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RpoS co-operates with other factors to induce *Legionella pneumophila* virulence in the stationary phase

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Summary

Legionella pneumophila **replicates within amoebae and macrophages and causes the severe pneumonia Legionnaires' disease. When broth cultures enter the post-exponential growth (PE) phase or experience amino acid limitation, *L. pneumophila* accumulates the stringent response signal (p)ppGpp and expresses traits likely to promote transmission to a new phagocyte. The hypothesis that a stringent response mechanism regulates *L. pneumophila* virulence was bolstered by our finding that the avirulent mutant Lp120 contains an internal deletion in the gene encoding the stationary phase sigma factor RpoS. To test directly whether RpoS co-ordinates virulence with stationary phase, isogenic wild-type, *rpoS-120* and *rpoS* null mutant strains were constructed and analysed. PE phase *L. pneumophila* became cytotoxic by an RpoS-independent pathway, but their sodium sensitivity and maximal expression of flagellin required RpoS. Likewise, full induction of sodium sensitivity by experimentally induced (p)ppGpp synthesis required RpoS. To replicate efficiently in macrophages, *L. pneumophila* used both RpoS-dependent and -independent pathways. Like those containing the *dotA* type IV secretory apparatus mutant, phagosomes harbouring either *rpoS* or *dotA rpoS* mutants rapidly acquired the late endosomal protein LAMP-1, but not the lysosomal marker Texas red–ovalbumin. Together, the data support a model in which RpoS co-operates with other regulators to induce *L. pneumophila* virulence in the PE phase.**

Introduction

Legionella pneumophila, a Gram-negative aquatic bacterium, causes the severe pneumonia Legionnaires' disease. Infection with *L. pneumophila* is estimated to be the third or fourth leading microbial cause of community-acquired pneumonia, comprising 2–15% of cases requiring hospitalization (for a review, see Swanson and Hammer, 2000). Unfortunately, the mortality rate can approach 30–40% if the pneumonia is not treated promptly with antibiotics effective against *Legionella* (Muder, 1989).

In the environment, *L. pneumophila* is found in fresh water, where it probably replicates within amoebae (Rowbotham, 1980) and multispeciated biofilms (Rogers and Keevil, 1992; Rogers *et al.*, 1994). If humans inhale aerosolized water from sources contaminated with *L. pneumophila*, such as cooling towers, potable water supplies and even vegetable sprayers, the bacteria can invade and replicate within alveolar macrophages and cause disease (Swanson and Hammer, 2000).

L. pneumophila is able to replicate within bactericidal phagocytes by blocking the immediate fusion of its phagosome with lysosomes and, instead, associating first with mitochondria and later with rough endoplasmic reticulum (Horwitz, 1983a, b; Horwitz and Maxfield, 1984; Swanson and Isberg, 1995). By >12 h after infection, when replication is under way, the majority of *L. pneumophila* vacuoles acquire lysosomal characteristics, including the late endosomal and lysosomal proteins LAMP-1 and cathepsin D, access to the endocytic pathway and an acidic pH. In addition, the bacteria are acid resistant (Sturgill-Koszycki and Swanson, 2000). Thus, maturation of the *L. pneumophila* phagosome into a replication vacuole resembles autophagy (Swanson and Isberg, 1995; Sturgill-Koszycki and Swanson, 2000), a ubiquitous eukaryotic process in which vesicles derived from the endoplasmic reticulum deliver host organelles and other cytoplasmic contents to the lysosomes (Dunn, 1994). In such a vacuole, the *L. pneumophila* would have access to endocytosed nutrients.

By 24 h after infection, *L. pneumophila* express traits that probably promote its transmission to a new host phagocyte. We predict that virulence expression by intracellular bacteria is a response to nutrient deprivation, based on studies of *L. pneumophila* broth cultures. Upon entry into stationary phase or after transfer to spent medium, the cultures become cytotoxic to macrophages, motile and competent to evade lysosomes, whereas

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Table 1. Strains, primers and plasmids used.

Strain	Relevant genotype/phenotype	Reference or source
E. coli		
ZK918	*rpoS::kan* λMAV103 (*bolA::lacZ* transcriptional fusion)	Bohannon *et al.* (1991)
JM109	F′ *traD36 proA*$^+$ *proB*$^+$ *lacI*^{q} *lacZΔM15/recA1 endA1 gyrA96* (Nal^r) *thi hsdR17 supE44 relA1 Δ(lac-proAB) mcrA*	Laboratory collection
DH5α λpir	F-*endA1 hsdR17* (r$^-$ m$^+$) *supE44 thi-1 recA1 gyrA* (Nal^r) *relA1 Δ(lacZYA-argF)*$_{U169}$ φ80dL*acZΔM15* λ*pir*RK6	Laboratory collection
L. pneumophila		
Lp02	Str^R, Thy$^-$, HsdR$^-$	Berger and Isberg (1993)
Lp03	Lp02 *dotA* ^{676}CAA → ^{676}TAA	Berger and Isberg (1993)
Lp120	Lp02 EMS mutant *rpoS* Δ215–283	Swanson and Isberg (1996)
MB 379	Lp02 *rpoS::kan*	This work
MB 380	Lp02 *rpoS* Δ215–283	This work
MB 381, 382	Lp03 *rpoS::kan*	This work
Primers		
rpoS 1	5′-GCGCGTTAATGCAGGGCAGG	Hales and Shuman (1999)
rpoS 2	5′-CCAAAGAACTACTGGCAAG	Hales and Shuman (1999)
rpoS N	5′-ATCCACCAGGTCGCATAAGTTGAAA	This work
Plasmids		
pBluescript KS+	*oriR* (ColE1) Amp^r	Stratagene
pUC4K	pUC4 containing Km^r GenBlock	Pharmacia
pKB5	*Eco*RI fragment containing *tdΔi in* pMMB67EH	Berger and Isberg (1993)
Lp01 genomic library	*Sau*3AI partially digested Lp01 genomic DNA fragments in pKB5	Berger and Isberg (1993)
pDEB2	2.4 kb *Kpn*I fragment containing *E. coli rpoS* in pUC19	Bohannon *et al.* (1991)
pKAS32	pGP704 *rpsL*	Skorupski and Taylor (1996)
pKAS46	pKAS32 Km^r	Skorupski and Taylor (1996)
pGEMT-Easy	MCS within coding region of β-lactamase α-fragment linearized with single-T overhangs, Amp^r	Promega
pMMBrelA	*Pst*I–*Eco*RI fragment containing *E. coli relA*, *Eco*RI fragment containing Gm^r, in pMMB67EH, Amp^s	Hammer and Swanson (1999)
pflaG	150 bp *flaAp* fragment fused to *GFPmut3* in pKB5 with pTac and lacI^q removed	Hammer and Swanson (1999)
pMB383	pKB5 with 6.8 kb *Sau*3AI–*Bam*HI *rpoS* fragment	This work
pMB384	pKB5 digested with *Age*I and ligated to remove *mobA* ATG site	This work
pMB385	*Sac*I–*Xba*I fragment containing 6.8 kb *rpoS* locus in pBluescript KS+	This work
pMB386	Km^r from pUC4K ligated into *Mlu*I, *Hpa*I-partial digest sites of pMB385 to generate *rpoS::kan* allele	This work
pMB387	*rpoS::kan* PCR product in pGEMT-Easy	This work
pMB388	*Not*I fragment of pMB387 containing *rpoS::kan* allele in pKAS32	This work
pMB389	pGEMT-Easy containing *rpoS-120* PCR product	This work
pMB390	*Not*I fragment of pMB389 containing *rpoS-120* in pKAS46	This work
pMB391 (pLP*rpoS*)	pMB383 digested with *Age*I and ligated to remove *mobA* ATG site	This work
pMB392 (pLP*rpoS*Δ)	pMB384 containing the 7.4 kb *rpoS::kan* locus from pMB386	This work

supplementation of the spent medium with amino acids abrogates virulence expression (Byrne and Swanson, 1998).

In *Escherichia coli*, limiting concentrations of amino acids are detected in the form of their cognate uncharged tRNAs by the ribosome-associated protein RelA. In response, RelA converts GTP to (p)ppGpp, a modified guanosine molecule that acts as a starvation signal for the cell and mediates a physiological change known as the stringent response (for a review, see Cashel *et al.*, 1996). In this rapid adaptation, synthesis of stable RNAs and proteins is inhibited, growth rate declines immediately, and traits that promote persistence in nutrient-poor environments are induced. Similar to the stringent response in *E. coli*, a (p)ppGpp signalling cascade in *L. pneumophila* induces traits that may promote transmission to a new host (Hammer and Swanson, 1999).

