (MB463, circles) or E phase csrA::kan mutants untreated (MB464; triangles, solid line) or induced with IPTG either at the time of macrophage infection (squares) or 48 h post infection (triangles, dashed line). Shown are the mean bacterial CFU ± SD determined at the times indicated for duplicate wells in one experiment; similar results were obtained in three to four independent experiments.
Therefore, either constitutive \( csrA \) production or deletion of \( letA \) prevented expression of PE phase traits necessary for efficient infection, but neither inhibited intracellular growth, consistent with the proposed role for CsrA as an essential repressor of transmissive phase traits and activator of replication. As the phenotypic patterns observed are identical to E phase wild-type bacteria, we postulate that excess CsrA, either from loss of LetA or induction of pcsrA, phase-locks \( L. pneumophila \) in the replicative form.

The reciprocal phenotype was observed when macrophages were infected with \( L. pneumophila \) that lack \( csrA \). Macrophages were incubated with E phase \( csrA \) mutants cultured in broth without IPTG. After 2 h, the percentage of viable and cell-associated \( csrA \) mutant bacteria was comparable to that observed in parallel infections with the virulent control, wild-type PE phase cultures (Fig. 4B). Thus, unlike E phase wild-type \( L. pneumophila \), E phase \( csrA \) mutants are highly infectious, consistent with the premature expression of genes necessary for \( L. pneumophila \) to efficiently enter macrophages and delay delivery to phagolysosomes. Nevertheless, in the absence of \( csrA \), the highly infectious \( L. pneumophila \) failed to replicate, even when incubated in macrophages for 72 h (Fig. 5B). In contrast, when IPTG was supplied at the time of macrophage infection, E phase \( csrA \) mutant bacteria mimicked wild-type PE phase cultures by infecting and also replicating efficiently (Fig. 5B). If instead E phase \( csrA \) mutant cells were incubated with IPTG both in broth culture and during macrophage infection, they behaved like wild-type E phase cultures (data not shown). We conclude that initially \( L. pneumophila \) must repress CsrA activity to infect macrophages efficiently; subsequently, CsrA activity must be induced to promote efficient replication.

\( csrA \) mutants are viable 48 h post infection

The fate of intracellular \( csrA \) mutants that persisted but did not replicate was examined in detail. Microscopy demonstrated that, by 48 h after infection, wild-type bacteria had replicated profusely (Fig. 6, column 1), whereas many of the \( csrA \) mutants persisted as single, intact rods (Fig. 6, column 2). In some macrophages, several either tightly packed or dispersed bacteria were seen, some of which were degraded. Those few \( csrA \) mutant bacteria that had replicated yielded 5–10 microbes per vacuole, appeared short and stubby, and stained poorly by DAPI (Fig. 6, column 2). To determine whether the \( csrA \) mutant cells that persisted in macrophages were viable, macrophages infected for 48 h were treated with IPTG, then incubated for an additional 12–16 h before microscopic analysis. Although a variety of phenotypes were again observed, many infected macrophages contained vacuoles of the replicative form of \( L. pneumophila \), as evidenced both by

![Fig. 6.](image-url)
their elongated form that stained brightly by DAPI or anti-
Legionella antibody and by the larger number of microbes
per vacuole (Fig. 6, columns 3 and 4), neither of which
was observed in the absence of IPTG treatment. More-
over, the csrA mutants that resided in macrophages for
48 h responded to IPTG treatment by replicating at
approximately the same rate as wild-type L. pneumophila,
as judged by the similar slope of the respective growth
curves (Fig. 5B). Therefore, based on results of both
microscopy and CFU assays, intracellular L. pneumophila
must express csrA to differentiate to the replicative form.

Discussion

This study demonstrates that L. pneumophila express the
global repressor CsrA during the replication period to
inhibit transmission traits and promote growth. When con-
ditions deteriorate, LetA/S relieves CsrA repression to
inhibit transmission traits and promote growth. When con-

First, the phenotypic profile of

Secondly, loss of CsrA bypassed the requirement for LetA
as an inducer of every PE phase trait examined. Specific-
ly, the letA mutant defects of cell shape, pigment pro-
duction, stress resistance, motility, cytotoxicity, sodium
sensitivity, and lysosomal evasion are a consequence of
constitutive CsrA-mediated repression as they can be
relieved by removal of CsrA (Table 1; Figs 2–4). Therefore,
we propose that the major role of the activated LetA two-
component response regulator is to counteract CsrA
repression. Nevertheless, LetA/S may also regulate other
as yet undefined traits independently of CsrA, as has been
demonstrated in other bacterial species (Blumer et al.,
1999; Suzuki et al., 2002).

