

## Role for RpoS but Not RelA of *Legionella pneumophila* in Modulation of Phagosome Biogenesis and Adaptation to the Phagosomal Microenvironment

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**The induction of virulence traits by *Legionella pneumophila* at the post-exponential phase has been proposed to be triggered by the stringent response mediated by RelA, which triggers RpoS. We show that *L. pneumophila* *rpoS* but not *relA* is required for early intracellular survival and replication within human monocyte-derived macrophages and *Acanthamoeba polyphaga*. In addition, *L. pneumophila* *rpoS* but not *relA* is required for expression of the pore-forming activity. We provide evidence that RpoS plays a role in the modulation of phagosome biogenesis and in adaptation to the phagosomal microenvironment. Thus, there is no functional link between the stringent response and RpoS in the pathogenesis of *L. pneumophila*.**

In aquatic environments, *Legionella pneumophila* replicates intracellularly within protozoa (11, 27). Upon transmission to humans, *L. pneumophila* replicates intracellularly within alveolar macrophages (26, 44). Ultrastructural and molecular studies have shown that intracellular replication of *L. pneumophila* in amoebae and macrophages exhibits similar features (1, 2, 39). After invasion of a eukaryotic host cell, *L. pneumophila* diverts its phagosome from the “default” endosomal-lysosomal pathway to establish an endoplasmic reticulum-derived replicative niche (18, 19, 34, 42, 45). The *L. pneumophila* Dot/Icm type IV secretion system has been shown to be essential for biogenesis of the *Legionella*-containing phagosome (LCP) in quiescent macrophages (38, 43). However, in gamma interferon-activated macrophages, the LCP fuses to lysosomes and the bacteria fail to replicate (35). Upon termination of intracellular replication, *L. pneumophila* disrupts the phagosomal membrane and continues to replicate in the cytoplasm prior to lysis of the plasma membrane and egress of the progeny (5, 22, 24, 25, 28, 46).

The stringent response is initiated in response to various nutritional stresses, including starvation for amino acids, with subsequent accumulation of the stringent response alarmone ppGpp, which is synthesized by RelA (9). Expression of virulence traits by *L. pneumophila* has been shown to be triggered at the post-exponential (PE) phase and coincides with accumulation of ppGpp and expression of the stationary-phase sigma factor RpoS (8, 15, 16, 29, 41, 47). The ppGpp alarmone has been proposed to trigger two parallel signaling pathways in *L. pneumophila*, one of which is controlled by RpoS while the other is controlled by the LetA/S two-component regulatory system (6, 15, 29, 30). The two pathways coordinate the induction of several virulence traits at the PE phase (7, 14, 29). The roles of RpoS and RelA in intracellular infection by *L. pneumophila* have been controversial. Although *L. pneumophila*

RpoS has been shown to be required for intracellular replication in both amoebae and primary mouse macrophages (7, 14, 29), it is dispensable for intracellular replication in the human macrophage-like cell line HL-60 (7, 15). A key role for ppGpp in expression of *L. pneumophila* virulence traits has been proposed based on expression of a plasmid-borne *relA* gene of *Escherichia coli* in *L. pneumophila* (7, 16). Based on these observations, a model of a signaling cascade at the PE phase has been proposed in which expression of virulence traits (including pore-forming activity/cytotoxicity, motility, and evasion of lysosomal fusion) is triggered by ppGpp synthesized by RelA in response to amino acid starvation (5, 8, 29, 33, 41). A *relA* mutant has not been utilized to test this proposed model (7, 16). However, Zusman et al. have shown that a *relA* mutant of *L. pneumophila* does not produce ppGpp but is not affected in intracellular replication in amoebae and HL-60 human macrophage-like cells (47). It is not known whether *L. pneumophila* *rpoS* and *relA* are required for intracellular replication in human primary macrophages. In addition, it is not documented whether there is a functional link between the stringent response and the virulence regulatory cascade regulated by RpoS in *L. pneumophila* pathogenesis.

The virulent clinical isolate of *L. pneumophila* strain AA100, its isogenic *dotA* mutant, and their cultivation have been described previously (4, 46). Human monocyte-derived macrophages (hMDMs) were prepared as described previously (36, 37). U937 macrophage-like cells, hMDMs, and *Acanthamoeba polyphaga* were maintained as we described previously (4, 46). The growth kinetics of *L. pneumophila* strains in these host cells was determined by using a multiplicity of infection (MOI) of 10 followed by 1 h of gentamicin treatment, as we described previously (13, 23, 46). Transmission electron microscopy of infected amoebae and caspase-3 enzymatic activity assays were carried out as described previously (12, 28).