The phenotype of one avirulent mutant predicts that *L. pneumophila* encodes a global transcriptional regulator of virulence that is under the positive control of (p)ppGpp. In response to nutrient limitation, strain Lp120 exits exponential growth phase and accumulates (p)ppGpp but, unlike wild type, it does not express any of six virulence traits measured (Hammer and Swanson, 1999). *E. coli* RpoS, a sigma factor that co-ordinates the expression of stationary phase traits, including resistance to oxidative, acidic and mutagenic stress, is under the positive control of (p)ppGpp (Gentry *et al.*, 1993; Hengge-Aronis, 1996).

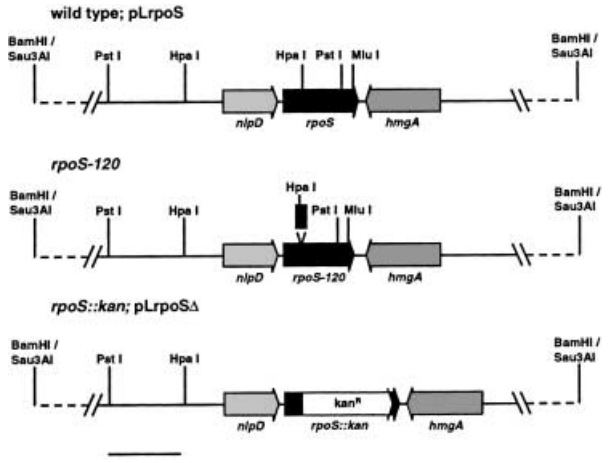

Fig. 1. Physical maps of the *rpoS* alleles studied. The wild-type *rpoS* allele is 1026 bp, encoding a 341-amino-acid protein with 71% similarity to *Pseudomonas aeruginosa* RpoS. The *rpoS-120* allele contains a 69 bp in frame deletion removing the *Hpa*I restriction site and is predicted to encode a protein lacking amino acids 72–94. The *rpoS* null allele, *rpoS::kan*, was constructed by replacing 693 bp of the coding region with a kan^R cassette. Arrows indicate the direction of transcription; dashed lines represent flanking regions contained within the 6.8 kb *Sau*3AI genomic locus (designated L) cloned into the *Bam*HI site of plasmids pLrpoS and pLrpoSΔ and used for complementation studies. Not pictured are the upstream *surE* and downstream *yebC* and *ruvC* genes, which are all transcribed from the same DNA strand as *rpoS*. Scale bar = 1 kb.

Therefore, we postulated that *L. pneumophila* virulence is co-ordinated with stationary phase by RpoS, a factor potentially lacking in mutant Lp120.

By constructing and characterizing an *rpoS* mutant, Hales and Shuman (1999) showed that *L. pneumophila* requires RpoS to grow within the protozoa *Acanthamoeba castellanii*, but not within the HL-60 or THP-1 macrophage-like cell lines. Whether *rpoS* mutations affect other traits correlated with virulence was not examined. However, other phenotypes of the *L. pneumophila rpoS* mutant were surprisingly different from the analogous *E. coli* mutants: PE phase cells remained resistant to acidic pH, hyperosmolarity and H_2O_2. All three of the transposon mutations analysed lay within the *rpoS* 3′ region, leaving open the possibility that each of the alleles encodes a protein with partial function. Nevertheless, taken together, the previously published data are consistent with the hypothesis that, rather than play a role in stress resistance, RpoS of *L. pneumophila* regulates traits required for persistence in its environmental niche.

To test this model, we examined the effect of an *rpoS* null mutation on co-ordinate expression of a panel of traits implicated in transmission, the stringent response-like pathway and the fate of *L. pneumophila* within primary mouse macrophages. Our findings demonstrate that RpoS mediates a subset of the *L. pneumophila* responses to nutrient limitation and the alarmone (p)ppGpp, including sodium sensitivity, maximal motility and evasion of the endosomal pathway within macrophages.

Results

Cloning and sequencing of the L. pneumophila rpoS *gene*

To isolate the *L. pneumophila rpoS gene*, an *L. pneumophila* genomic library (Table 1) was screened for clones that complement an *E. coli rpoS* mutation. Transformants of ZK918, an *E. coli* strain that contains an *rpoS::kan* null allele and an RpoS-dependent *bolAp1::lacZ* reporter fusion (Table 1), were plated on MacConkey agar; then complemented mutants were identified by their capacity to ferment lactose and thereby generate red colonies. Restoration of RpoS function in each candidate strain was confirmed by a visual test of catalase activity, a second RpoS-dependent trait (Bohannon *et al*., 1991; Yildiz and Schoolnik, 1998). A single β-galactosidase-positive, catalase-positive clone was identified, and the library plasmid pMB383 was purified.

Nucleotide sequencing of the 6.8 kb genomic fragment of pMB383 revealed a 1026 bp open reading frame (ORF) encoding a 342-amino-acid RpoS protein, as judged by three criteria. The predicted protein was 71% identical to *Pseudomonas aeruginosa* RpoS. Similar to *Vibrio cholerae and E. coli*, the *L. pneumophila rpoS* locus lies within an operon with *nlpD* (Fig. 1), which encodes a novel lipoprotein. Finally, using a similar strategy, Hales and Shuman (1999) identified the same locus (GenBank accession no. AF117715), which they demonstrated encodes a protein recognized by antiserum to the RpoS protein of *E. coli*.

Avirulent mutant Lp120 encodes an RpoS protein lacking 23 amino acids

Originally identified as defective for growth in macrophages, mutant Lp120 was later shown to have a pleiotropic phenotype (Swanson and Isberg, 1996; Hammer and Swanson, 1999). Based on its defective contact-dependent cytotoxicity, evasion of the macrophage endosomal pathway, sodium sensitivity, motility and flagellin or *flaA* expression, we speculated that mutant Lp120 lacked a global regulator of virulence. To begin to address the role of RpoS in virulence regulation, we tested whether a plasmid-borne *rpoS* locus complemented the virulence defects of mutant Lp120.

Some, but not all, of the virulence defects of Lp120 were complemented by *rpoS*. In the PE phase, Lp120 transformants carrying the cloned *rpoS* locus (pMB383) were ≈75-fold more sodium sensitive than the mutant parent, and their expression of a *flaAgfp* reporter (Hammer and Swanson, 1999) was restored, as judged

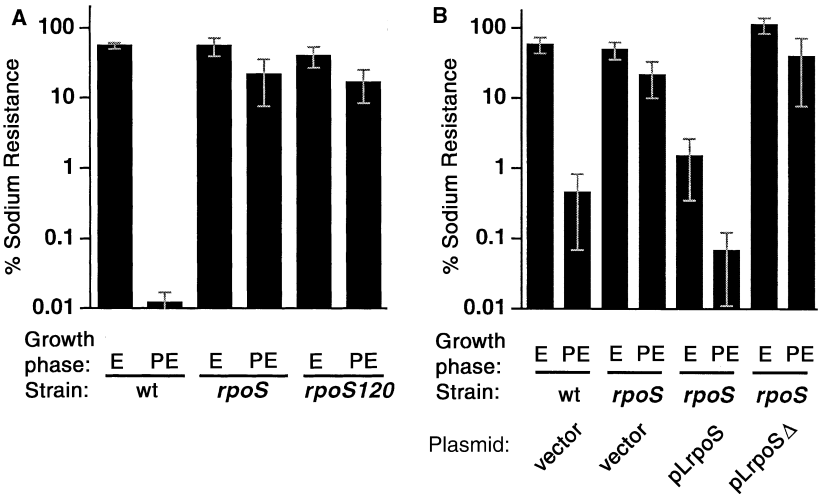

Fig. 2. RpoS is required for the expression of growth phase-dependent sodium sensitivity. A. Sodium sensitivity of exponential (E) or post-exponential (PE) phase broth cultures of *L. pneumophila* wild-type Lp02 (wt), *rpoS* null mutant MB379 or *rpoS-120* mutant MB380 strains. The values shown represent the mean ± standard error determined in at least three independent experiments for each strain. B. A plasmid-borne *rpoS* locus restores growth phase-regulated sodium sensitivity. Sodium sensitivity of E and PE cultures of wild type Lp02 (wt) or *rpoS* null mutant MB379 containing the vector pMB384 or the *rpoS* null mutant strain MB379 containing either pLrpoS (pMB391) or pLrpoSΔ (pMB392) are plotted as the mean ± standard error determined in three independent experiments. Sodium sensitivity was determined in (A) and (B) by plating duplicate samples on CYET and CYET supplemented with 100 mM NaCl and calculating percentage sodium-resistant cfu.

by fluorescence microscopy (data not shown). However, unlike wild type, *rpoS*-containing transformants of Lp120 failed to grow within macrophages or to express contact-dependent cytotoxicity.