The canonical Csr post-transcriptional regulatory sys-
tem consists of both the protein CsrA and the non-coding
inhibitory RNA CsrB. In other Gram-negative bacteria,
LetA/S homologues induce expression of the CsrB regu-

latory RNA, which binds multiple copies of CsrA to relieve
repression. Given that L. pneumophila encodes a LetA/S
two-component system that antagonizes CsrA, it is also
likely to utilize a CsrA-binding regulatory RNA. However,
when CsrB and CsrC of E. coli or the functionally similar
CsrB-like RNA species PrpB and RsmZ from Pseudomon-
as fluorescens (Aarons et al., 2000; Heeb et al., 2002;
Weibacher et al., 2003) were used as query sequences in a
BLASTN homology search of the unfinished Legionella
genome, no L. pneumophila csrB homologues were iden-
tified, consistent with previous results (Fettes et al., 2001).
The primary sequence of csrB is not well conserved;
instead, its secondary structure and CsrA protein binding
sites are likely critical for its activity. Therefore, functional
approaches will be needed to identify a putative L. pneu-

ora phila CsrB that is induced by LetA to counteract CsrA
activity.

Even when CsrA repression is alleviated, L. pneumo-

phila require the sigma factor FliA (sigma 28) to express
the transmission traits of motility, infectivity, and cytotoxic-
ity (Table 1; Fig. 4; data not shown). FliA is also necessary
for L. pneumophila growth in certain amoebae (Heuner
et al., 2002). Genetic data presented here and elsewhere
indicate that letA mutation results in excess CsrA activity
and loss of the RNA encoding the FliA sigma factor
(Fettes et al., 2001). FliA is known to activate several class
I flagellar genes involved in the terminal stages of flagel-

Figure 7). In
response to elevated ppGpp, the two-component system
LetA/S is activated and represses the activity of CsrA,
likely by inducing the expression of an unidentified CsrB
homologue. Relief of CsrA repression is sufficient to
induce expression of PE phase traits such as cell short-
ening, pigment production, and heat and osmotic resis-
tance. Additionally, loss of CsrA repression activates
the expression of a number of virulence-associated traits,
including cytotoxicity, motility, and evasion of phagosome-
lysosome fusion, resulting at least in part from activation
of the class II flagellar sigma factor FliA. CsrA may directly
repress fliA mRNA stability or translation, or there may be
an unidentified upstream activator of fliA expression that
CsrA targets. In parallel, the stationary-phase sigma factor
RpoS is also activated by ppGpp to induce a subset of PE
phase traits independently of the LetA/S-CsrA pathway.

© 2003 Blackwell Publishing Ltd, Molecular Microbiology, 50, 445–461
including motility, sodium-sensitivity, intracellular growth and endosomal evasion (Bachman and Swanson, 2001).

*Legionella pneumophila* require CsrA to replicate efficiently in broth culture and in macrophages (Supplementary material, Fig. S1; Fig. 5). Similarly, *S. typhimurium* csrA mutants grow slowly in culture (Altier et al., 2000a). *Legionella pneumophila* csrA mutants' premature expression of an array of virulence-associated traits may inhibit growth. However, two lines of evidence support the alternate hypothesis that CsrA is integral to *L. pneumophila* metabolism, independent of its repression of transmission factors. First, neither of two pleiotropic mutations, fliA and dotA, suppressed the growth defect of csrA mutants on solid or liquid media or in macrophages (data not shown). Second, attempts to recover by standard methods csrA null mutants in strains that lacked letA, rpoS, fliA or letA rpoS were unsuccessful, indicating that genetic inactivation of several transmission traits by any of these pleiotropic mutations is not sufficient to overcome the growth deficiency caused by a csrA mutation. Therefore, the poor growth of csrA mutants is not likely due to expression of virulence factors during the replication phase. Instead, by analogy to *E. coli* csrA or uvrY (letA homologue) mutants, we favour the hypothesis that *L. pneumophila* csrA mutants grow poorly due to imbalances in carbon flux and/or amino acid uptake (Wei et al., 2000; Pernestig et al., 2003). In *E. coli*, CsrA is a pro-glycolytic, antiguconeogenic global regulator, controlling numerous steps of carbon flux (reviewed in Romeo, 1998), and *L. pneumophila* is a fastidious bacterium that utilizes amino acids as its primary energy source (George et al., 1980; Hoffman, 1984). Perhaps the intracellular environment is especially stringent and prevents replication of *L. pneumophila* csrA mutants, whereas nutrient-rich broth culture supports slow replication of the microbes.