To analyse more directly the RpoS function of mutant Lp120, its *rpoS* allele (*rpoS-120*) was amplified, and its nucleotide sequence was determined. As expected, Lp120 contained an *rpoS* mutation: the locus had sustained a large in frame deletion and was predicted to produce a protein that lacked amino acid residues 72–94.

Construction of rpoS *null and* rpoS-120 *mutants*

The partial complementation of mutant Lp120 by *rpoS* could be explained by one of several mechanisms: ectopic expression of *rpoS* may not fully complement an *rpoS* mutation (Iriarte *et al*., 1995; Yildiz and Schoolnik, 1998; Hales and Shuman, 1999); chemical mutagenesis may have generated multiple mutations in strain Lp120; *rpoS-120* may encode a dominant-negative mutant protein. To examine more directly how RpoS contributes to *L. pneumophila* virulence regulation, an *rpoS* null allele and the *rpoS-120* allele were recombined onto the wild-type Lp02 chromosome (Fig. 1; *Experimental procedures*; data not shown). In broth, the *rpoS* null mutant replicated with similar kinetics to wild type, although a modest decrease (≈ two- to fourfold) in recoverable colony-forming units (cfu) was seen for PE phase cultures (data not shown). The basis for the observed disparity between OD_{600} and cfu for cultures of *rpoS* null mutants is not understood.

RpoS is not required for post-exponential phase stress resistance

Previously, Hales and Shuman (1999) reported that PE phase cultures of an *L. pneumophila rpoS* transposon mutant resembled wild type in their resistance to a variety of stresses, including 5 M NaCl, pH 3 citric acid or 10 mM H_2O_2. To verify that resistance of *L. pneumophila* to certain environmental stresses was RpoS independent, we subjected our wild-type and *rpoS* mutant strains to these same harsh conditions for 1 h, then measured their viability. In all cases, the *rpoS* strain survived as well as wild type (data not shown), confirming the assertion that at least some stress resistance of PE phase *L. pneumophila* is *rpoS* independent.

RpoS regulates sodium sensitivity and flagellin expression, but not contact-dependent cytotoxicity

As entry into the PE phase or accumulation of (p)ppGpp induces several *L. pneumophila* virulence traits, we examined whether *rpoS* is required for *L. pneumophila* virulence. Sodium sensitivity of *L. pneumophila* correlates with virulence (Byrne and Swanson, 1998) and a functional Dot/Icm secretion system (Sadosky *et al*., 1993; Vogel *et al*., 1996), which is required for proper trafficking of *L. pneumophila* within macrophages (for a review, see Vogel and Isberg, 1999). Whereas exponential (E) phase cells readily formed colonies on medium containing 100 mM NaCl, PE bacteria exhibited an ≈1000-fold decrease in colony formation. In contrast, PE phase *rpoS* null and *rpoS-120* mutant strains remained sodium resistant (Fig. 2A). As expected, a plasmid-borne *rpoS* locus (pLrpoS; Table 1; Fig. 1) restored sodium sensitivity to the null mutant, whereas pLrpoSΔ, a plasmid constructed in parallel that lacked an intact *rpoS* gene (Table 1; Fig. 1), did not (Fig. 2B). Why carriage of the RSF1010 vector reduced sodium sensitivity (e.g. compare Fig. 2A and B) is not understood.

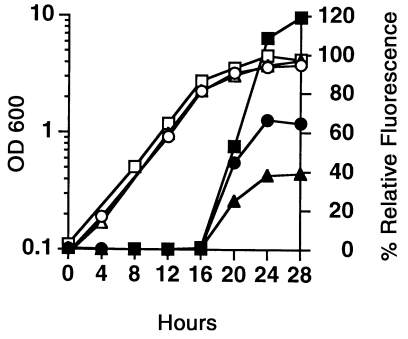

Fig. 3. Growth phase-dependent transcriptional activation of a flagellin promoter fusion (*flaAgfp*) by wild-type and *rpoS* mutant cultures. At the times shown, cultures of *L. pneumophila* wild-type Lp02 (squares), *rpoS* null mutant MB379 (circles) and *rpoS-120* mutant MB380 (triangles) strains containing pflaG were sampled, and their OD_{600} (open symbols) and fluorescence (closed symbols) were measured. Fluorescence units are relative to a PE phase Lp02 reference culture containing the *flaAgfp* reporter construct. The values shown are representative of three independent experiments.

Ectopic *rpoS* expression also increased sodium sensitivity in E phase, perhaps as a result of multiple gene copies. Thus, the *rpoS* null phenotype is an exception to the general rule that salt resistance is conferred by mutations within loci encoding the Dot/Icm secretion apparatus. Whatever its mechanism, growth phase-dependent expression of sodium sensitivity required RpoS.

To examine whether *L. pneumophila* RpoS regulates flagellin synthesis and motility, expression of a *flaAgfp* reporter (Hammer and Swanson, 1999) by broth cultures of the wild-type and mutant strains was monitored at 4 h intervals. E phase wild-type cells were not fluorescent; upon entering the PE phase, the wild-type strain reached 120% relative fluorescence. In contrast, neither the *rpoS-120* nor the *rpoS* null mutant fully induced *flaAgfp* expression in the PE phase: the mutants emitted only one-third and one-half of the wild-type fluorescence respectively (Fig. 3). Furthermore, the *rpoS* mutants were less motile than wild type, as judged by light microscopy of PE phase cultures (data not shown). Thus, RpoS contributes to the PE phase induction of *flaA* expression by *L. pneumophila*.

A contact-dependent cytotoxicity of *L. pneumophila* for macrophages is co-ordinately expressed with sodium sensitivity and motility in the PE phase (Byrne and Swanson, 1998). Insertion of pores by *L. pneumophila* into phagocyte membranes probably causes osmotic lysis (Kirby *et al.*, 1998) and may promote bacterial escape from host cells (Byrne and Swanson, 1998; Alli *et al.*, 2000). To assess the cytotoxicity of the *rpoS* mutants, PE phase cultures were incubated with macrophages for 1 h; then metabolic reduction of the colorimetric dye Alamar blue by viable macrophages was measured. Surprisingly, PE phase *rpoS-120* and *rpoS* null mutants were each as cytotoxic as wild type (Fig. 4). Therefore, the cytotoxicity of *L. pneumophila* is co-ordinated with the PE phase by an RpoS-independent mechanism.

Interestingly, expression of multiple copies of the wild-type *rpoS* gene inhibited the expression of some virulence traits. For example, the *rpoS* mutant carrying pLrpoS was defective for cytotoxicity to macrophages, and it also lost the residual motility seen in *rpoS* null mutants. Similarly, wild-type bacteria transformed with pLrpoS were somewhat less motile than control cultures (data not shown). These dominant-negative effects were attributable solely to ectopic RpoS expression, as the *rpoS* mutant containing the pLrpoSΔ control plasmid remained cytotoxic and somewhat motile (data not shown).

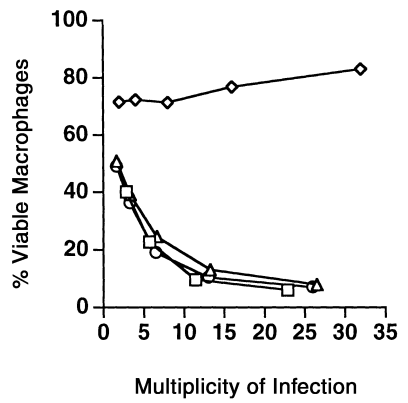

Fig. 4. Cytotoxicity to macrophages of PE phase *L. pneumophila* is RpoS independent. Macrophages were incubated for 1 h with wild-type Lp02 exponential (E) (diamonds) or post-exponential cultures (PE) (squares) or *rpoS* mutant MB379 PE (triangles) or *rpoS-120* mutant MB380 PE cultures (circles); then macrophage viability was assessed by quantifying reduction of the colorimetric dye Alamar blue. Means are shown for triplicate samples from one representative experiment of two to five performed for each *rpoS* mutant strain. All standard errors were less than 11% of the values plotted.

Induction of sodium sensitivity by ppGpp requires RpoS

The stringent response model of virulence postulates that the second messenger (p)ppGpp co-ordinates virulence expression with entry into the PE phase, in part by positively regulating RpoS. Yet, two observations argue that an RpoS-independent factor or factors must also mediate the physiological response to (p)ppGpp. In response to gratuitous RelA expression, the *rpoS* mutant Lp120 exits exponential phase (Hammer and Swanson, 1999). Also, in the PE phase or in response to (p)ppGpp, wild-type *L. pneumophila* become cytotoxic, a trait that is RpoS independent (Fig. 4).

To determine whether (p)ppGpp can also induce sodium sensitivity by an RpoS-independent pathway, E phase cultures of wild-type and *rpoS* mutant strains containing the inducible *E. coli relA* plasmid pMMBrelA

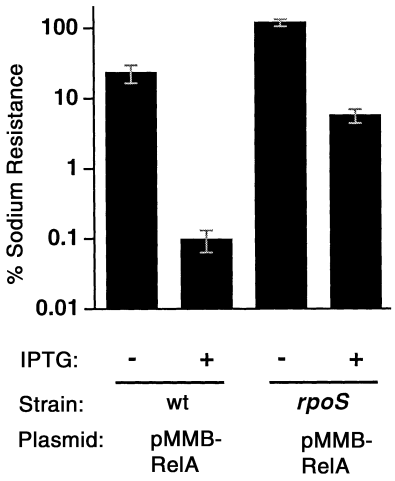

Fig. 5. Induction of maximal sodium sensitivity by experimentally induced RelA expression is RpoS dependent. Wild-type Lp02 (wt) and *rpoS* null mutant MB379 strains containing pMMBRelA were cultured to $OD_{600} \approx 0.4$, either induced for 4.5 h with IPTG (+) or not (–); then their sodium sensitivity was determined by plating duplicate samples on CYET and CYET supplemented with 100 mM NaCl. The values plotted represent the mean ± standard error determined in two independent experiments.

were analysed. As expected, after induction for 4.5 h with 200 μM IPTG, the apparent growth rate of the wild-type and *rpoS* mutant strains slowed, and both strains became cytotoxic (data not shown). However, relative to the controls, *rpoS* mutant cultures became only somewhat salt sensitive (Fig. 5). Therefore, RpoS and perhaps another factor(s) are required to induce maximal sodium sensitivity in response to the starvation signal ppGpp.

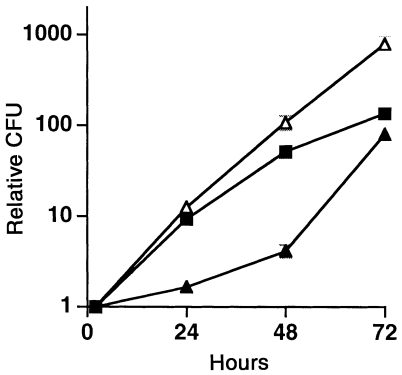

Fig. 6. Robust intracellular growth of *L. pneumophila* in macrophages is RpoS dependent. Macrophages were infected for 2 h at an MOI of 0.1–0.5 with PE phase wild-type Lp02 containing the vector pMB384 (squares) or *rpoS* mutant MB379 containing either the same vector (closed triangles) or pMB391 (pLrpoS, open triangles); then macrophages were washed to remove extracellular bacteria. At the times indicated, macrophages were lysed with PBS + 0.05% saponin and plated to quantify cfu. The values plotted represent the mean cfu ± standard error from duplicate or triplicate wells at each time point and are representative of two independent experiments.

L. pneumophila *requires RpoS to establish a replicative niche in bone marrow-derived macrophages*

Previously, an *rpoS* transposon insertion mutant was shown to replicate within the THP-1 and HL-60 macrophage-like cell lines, but not the protozoa *A. castellanii*, suggesting a species-specific role for RpoS (Hales and Shuman, 1999). On the other hand, the avirulent *L. pneumophila* strain Lp120, which at a minimum contains an *rpoS* mutation, is attenuated for intracellular growth and evasion of lysosomes in primary mouse macrophages (Swanson and Isberg, 1996). Therefore, we next examined whether RpoS regulates traits required for the infection of primary macrophages.

Murine bone marrow-derived macrophages were infected with either the wild-type or the *rpoS* mutant strain; then the relative increase in cfu of each strain during a 3 day infection period was compared (Fig. 6). By 48 h, the number of wild-type *L. pneumophila* increased ≈ 50-fold, whereas the yield of the *rpoS* mutant cfu had increased only fourfold. The defective intracellular growth phenotype of the mutant strain was a consequence of its *rpoS* null mutation: a similar growth defect was observed for each of three independent *rpoS::kan* mutant isolates (data not shown), and an *rpoS* null strain containing the pLrpoS plasmid replicated similarly to wild type (Fig. 6). Interestingly, from 48 to 72 h, we consistently observed an increase in the apparent replication rate of the *rpoS* mutant strains, so that their yield at 72 h was nearly identical to that of wild type (see *Discussion*).

L. pneumophila rpoS *mutants persist but rarely replicate within macrophages*

The observation that *rpoS* mutants initially replicate poorly in macrophages could reflect any of three types of defects: each intracellular *rpoS* bacterium replicates more slowly than wild type; a subpopulation of bacteria persists without replication; or a subpopulation of bacteria are killed by the macrophage. To distinguish between these possibilities, the number of bacteria per macrophage vacuole was assessed microscopically during the primary infection period. By fluorescently staining infected macrophages with an anti-*Legionella* antibody, both intact and degraded bacteria could be quantified. By 18 h after infection, PE phase wild-type *L. pneumophila* replicated to high numbers; vacuoles often contained more than 20 bacteria (Fig. 7A, images 4–6, and B). In contrast, E phase wild-type cells, which do not evade lysosomal fusion (Byrne and Swanson, 1998), were degraded and distributed throughout the lysosomal network (Fig. 7A, images 1–3, and B).

Even 18 h after infection, most *rpoS* mutant bacteria remained as single intact rods (Fig. 7A, images 7–9):

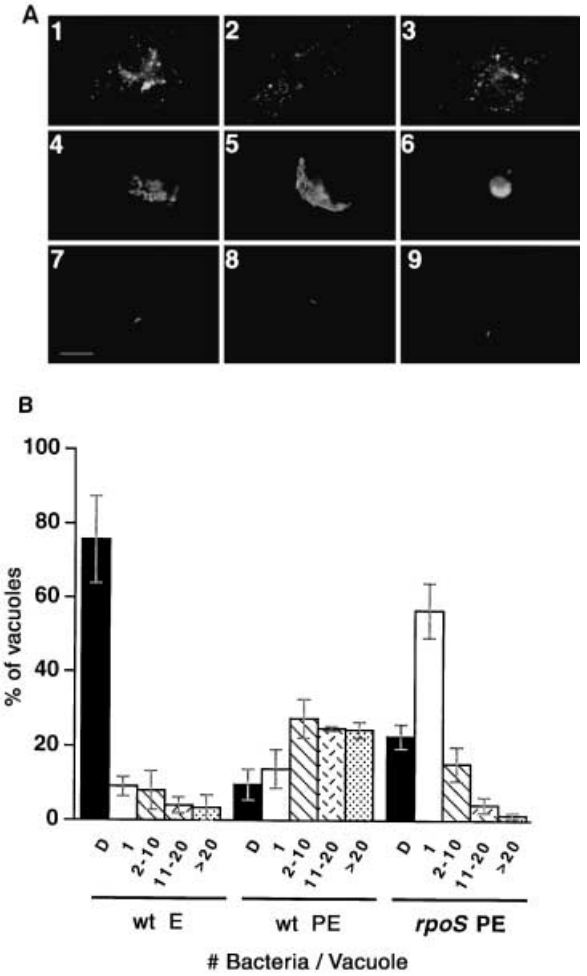

Fig. 7. The majority of intracellular *rpoS* bacteria are arrested at the single-cell stage.
A. Fluorescence micrographs of mouse bone marrow-derived macrophages 18 h after infection with *L. pneumophila*. Macrophages plated on coverslips were infected for 2 h at a low MOI to ensure singly infected macrophages (MOI of 0.5–2 for E phase wild type and MOI of 0.1–0.5 for PE phase wild type or *rpoS* mutant MB379). At 18 h, cultures were fixed, stained fluorescently with *L. pneumophila* specific antibody, and the number of bacteria per vacuole were scored. The predominant growth phenotypes seen for each strain in four independent experiments are represented: E phase wild type that have been degraded (1–3), PE phase wild type that have replicated (4–6) and PE phase *rpoS* mutant bacteria that persist as single cells (7–9). Scale bar = 10 μM.
B. The frequency of *L. pneumophila* replication observed within primary mouse macrophages 18 h after infection. Each vacuole was scored as one of four categories based on the number of intact rods it contained: 1 [see (A) images 7–9], 2–10, 11–20 and > 20. The > 20 category represents vacuoles containing bacteria too numerous to count accurately [see (A) images 4–6]; based on their size, we estimated that vacuoles in this class may contain as many as 50 bacteria. Antibody-positive, non-rod-shaped particles were scored as degraded (D) bacteria [see (A) images 1–3]. Values plotted are the mean frequency of each growth category ± standard error, scored for 50 vacuoles per strain in three independent experiments.