A remarkable finding is that even after extended incubations in macrophages, csrA mutants remain competent to replicate, provided csrA expression is first restored (Figs 5 and 6). In contrast, dotA conditional mutants induced to express dotA subsequent to macrophage infection do not replicate (Roy et al., 1998). Likewise, csrA dotA double mutants induced to express CsrA after macrophage infection failed to grow (data not shown). *Legionella pneumophila* must traffic immediately to an appropriate compartment to be competent to replicate (Roy et al., 1998). Whereas the Dot/Icm type IV secretion system is essential during phagocytosis but dispensable for replication (Coers et al., 1999), CsrA must be repressed for *L. pneumophila* to evade immediate phagosome-lysosome fusion, but must be active for intracellular replication that occurs in an acidic lysosomal compartment (Sturgill-Koszycki and Swanson, 2000).

While residing in macrophages for 48 h, csrA mutants acquire an atypical, condensed structure that stains...
poorly with DAPI and antibody (Fig. 6). After prolonged infection of HeLa epithelial cells, *L. pneumophila* differentiates into a spor-like Mature Intracellular Form (MIF) that is extraordinarily resilient and infectious (Faulkner and Garduno, 2002; Garduno et al., 2002). Because loss of *csrA* is predicted to lock *L. pneumophila* in the transmissive phase, their unusual morphology may indicate that during extended incubations in macrophages *csrA* mutants differentiate to MIFs.

Although in broth cultures a stringent response-like pathway is sufficient to activate numerous virulence traits of *L. pneumophila* (Hammer and Swanson, 1999), other signal transduction pathways are also likely to contribute to virulence expression. For example, even when CsrA activity was lacking in exponential phase cultures, *L. pneumophila* did not produce pigment nor become fully heat resistant or sodium-sensitive, indicating that these traits may also be regulated independently of CsrA. Furthermore, if the RelA alarmone acts solely via LetA/S to de-repress virulence traits, the *letA* and *relA* mutant phenotypes should be similar. *relA* mutants are partially defective for pigment production and motility, but unlike *letA* mutants, they are as sodium sensitive and cytotoxic as wild-type *L. pneumophila* (Hammer et al., 2002; Zusman et al., 2002). It is likely that *L. pneumophila* encodes redundant and overlapping mechanism(s) to regulate the transmissive phenotype, some of which may be independent of *relA* and the stringent-like response.

An unexpected result was the partial suppression of the sodium resistance of *dotA* mutants by a *csrA* mutation. The mechanism of sodium sensitivity is not understood, but the phenotype is linked to a functional type IV secretion system (Sadosky et al., 1993; Vogel et al., 1996) and the PE phase (Byrne and Swanson, 1998). However, not all sodium-sensitive microbes infect macrophages well (e.g. *fliA* mutants), nor do all sodium-resistant microbes fail to replicate (e.g. *letA* mutants), so the sodium sensitivity phenotype cannot be interpreted precisely. Perhaps loss of *csrA* restores partial function of the type IV secretory apparatus by activating compensatory genes in *lvh*, a region that encodes an alternate type IV secretion system that is dispensable for intracellular replication but contributes to conjugation (Segal et al., 1999). Interestingly, a *csrA* homologue *lvhC* maps within the *lvh* type IV secretion region (Segal et al., 1999), but its function is not known. Therefore, connections between CsrA, *Icm/ Dot*, and *LvH* may warrant further consideration.

Here we have demonstrated that CsrA functions at a regulatory crux, repressing transmission traits while activating replication of the intracellular pathogen *L. pneumophila*. Other pathogens may utilize a similar strategy to achieve reciprocal expression of two physiological states. In this model, intracellular pathogens are designed to either replicate efficiently or to promote their transmission to a new host, but not both. By doing so, microbes would minimize energy loss from production of extraneous transmission traits during the replication period and maximize rapid and coordinated differentiation to a transmissive form in response to environmental cues, thereby linking virulence intimately with general bacterial physiology.