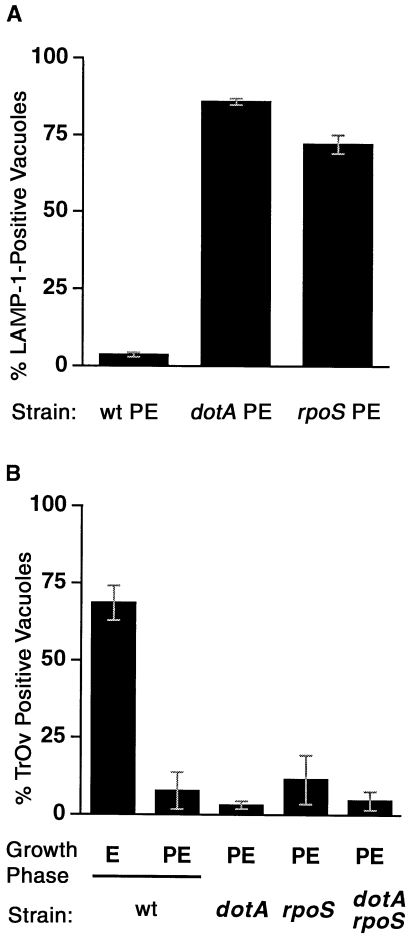

Fig. 8. *L. pneumophila* requires RpoS to establish a vacuole that does not acquire the late endosomal protein LAMP-1.
A. Co-localization of the late endosomal protein LAMP-1 with vacuoles containing wild-type Lp02, *rpoS* mutant MB379 or *dotA* mutant Lp03 PE phase bacteria. Macrophages plated on coverslips were infected at an MOI of 0.1–2 for 2 h; then the late endosomal and lysosomal compartments were stained with the anti-LAMP-1 antibody 1BD4 and an Oregon green-conjugated secondary antibody, and intact bacteria were labelled with DAPI. Co-localization of *L. pneumophila* with LAMP-1 was scored for 50 bacterial vacuoles per coverslip. The values are the mean ± standard error for three (*dotA*) or five (wt, *rpoS*) independent experiments.
B. Post-exponential phase *L. pneumophila* evade lysosomes by an RpoS- and DotA-independent mechanism. Macrophages whose lysosomes were labelled fluorescently by endocytosis of Texas red–ovalbumin (TROv) were infected at an MOI of 0.5–2 for 2 h with E and PE phase wild-type Lp02 and PE phase *rpoS* null mutant MB379, *dotA* mutant Lp03 and *dotA rpoS* double mutant MB381 bacteria. After fixation, the bacteria were stained with an anti-*Legionella* mouse monoclonal antibody and an Oregon green-conjugated anti-mouse secondary antibody, and co-localization of *L. pneumophila* with TROv was scored by fluorescence microscopy for 50 bacterial vacuoles per coverslip in two to five independent experiments. Similar results were obtained with a second *dotA rpoS* mutant clone.

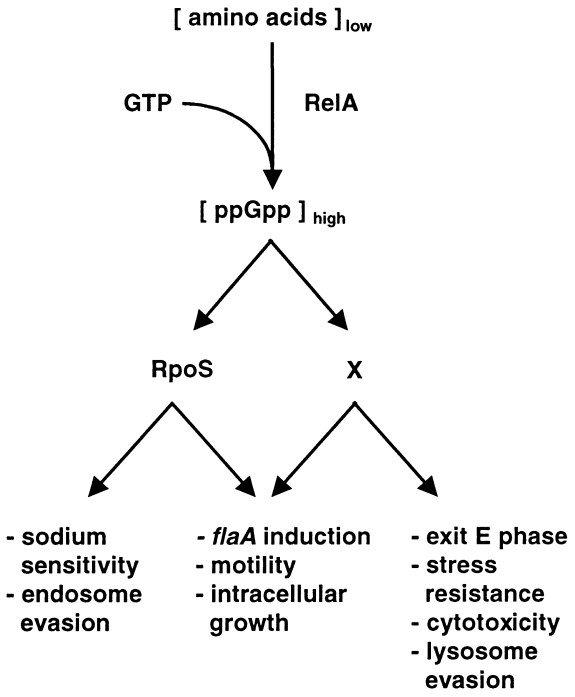

Fig. 9. Stringent response model of *L. pneumophila* virulence regulation. Three categories of phenotype were observed: RpoS-dependent traits, RpoS-independent traits and traits regulated by RpoS and another factor (X) in response to (p)ppGpp accumulation.

57% of the vacuoles contained a single bacterium, and only 5% of the vacuoles contained more than 10 (Fig. 7B). This latter subpopulation of replicating mutants can account for the modest increase in *rpoS* mutant cfu observed during the first 48 h of infection (Fig. 6). Thus, an RpoS-dependent factor(s) promotes either proper trafficking of *L. pneumophila* vacuoles or some subsequent stage of bacterial replication.

An rpoS *mutant traffics rapidly to a late endosome-like compartment*

The persistence of single, intact *rpoS* mutant bacteria 18 h after infection may reflect a failure to construct a replication vacuole, a defect in nutrient acquisition or an intolerance to stress encountered as the *Legionella* vacuole matures into an acidic, lysosomal compartment (Sturgill-Koszycki and Swanson, 2000). To begin to distinguish between these possibilities, the capacity of mutant and wild-type phagosomes to evade the endosomal pathway was assessed by scoring co-localization of intracellular bacteria with the late endosomal and lysosomal protein LAMP-1 and with lysosomal Texas red–ovalbumin (TROv). E phase wild-type cells fail to evade the lysosomal network: ≈75% of these phagosomes contained LAMP-1 (Joshi *et al.*, 2001), and 68% were TROv positive (Fig. 8B). As expected (Swanson and Isberg, 1996; Byrne and Swanson, 1998; Roy *et al.*, 1998; Joshi *et al.*, 2001; Sturgill-Koszycki and Swanson, 2000), in the first 2 h of infection, vacuoles containing PE phase wild-type *L. pneumophila* rarely acquired LAMP-1 (4%; Fig. 8A) or TROv (8%; Fig. 8B). In contrast, phagosomes harbouring the *dotA* type IV secretion mutant Lp03 rapidly acquired LAMP-1, but not TROv (Fig. 8A and B; Roy *et al.*, 1998; Joshi *et al.*, 2001). The trafficking of *rpoS* mutants was strikingly similar to that of *dotA* mutants: 72% of 2-h-old *rpoS*-infected vacuoles had accumulated LAMP-1 (Fig. 8A) but not TROv (Fig. 8B). The large percentage of *rpoS* mutant phagosomes that had mistargeted at 2 h corresponds well with the observation that 57% of vacuoles contained only a single bacterium at 18 h (Figs 7B and 8A).

A previous study predicted that *L. pneumophila* requires both Dot-dependent and -independent factors to evade lysosomes (Joshi *et al.*, 2001). To determine whether the expression of one or both of these pathways requires RpoS, we constructed an *rpoS dotA* double mutant and examined its fate in macrophages. The double mutant behaved similarly to the *rpoS and dotA* single mutants: unlike the majority of phagosomes containing E phase wild-type bacteria, only 5% of *rpoS dotA* vacuoles accumulated lysosomal TROv (Fig. 8B), although the majority were LAMP-1 positive (data not shown). Therefore, upon entry to PE, *L. pneumophila* expresses two classes of factors that block phagolysosome formation: an RpoS- and Dot-dependent activity(ies) prevents accumulation of LAMP-1, and an RpoS- and Dot-independent factor(s) blocks merger with the lysosomes.

Discussion

During its natural life cycle, *L. pneumophila* encounters at least two different habitats: the bacteria replicate within amoebae, then infectious planktonic forms are released into water. Safe transmission of progeny from one host phagocyte to the next may be promoted by the pathogens' capacity to lyse host membranes, to survive hypotonic shock, to swim and to evade lysosomal degradation. Detailed studies of broth cultures indicated that replicating *L. pneumophila* convert to a transmissible form when amino acids are limiting (Byrne and Swanson, 1998). In a manner similar to the *E. coli* stringent response, the accumulation of the alarmone (p)ppGpp by *L. pneumophila* co-ordinates entry into the PE phase with the expression of a number of virulence traits (Hammer and Swanson, 1999). Here, analysis of the virulence profile of strains lacking the stationary phase sigma factor RpoS has allowed further refinement of this model of virulence regulation (Fig. 9). In response to amino acid depletion, the second messenger (p)ppGpp induces RpoS activity,

which then activates the expression of genes that confer sodium sensitivity (Figs 2 and 5) and motility (Fig. 3), the capacity to evade the endosomal pathway (Fig. 8) and the ability to replicate intracellularly (Figs 6 and 7). In parallel, an RpoS-independent factor(s) (Fig. 9, depicted as X) responds to (p)ppGpp by triggering exit from the E phase and inducing cytotoxicity and evasion of lysosomes (Fig. 4). Also, unlike other species of Gram-negative bacteria, *L. pneumophila* becomes resistant to stress in the PE phase by an RpoS-independent mechanism (Hales and Shuman, 1999; data not shown). RpoS and a second pathway appear to co-operate to induce maximal *flaA* expression, motility and intracellular growth, as these activities were diminished but not eliminated in *rpoS* mutants. Thus, the model predicts that, in the PE phase, RpoS acts in parallel with additional transcriptional regulators to co-ordinate the expression of traits that promote survival and transmission of *L. pneumophila*.

Other pathogenic bacteria also rely on RpoS to survive and flourish within varied host environments. For example, the food-borne enteric pathogens *Salmonella typhimurium* and *Shigella flexneri* require RpoS to survive low pH, and *S. typhimurium rpoS* mutants have a lethal dose in mice that is ≈1000-fold greater than wild type (Fang *et al*., 1992; Small *et al*., 1994). Similarly, *V. cholerae* need RpoS for efficient intestinal colonization (Merrell *et al*., 2000). In contrast, RpoS does not appear to control *Yersinia enterocolitica* virulence, as judged by tissue culture assays of epithelial cell invasion and mouse models of infection (Badger and Miller, 1995), but this sigma factor does induce stress resistance. Unlike *Y. enterocolitica*, *L. pneumophila* requires RpoS for proper trafficking within host cells, but not for stress resistance.

To establish a replication niche in phagocytes, *L. pneumophila* must avoid immediate delivery to degradative lysosomes. To do so, as the bacteria enter the PE phase, they deploy both Dot-dependent and -independent factors (Vogel and Isberg, 1999; Joshi *et al*., 2001). In macrophages, *L. pneumophila* RpoS was required for robust intracellular growth (Fig. 6). The dramatic failure of *rpoS* mutants to replicate was documented directly by fluorescence microscopy of macrophages 18 h after infection: wild-type *L pneumophila* replicated to large numbers (Fig. 7A, images 4–6), but the majority of *rpoS* mutant vacuoles contained only a single bacterium (Fig. 7A, images 7–9). As expected, the growth defect in *L pneumophila rpoS* mutants correlated with a failure to block phagosome maturation (Fig. 8).

Interestingly, the trafficking of *rpoS* mutants resembled that of strains lacking a functional Dot/Icm secretory apparatus. By 2 h after infection, the majority of *rpoS* and *dotA* null mutants resided within LAMP-1-positive compartments, yet neither cell type progressed to the lysosomes, as judged by their integrity and failure to co-localize with endocytosed TROv. Moreover, an *rpoS dotA* double mutant had an intracellular fate indistinguishable from that of either single mutant: persistence within a late endosome-like compartment as an intact rod of unknown viability (Fig. 8). In contrast, phagosomes harbouring E phase *L. pneumophila* rapidly acquire lysosomal markers, and the cells are degraded (Joshi *et al*., 2001; Fig. 7A). Accordingly, in the PE phase, RpoS may induce components of the type IV pathway to isolate *L. pneumophila* from the LAMP-1 endosomal compartment; in parallel, another PE factor(s) is expressed by an RpoS- and Dot-independent mechanism to block its delivery to the degradative lysosomes.

Based on the similar fate of *dotA* and *rpoS* mutants in macrophages, one could postulate that RpoS regulates the expression of either the Dot/Icm apparatus or its putative effectors. However, analysis of transcriptional fusions to 10 of the 24 *icm* genes showed no difference in the expression of these Dot/Icm structural genes between E and PE phase, and the expression of an *icmX::lacZ* reporter was independent of RpoS (Hales and Shuman, 1999). Furthermore, unlike RpoS, Dot/Icm function appears to be crucial for replication, not only in primary macrophages and amoebae, but also in more permissive monocytic cell lines (Segal and Shuman, 1999; Vogel and Isberg, 1999). Finally, the *rpoS* null mutant did not display the defect in conjugation seen for a *dotA* mutant (Vogel *et al*., 1998; data not shown). Therefore, RpoS appears to regulate either substrates of the secretion apparatus or accessory factors that are needed for maximal virulence, but not the Dot/Icm structural components themselves.

After 48 h within macrophages, the *rpoS* mutants consistently exhibited an increase in their apparent growth rate (Fig. 6). It is unlikely that the minor population of cells that replicate in the primary infection period (Fig. 7B) are sufficient to account for this phenomenon, as we expect their defect in establishing a replication vacuole to be reiterated in each successive ≈24 h infection cycle. Perhaps macrophage defence mechanisms are eventually depleted, enabling the otherwise crippled *rpoS* mutants to flourish. For example, in an *S. typhimurium* infection model, nitric oxide produced by interferon (IFN)-γ-activated peritoneal macrophages is bacteriostatic (Vazquez-Torres *et al*., 2000), but its production is probably maintained only for a few days and only if the inductive stimulus and L-arginine substrate are continually available (MacMicking *et al*., 1997). Alternatively, prolonged residence of the *L. pneumophila* mutant bacteria within LAMP-1 vacuoles may induce a second regulatory pathway that compensates for the loss of RpoS function.

In contrast to their poor growth in A/J murine macrophages and in *A. castellanii*, *rpoS* mutants replicated like wild-type *L. pneumophila* within two monocytic cell lines,

HL-60 and THP-1 cells (Figs 6 and 7; Hales and Shuman, 1999). In direct comparisons with macrophage-like cell lines, primary murine macrophages and amoebae are more restrictive hosts for *L. pneumophila* (Gao *et al*., 1997; Joshi and Swanson, 1999). Therefore, we speculate that RpoS is crucial only in stringent environments, including cultured amoebae or primary macrophages. Recent studies indicate that *L. pneumophila* resides in biofilms (Rogers and Keevil, 1992; Rogers *et al*., 1994), suggesting that more complex habitats may contribute to the evolution of multiple pathways to promote *L. pneumophila* survival and replication in the environment.

The avirulent mutant Lp120 was found to contain a large in frame deletion in *rpoS,* but only some of its defects were attributable to this mutation. Once backcrossed onto a wild-type chromosome, the *rpoS-120* allele did not confer the severely defective cytotoxicity (Fig. 4), motility or intracellular growth phenotypes observed for the parent strain, mutant Lp120 (Swanson and Isberg, 1996; Hammer and Swanson, 1999). Moreover, unlike wild type and the isogenic *rpoS* null mutant, PE phase Lp120 remained sensitive to osmotic shock and to prolonged starvation in broth (Hammer and Swanson, 1999). As predicted, the expression of a plasmid-borne *rpoS* locus by mutant Lp120 complemented defects in sodium sensitivity and *flaAgfp* expression, but not in cytotoxicity, motility or growth in macrophages (M. A. Bachman and M. S. Swanson, unpublished). Therefore, in addition to its *rpoS-120* allele, strain Lp120 appears to harbour another mutation(s) that attenuates its virulence. An intriguing possibility is that this mutation may identify a locus that acts in parallel to RpoS to induce stress resistance and virulence of PE phase *L. pneumophila*.