### Experimental procedures

#### Bacterial strains and culture

*Legionella pneumophila* Lp02 (MB110), a virulent thymine auxotroph derived from the Philadelphia 1 strain (Berger and Isberg, 1993), was the parent for all the strains analysed (*Supplementary material, Table S1*). *Legionella pneumophila* was cultured in N-(2-acetamido)-2-aminoethanesulfonic acid (ACES, Sigma)-buffered yeast extract (AYE) broth at 37°C with agitation or on ACES-buffered charcoal yeast extract (CYE) agar at 37°C, both supplemented as necessary with 100 μg ml⁻¹ thymidine (AYET, CYET). Bacteria obtained from colonies <2 weeks old were cultured in broth overnight, then subcultured in fresh AYET for an additional 16–24 h before experimentation. Exponential phase cultures (E) are defined as OD₆₀₀nm 0.3–2.0 and post-exponential cultures (PE) as OD₆₀₀nm 3.0–4.4. Where indicated, kanamycin (kan) was added to a final concentration of 25 μg ml⁻¹, gentamicin (gent) to 10 μg ml⁻¹, chloramphenicol (cam) to 5 μg ml⁻¹, and isopropyl-beta-D-thiogalactopyranoside (IPTG) to 200 μM (*in vitro* culture) or 1 mM (macrophage culture). To ascertain colony-forming units (CFU), serial dilutions of bacteria were incubated on CYET for 4 days and resultant colonies counted. All cloning was done in the *E. coli* DH5α strain using standard molecular techniques.

#### Macrophage cultures

Bone marrow-derived macrophages were isolated from the femur exudates of A/J mice (Jackson Laboratory) and cultured as described (*Supplementary material, Appendix A1; Swanson and Isberg, 1995*).

#### Phenotypic analysis of PE phase traits

Cytotoxicity of *L. pneumophila* for bone marrow-derived macrophages was quantified by incubating microbes in RPMI/FBS with macrophages for 1 h at various multiplicities of infection (MOI), then removing microbes and adding RPMI/FBS + 10% Alomar Blue (AccuMed) for 6–12 h (*Supplementary material, Appendix A1; Byrne and Swanson, 1998; Hammer and Swanson, 1999*). Sodium sensitivity was calculated by plating 10-fold serial dilutions of broth cultures into PBS onto CYET agar with or without 100 mM NaCl, then enumerating CFU after a 5–6 day incubation as described (Byrne and Swanson, 1998). Infectivity is a gauge of the ability of *L. pneumophila* strains to bind, enter, and survive inside murine bone marrow-derived macrophages during a 2-h incubation, as previously described (*Supplementary material, Appendix A1; Byrne and Swanson, 1998*). The ability of *L. pneumophila* strains to withstand a heat stress or osmotic shock was...
were done for 1 h at 37°C diluted 1:2000 (Molecular Probes). Incubations with antibody washes in 2% goat serum in PBS to reduce non-specific binding were performed. To gauge promoter activity, GFP production by several independent clones of pcsrAgfp, pflaAgfp, or pTPL6-flaAgfp containing L. pneumophila was quantified by fluorometry, as described (Supplementary material, Appendix A1; Hammer and Swanson, 1999).

Promoter activity of csrA and flaA in broth cultures

To assess csrA expression, the csrA promoter was fused to a promoterless gfp to generate the plasmid pcsrAgfp (Supplementary material, Appendix A1), which was transferred to Lp02 by electroporation. To gauge promoter activity, GFP production by several independent clones of pcsrAgfp, pflaAgfp, or pTPL6-flaAgfp containing L. pneumophila was quantified by fluorometry, as described (Supplementary material, Appendix A1; Hammer and Swanson, 1999).