To determine the consequence of loss of RpoS residues 72–94, isogenic *rpoS-120* and *rpoS* null mutant strains were constructed and compared. The mutant strains were similarly defective in assays of sodium sensitivity (Fig. 2A) and intracellular growth (data not shown). However, in each of three independent experiments, *flaAgfp* expression was lower in the *rpoS-120* cells than in the null mutant bacteria. As judged by CLUSTALW alignment (data not shown), the RpoS region deleted in the *rpoS-120* strain corresponds to the *E. coli* RpoD conserved region termed 1.2, a domain required for open complex formation and subsequent transcription initiation (Wilson and Dombroski, 1997). However, RpoD molecules lacking this domain still compete with full-length RpoD for core polymerase binding (Wilson and Dombroski, 1997). If the *rpoS-120* allele encodes a stable protein, perhaps it fails to activate the majority of its target promoters, but interferes with the transcription of other promoters, including those influencing motility.

The prediction that, under particular conditions, RpoS proteins can repress the expression of some PE phase traits is bolstered by the phenotype of cells that contain multiple copies of the *rpoS* locus. When either wild-type or *rpoS* mutant cells carried pLrpoS, their motility and cytotoxicity was inhibited. As has been reported previously for *E. coli* (Farewell *et al*., 1998), *L. pneumophila* RpoS may compete with other sigma factors, such as RpoD, for binding to RNA polymerase. By this mechanism, genes normally regulated by other transcription factors would be repressed by RpoS, either directly or indirectly.

RpoS appears to be the first of multiple transcription factors likely to regulate *L. pneumophila* virulence in response to growth conditions. In particular, the observation that RpoS induces some post-exponential phase traits but not others suggests that the regulatory circuit that governs the virulence of this phagocyte pathogen is likely to be sophisticated and complex. Defined regulatory mutants will probably serve as powerful genetic tools to identify the effectors of *L. pneumophila* virulence. Therefore, identification of its full complement of regulatory proteins and their respective environmental and cellular signals will be a valuable step towards understanding the dramatic physiological changes that equip intracellular *L. pneumophila* to search out and exploit a new replicative niche.

Experimental procedures

Bacterial strains and culture conditions

L. pneumophila Lp02, a virulent thymine auxotroph (Berger and Isberg, 1993), Lp03, a *dotA* mutant, and *rpoS* and *rpoS-120* strains derived from Lp02 were all cultured in N-(2-acetamido)-2-aminoethanesulphonic acid (ACES; Sigma)-buffered yeast extract broth supplemented with 100 μg ml^{-1} thymidine (AYET) at 37°C. Bacteria were plated on ACES-buffered charcoal–yeast extract agar supplemented with 100 μg ml^{-1} thymidine (CYET). Where indicated, kanamycin was added to a final concentration of 25 μg ml^{-1} and streptomycin to 2.5 mg ml^{-1}. *E. coli* ZK918 (Bohannon *et al*., 1991) was cultured on Luria–Bertani or MacConkey agar (Difco) and, where indicated, supplemented with ampicillin to a final concentration of 100 μg ml^{-1} or with kanamycin to 25 μg ml^{-1}.

Primers and plasmids

The cloned *rpoS* locus was isolated from an *L. pneumophila* genomic library composed of 5–10 kb *Sau*3AI fragments from wild-type strain Lp01 ligated into the *Bam*HI site of pKB5 (see Table 1). Sequence analysis was performed by subcloning the *rpoS* locus into pBluescript KS (Stratagene). Polymerase chain reaction (PCR) products were cloned using pGEMT-Easy (Promega); allelic exchange was performed using the suicide vectors pKAS32 and 46 (Skorupski and Taylor, 1996). Oligonucleotide primers rpoS1 and rpoS2 used to amplify the *rpoS* coding region have been described

previously (Hales and Shuman, 1999). To amplify the deletion region in the *rpoS-120* allele, primer rpoSN was designed using PRIMER SELECT (DNAstar); all primers were synthesized by the University of Michigan Biomedical Research Core Facilities; Table 1 lists their sequences. Plasmid pMMBrelA, used for the expression of *E. coli relA* in *L. pneumophila*, and the reporter plasmid pflaG, containing the *flaAgfp* reporter fusion, have been described previously (Hammer and Swanson, 1999; Table 1).

Because conjugation functions of the RSF1010 plasmids attenuate *L. pneumophila* virulence (Segal and Shuman, 1998), the *mobA* gene sequences, located between two *Age*I sites, were deleted from pKB5 and pMB383, generating pMB384and pMB391 (pLrpoS) respectively. As complementation of *rpoS* mutants in other species has been confounded by a requirement for upstream sequences (Yildiz and Schoolnik, 1998), including the *rpoS* promoter within the upstream *nlpD* locus (Lange and Hengge-Aronis, 1994; Lange *et al*., 1995), the entire 6.8 kb *rpoS* was used for complementation studies. To assess whether other genes linked to the *rpoS* locus affected virulence, the *rpoS::kan* allele from pMB386 was cloned into pMB384 to create pMB392 (pLrpoSΔ).

Antibodies and reagents

The late endosomal and lysosomal marker LAMP-1 was detected with monoclonal antibody (mAb) 1D4B obtained from the Developmental Studies Hybridoma Bank of the Department of Pharmacology and Molecular Sciences, Johns Hopkins School of Medicine, Baltimore, MD, USA, and the Department of Biology, University of Iowa, IA, USA. The antibody Lp1 mAb1 against an *L. pneumophila* serogroup 1 surface antigen was obtained from the American Type Culture Collection (ATCC no. CRL1765). Anti-*Legionella* rabbit serum was a kind gift from R. Isberg (Tufts University School of Medicine, Boston, MA, USA). Secondary antiserum, Texas red–ovalbumin and the nucleotide stain 4′,6-diamidino-2-phenylindole (DAPI) were obtained from Molecular Probes.

Colony PCR

To obtain either chromosomal or plasmid DNA rapidly for PCR analysis, a portion of a colony was transferred on an autoclaved P200 pipette tip to 100 μl of autoclaved water in a microcentrifuge tube. The samples were boiled for 2 min, centrifuged at 15 800 *g* for 10 s to pellet cellular debris, then 20 μl of the cleared suspension was used as a DNA template in each 100 μl PCR reaction (Roche high-fidelity PCR kit).

Cloning and sequencing of L. pneumophila rpoS

The *E. coli rpoS* mutant strain ZK918 was transformed with a *Sau*3A *L. pneumophila* genomic library. Transformants were selected on MacConkey agar containing ampicillin and kanamycin and screened for colony colour. Strain ZK918 carrying the *E. coli rpoS* on the plasmid pDEB2 served as a positive control for the screen. To confirm the colony colour, red colonies were restreaked on MacConkey agar. Next, candidates were tested for catalase by patching onto LB agar containing ampicillin and kanamycin, incubating for 24 h at 37°C, then dropping 10 μl of 3% H_2O_2 onto the patch and comparing its bubbling with that seen for ZK918 carrying pDEB2. A single β-galactosidase-, catalase-positive clone was identified, and its library plasmid was purified.

Restriction analysis of the complementing plasmid pMB383 revealed a 6.8 kb chromosomal insert. To determine its nucleotide sequence, this chromosomal locus was subcloned into pBluescript KS (pMB385). The entire *rpoS* ORF as well as portions of the flanking sequence were sequenced by the University of Michigan DNA Sequencing Core using T3, T7 and custom primers. The *rpoS* allele from mutant Lp120 was amplified by colony PCR using primers rpoS1 and rpoS2 (see Table 1). This PCR product was ligated into pGEMT-Easy and sequenced using custom primers derived from known *rpoS* sequence as described above.

Construction of rpoS *null and* rpoS-120 *alleles*

To construct an *rpoS* null allele, pMB385 was digested to completion with *Mlu*I and partially with *Hpa*I, deleting 693 bp of the *rpoS* ORF (Fig. 1), then made blunt with Klenow fragment (New England Biolabs). In parallel, the Tn*903* kanamycin^R allele was isolated as a *Pst*I fragment from pUC4K, gel purified, made blunt with Klenow, then ligated to the pMB385 blunt-end fragment to create the *rpoS::kan* allele on pMB386. The *rpoS::kan* allele was amplified using primers rpoS1 and rpoS2 and cloned into pGEMT-Easy (Promega) creating pMB387. Next, the *rpoS::kan* allele was isolated as a *Not*I fragment and cloned into the suicide vector pKAS32, generating pMB388 in the *E. coli* host strain DH5αλpir whose phage-encoded pi replicon allows plasmid propagation.