Fluorescence microscopy

The activity of the csrA and flaA promoters in vivo was quantified by fluorescence microscopy. Macrophages were infected at an MOI of <1.0 with PE phase cultures of Lp02 pcsrAgfp (MB469) and Lp02 pflaAgfp (MB355). To synchronize the infection, microbes were centrifuged onto prechilled coverslips monolayers for 10 min at 400 g, and then incubated at 37°C for an additional 10–30 min. Next, extracellular microbes were washed away and fresh media was added. At desired time points, coverslips were fixed for 30 min with prewarmed periodate-lysine-2.5% paraformaldehyde (McLean and Nakane, 1974), then washed extensively with PBS. Fixed cells were permeabilized with ice-cold methanol. Anti-Legionella rabbit serum (gift of Dr R. Isberg, Tufts University School of Medicine, Boston, MA, USA) was diluted 1:2000 into 2% goat serum in PBS and was detected by fluorescein isothiocyanate-conjugated rabbit antirabbit secondary antibodies diluted 1:2000 (Molecular Probes). Incubations with antibody were done for 1 h at 37°C and were followed by several washes in 2% goat serum in PBS to reduce non-specific binding. Macrophage and bacterial DNA was stained with 4′,6-diamidine-2-phenylindole (DAPI) at 0.5 μg ml⁻¹. Pilot experiments determined that 16 h post infection was optimal for visualizing vacuoles of replicating L. pneumophila, which were defined as tightly associated groups of five or more bacteria. Vacuoles were scored as GFP positive if >50% of the bacteria emitted green fluorescence above background. Duplicate coverslips were examined for each sample, and >50 replicative vacuoles were scored. Microscopy was performed with a Zeiss Axioplan 2 fluorescence microscope equipped with a 100× Plan-Neofluor objective of numerical aperture 1.3. Images were captured on a Spot digital camera (Diagnostics Instruments).

The percent of intact microbes after a 2 h incubation in macrophages was determined by fluorescence microscopy (Supplementary material, Appendix A1; Bachman and Swanson, 2001). csrA mutant strains and wild-type controls were also visualized by fluorescence microscopy after extended periods inside macrophages. At the desired time post infection, preparations were fixed and processed for fluorescence microscopy as described above for the promoter-gfp fusion experiments.

Construction of csrA conditional null alleles

To create two csrA null alleles, first the csrA coding sequence was deleted and replaced with the kan or gent antibiotic resistance cassettes to create pGEM-ΔcsrA-Kan and pGEM-ΔcsrA-Gent (Supplementary material, Appendix A1). Next, the entire 4.2 kb or 3.6 kb mutant csrA genomic region from each plasmid was amplified by PCR using primers csrAup and csrAdown and the High Fidelity PCR kit (Roche), then the mutant allele was transferred by natural transformation (Stone and Abu Kwaik, 1999) into the wild-type Lp02 or the desired mutant background using the method of Dr Joseph Vogel (Washington University, St Louis, MO, USA), as described previously (Supplementary material, Appendix A1; Bachman and Swanson, 2001).

To transfer the csrA mutant allele onto the wild-type Lp02 chromosome, several independent natural competence experiments were performed with DNA amplified from pGEM-ΔcsrA-Kan and pGEM-ΔcsrA-Gent. Of ~50 independent antibiotic resistant colonies screened by colony PCR for the presence of a mutant-sized csrA locus, all colonies screened retained the wild-type sized csrA allele, whereas a control experiment performed in parallel yielded three of three desired homologous recombinants. When creation of a csrA mutant was attempted in letA-22::kan (MB414), tflA-35::kan (MB410), rpoS120 (MB380), and letA-22::kan rpos120 (MB461) mutant backgrounds, no csrA mutants were identified among the 5–10 colonies of each that were screened. Therefore, to bypass the apparent slow growth and/or lethality of csrA mutants, a conditional null strategy was adopted.

To generate a strain in which csrA expression could be induced, we recombined the mutant csrA chromosomal locus into a wild-type strain transformed with pMMP206:mob-csrA (p206-csrA), a plasmid encoding a tightly regulated csrA ORF whose expression could be induced by IPTG (see Supplementary material, Appendix A1 for details).

Lp02 p206-csrA (MB477) was cultured with IPTG and 1 μg amplified DNA from either pGEM-ΔcsrA-Gent or pGEM-ΔcsrA-Kan, then transformants were selected on media that contained IPTG and the appropriate antibiotic. Several independent csrA conditional null clones containing either the gent (csrA::gent p206-csrA; MB465) or kan (csrA::kan p206-csrA; MB464) csrA alleles were isolated, verified by PCR and antibiotic resistance tests, then analysed in phenotypic assays. The conditional mutants were maintained on solid medium with appropriate antibiotics and 200 μM IPTG, as uninduced bacteria grew very poorly, and stable suppressors of the slow growth phenotype eventually arose after >5 days. When cultured in broth without IPTG, all clones displayed similar slow growth and premature motility and cytotoxicity during exponential phase, indicating that the phenotypes observed were due to the disrupted csrA locus and not unknown second site mutation(s). Conditional mutants maintained for 3–4 days in the exponential phase through serial backdilutions of broth cultures did not increase...
in growth rate, indicating suppressors of the slow growth phenotype did not readily arise in broth culture. To examine the effects of a csrA null phenotype in broth cultures, IPTG, which induces expression of csrA from the p206-csrA plasmid, was withdrawn as described elsewhere.

csrA double mutants were created by a similar strategy using the csrA::gent conditional null strain (MB465) or csrA::kan strain (MB460) cultured with IPTG as the recipient and fliA, letA, and dotA mutant PCR products (Supplementary material, Appendix A1) as the donor alleles. Several independent colonies of the putative csrA double mutants were verified by PCR to contain fliA, letA, or dotA mutant alleles, respectively, in combination with the csrA mutant locus. Appropriate antibiotic resistances were verified for all double mutants. Additionally, two independent isolates of each double mutant were assayed for broth growth, cytotoxicity and heat resistance; in all cases, the two isolates behaved similarly. These strains were maintained on CYET + IPTG to ensure expression of plasmid-borne csrA.

To study the phenotype of csrA single or double mutants, colonies were inoculated in AYET broth that contained IPTG/ cam, cultured overnight to E phase, then washed and sub-cultured in AYET/cam broth without IPTG for 16–24 h to allow intracellular stores of CsrA to deplete before experiments were performed. The wild-type control for all csrA mutant experiments was Lp02 p206-invcsrA (MB463). For infectivity, cytotoxicity, sodium-sensitivity, heat-resistance, and intracellular growth assays, CFU were plated on CYET/ IPTG. As a test of genetic stability, dilutions were also plated on CYET/IPTG + cam, kan, or gent; no difference in CFU yield on medium ± antibiotics was noted, indicating stable maintenance of the csrA, csrA fliA, csrA letA, and csrA dotA alleles and the p206-csrA plasmid.

CsrA constitutive expression plasmid

To complement csrA conditional null studies, a plasmid was engineered to yield constitutive expression of csrA when induced with IPTG. The ~1 kb Ndel-EcoRI fragment containing 350 bp 5’ and 400 bp 3’ to the csrA ORF obtained from pGEM-csrA was cloned into the MCS of pMMBGent-Δmob (Hammer and Swanson, 1999), a derivative of pMMB67EH (Frey et al., 1983). Similar to pMNB206, pMMBGent is an RSF1010 plasmid, but is marked with gent resistance and has a Pmac IPTG responsive promoter known to drive high levels of expression of the downstream gene (Hammer and Swanson, 1999). The resultant plasmid pMMBGent-Δmob-csrA (pcsrA) was electroporated into wild-type Lp02 (MB472), letA-22.Δkan (MB476), fliA-35.Δkan (MB475), and rpoS120 (MB474) (Hammer et al., 2002). Plasmid construction was verified by diagnostic restriction digest and complementation of an E. coli csrA mutant. pcsrA strains were cultured in AYET/gent + IPTG to maintain constitutive csrA expression. In all experiments with Lp02 pcsrA (MB472), wild-type Lp02 containing the empty pMMBGent-Δmob vector (MB473) was used as a control strain.

Acknowledgements

We thank Dr Mike Bachman and Brenda Byrne for their helpful discussions and insight, and J. D. Sauer, K. T. Young, and Anna Molofsky for review of the manuscript. This work was supported by the University of Michigan Genetics Training Grant, NIH grant AI 44212–01, and the University of Michigan Medical Scientist Training Program.

Supplementary material

The following material is available from http://www.blackwellpublishing.com/products/journals/suppmat/mmi/mmi3706/mmi3706sm.htm

Fig S1. L. pneumophila requires the CsrA repressor to grow efficiently in broth.

Fig S2. LetA induces and CsrA represses cocccoid cell morphology.

Fig S3. LetA/S activates and CsrA represses pigment production by PE phase L. pneumophila.

Table S1. Bacterial strains, plasmids and primers.


References


McLean, I.W., and Nakane, P.K. (1974) Periodate-lysine-