To perform the allelic exchange, pMB388 was transferred by electroporation to Lp02 (Str^R); then transformants that had integrated the plasmid onto the chromosome were selected on CYET containing kanamycin. To select isolates that had excised by recombination vector sequences marked genetically by a *Bacillus subtilis rpsL* allele that confers streptomycin sensitivity, clones were restreaked onto CYET containing kanamycin and streptomycin. Candidate mutant clones were colony purified on CYET containing kanamycin, then screened for the presence of the *rpoS::kan* allele by colony PCR using primers rpoS1 and rpoS2 (Table 1), in which a 569 bp size increase was diagnostic for the mutant allele.

Allelic exchange was confirmed phenotypically by patching candidates onto CYET, CYET/Kan and CYET/Kan/Str and comparing growth after 48 h at 37°C. The molecular structure of the null allele and loss of pKAS32 sequences was confirmed by Southern analysis using *Pst*I-digested genomic DNA and two DNA probes labelled by random priming (Roche digoxigenin non-radioactive nucleic acid labelling and detection system): an *Hpa*I fragment from pMB383 that contained 5′ *rpoS* sequence and a *Bam*HI–*Sal*I fragment from pKAS32 containing the *bla* allele. Clones containing the *rpoS::kan* insertion had lost a *Pst*I site, resulting in a hybridizing fragment of 6.5 kb versus 3.2 kb for the wild-type locus (see Fig. 1) To create an *rpoS dotA* double mutant, allelic exchange and Southern analysis was performed by transforming *dotA* mutant strain with pMB388 exactly as described above.

To cross the *rpoS* allele from Lp120 onto a wild-type chromosome, the *rpoS* allele from mutant Lp120 (Swanson and Isberg, 1996) was amplified by colony PCR, then shuttled into pKAS46 (*kan*^{R}, *rpsL*), as described for the *rpoS::kan* allele, to generate pMB390. Allelic exchange was performed exactly as before, except that counterselection was performed on CYET containing streptomycin only to permit loss of the *kan*^{R}-encoding plasmid sequence. Construction of the *rpoS-120* allele was confirmed by Southern analysis, using the same probes as above, where loss of an *Hpa*I restriction site in the mutant allele increased the size of the complementary genomic fragment to 12 kb from the wild-type size of 1.6 kb (Fig. 1).

Sodium sensitivity

Sodium sensitivity was determined by culturing *L. pneumophila* in broth to exponential (E) or post-exponential (PE) phase, diluting samples in AYE broth and plating duplicate samples on CYET and CYET containing 100 mM NaCl. The percentage of bacteria that were sodium resistant was calculated as (cfu ml^{-1} on CYET + 100 mM NaCl)/(cfu ml^{-1} on CYET) $\times$ 100.

To quantify (p)ppGpp-induced sodium sensitivity, E cultures of Lp02 and *rpoS* strains containing pMMBRelA at an optical density of $\approx$0.4 at 600 nm (OD_{600}) were divided: one culture was supplemented to 200 μM IPTG and the other was not. After incubation for an additional 4.5 h, motility, sodium sensitivity and OD_{600} were assessed.

Stationary phase stress resistance

The resistance of *L. pneumophila* to a variety of stresses was assayed as described previously (Hales and Shuman, 1999). Briefly, bacteria cultured to E (OD_{600} $\approx$1) and PE phase (OD_{600} $\approx$3–4) were collected by centrifugation and resuspended in 1$\times$ M63 salts or an equal volume of 5 M NaCl, 1 mM citric acid, pH 3, or 10 mM H_2O_2. After incubation for 1 h in a 37°C water bath, cultures were diluted serially in 1$\times$ M63 salts, then plated onto CYET plates to determine cfu.

Quantification of flaAgfp *fluorescence*

Transcription of *flaA* was quantified using a *flaAgfp* reporter plasmid (pflaG; Table 1) as described previously (Hammer and Swanson, 1999). At the times indicated, the OD_{600} of an aliquot was measured, then cells were collected by centrifugation and resuspended to an OD_{600} of $\approx$0.1 to ensure similar bacterial concentrations for fluorometry measurements. At the beginning of the experiment, a reference culture of PE phase wild-type Lp02 cells expressing the *flaAgfp* fusion was used to establish the parameters for detection of maximal fluorescence. The fluorescence of each sample was measured relative to this positive control on an SPF-500C spectrophotometer (SLM Instruments) using an excitation of 488 nm with a bandpass width of 2.5 nm and emission of 510 nm with bandpass width of 5 nm.

Intracellular growth

Intracellular growth of *L. pneumophila* was assayed in bone marrow-derived macrophages from A/J mice. Strains were cultured to the PE phase, as judged by their OD_{600} and motility. Motile cultures of similar density were diluted into RPMI + 10% fetal bovine serum (FBS) to achieve a multiplicity of infection (MOI) of $\approx$0.2; then 250 μl was added to 2.5 $\times$ 10^5 macrophages plated well^{-1} of a 24-well plate. After incubation at 37°C and 5% CO_2 for 2 h, monolayers were washed with RPMI + 10% FBS to remove extracellular bacteria; then samples were lysed with PBS + 0.05% saponin, diluted serially in AYE and plated for cfu. At 24, 48 and 72 h, supernatants were collected, monolayers were lysed and pooled with supernatants, then aliquots were plated for cfu as described. For each time point, duplicate or triplicate samples were measured for each strain. Relative intracellular growth was calculated by dividing the cfu obtained at 24, 48 and 72 h by the cfu at 2 h.

Fluorescence microscopy

Fluorescence micrographs of *L. pneumophila*-infected bone marrow macrophages 18 h after infection were obtained using a Zeiss Axioplan 2 fluorescence microscope equipped with 100$\times$ Plan-Neofluor objective of numerical aperture 1.3 and a Spot digital camera. Macrophages plated on coverslips at a density of 1–2 $\times$ 10^5 were infected at an MOI that had been determined empirically to ensure that a majority of macrophages infected for 2 h contained a single bacterium; see the legend to Fig. 7 for values. After 2 h, cultures were washed with RPMI + 10% FBS to remove extracellular bacteria. At 2 and 18 h, coverslips were removed, fixed and permeabilized as described previously (Swanson and Isberg, 1996). To reduce non-specific binding by antibodies, samples were incubated for 0.5 h with PBS−5% sucrose + 2% goat serum. Intact (wild type and *rpoS* PE) and degraded (wild type E) bacteria were stained using the anti-*Legionella* mouse monoclonal antibody Lp1 mAb1 (ATCC) diluted 1:20 and an Oregon green-conjugated anti-mouse secondary antibody diluted 1:2000 (Molecular Probes).

To determine the number of bacteria per vacuole after 18 h, all infected macrophages in a given macroscopic field were scored as one of five categories: infected with a single bacterium; 2–10 bacteria; 11–20 bacteria; $>$20 bacteria; or degraded bacteria. Degraded bacteria were defined as Oregon green-stained, non-rod-shaped particles. For each coverslip, 50 infected macrophages were scored.

Fusion of L. pneumophila *phagosomes with late endosomes and lysosomes*

Fusion of *L. pneumophila* phagosomes with late endosomes and lysosomes was assessed by quantifying their co-localization with the membrane glycoprotein LAMP-1. After a 2 h infection, macrophages were washed with RPMI + 10% FBS to remove extracellular bacteria, then fixed and permeabilized as described above. Next, LAMP-1 was stained using mAb 1D4B neat, followed by Oregon green-conjugated anti-rat serum diluted 1:2000. *L. pneumophila* were stained with 600 μM DAPI in PBS + 5% sucrose.

To assess the fusion of *L. pneumophila* phagosomes with lysosomes, uninfected macrophages were allowed to endocytose 100 μg ml^{-1} Texas red–ovalbumin (TROv) for 0.5 h. Next, macrophages were washed three times, then incubated for an additional 0.5 h to allow trafficking of TROv to lysosomes. Macrophages were then infected, fixed, permeabilized and stained for *L. pneumophila* using Lp1 mAb1 as described above.

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