The *L. pneumophila* *rpoS* and *relA* genes were amplified by PCR from the genomic DNA of the parental strain AA100 and were cloned individually into the pBC-sk<sup>+</sup> plasmid to generate plasmids pBRO and pRL-4, respectively. Mutagenesis of the cloned *rpoS* gene was carried out using an EZ::TN<KAN-2>

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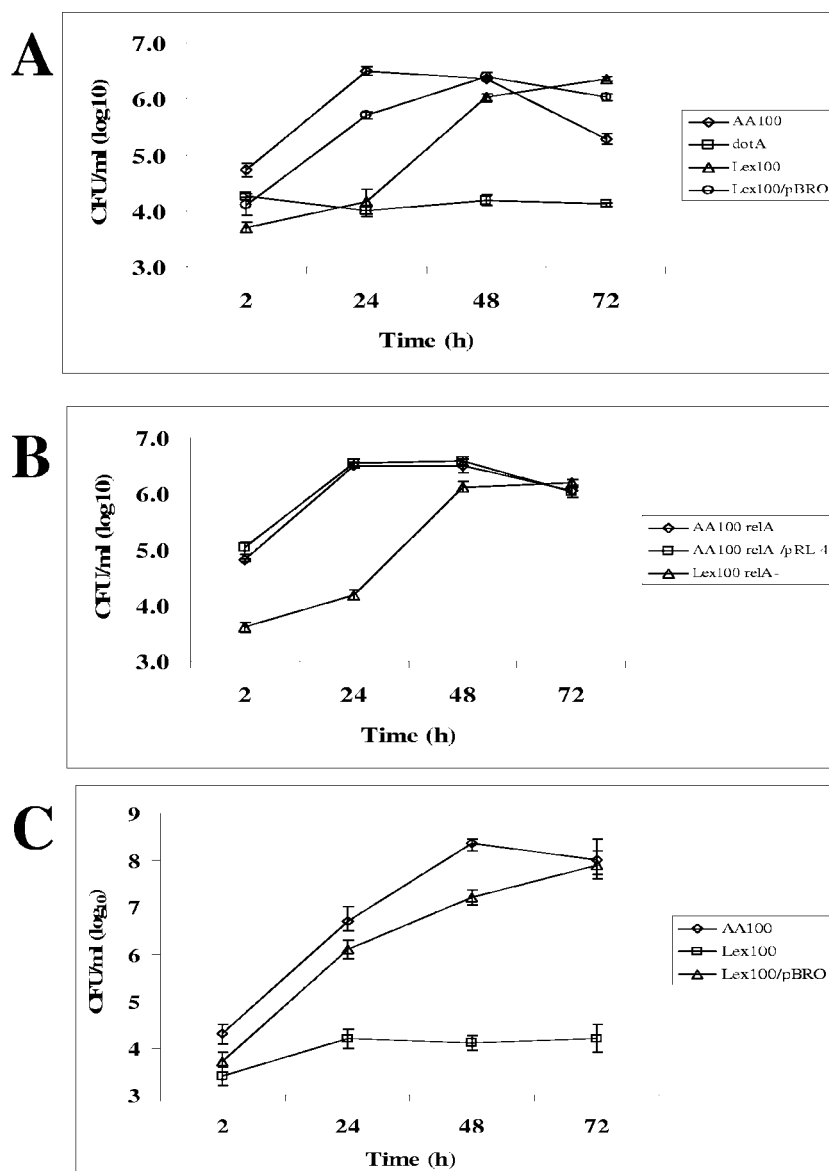


FIG. 1. Role of *L. pneumophila rpoS* and *relA* in intracellular replication in hMDMs and *A. polyphaga*. (A) Growth kinetics of AA100, *dotA*, Lex100, and Lex100/pBRO in hMDMs. (B) Growth kinetics of AA100*relA*<sup>-</sup>, AA100*relA*<sup>-</sup>/pRL-4, and Lex100*relA*<sup>-</sup> in hMDMs. (C) Growth kinetics in *A. polyphaga*. The infection was carried out in triplicate with an MOI of 10 for 1 h, followed by 1 h of gentamicin treatment to kill extracellular bacteria. The infected monolayers were lysed and plated onto agar plates for colony enumeration. The data are representative of three independent experiments. Error bars, standard deviations.

insertion kit as described by the manufacturer (Epicentre Technologies, Madison, WI). The plasmid generated was designated pBRT. Mutagenesis of the cloned *relA* gene was carried out by inserting a gentamicin-resistant gene, and the plasmid generated was designated pRL-44. The *rpoS* mutant strain Lex100 and the *relA* mutant strain AA100*relA*<sup>-</sup> were generated by natural transformation using the pBRT and pRL-44 plasmids, respectively, as we described previously (40). The *rpoS relA* double-mutant strain Lex100*relA*<sup>-</sup> was generated from the *rpoS* mutant strain Lex100 by natural transformation using the pRL-44 plasmid. Insertion of the EZ::TN<R6K $\gamma$ ori/KAN-2> transposon and the gentamicin-resistant cassette within the chromosomal *rpoS* and *relA* genes, respectively, was confirmed by PCR and Southern blot analysis.

***L. pneumophila rpoS* is essential for intracellular replication in amoebae and human monocyte-derived macrophages.** *L. pneumophila rpoS* has been shown to be essential for induction of some PE phase-induced virulence traits such as evasion of lysosomal fusion and intracellular replication in mouse bone marrow-derived macrophages (7, 15). However, it has been shown that *L. pneumophila* RpoS is not required for intracellular replication in the human macrophage-like cell line HL-60 (15). Similarly, our data show that within U937 macrophages, the *L. pneumophila rpoS* mutant Lex100 replicated similarly to the parental strain AA100 (data not shown). The intracellular growth kinetics in hMDMs showed that by 24 h after infection, the *rpoS* mutant strain Lex100 was defective in intracellular

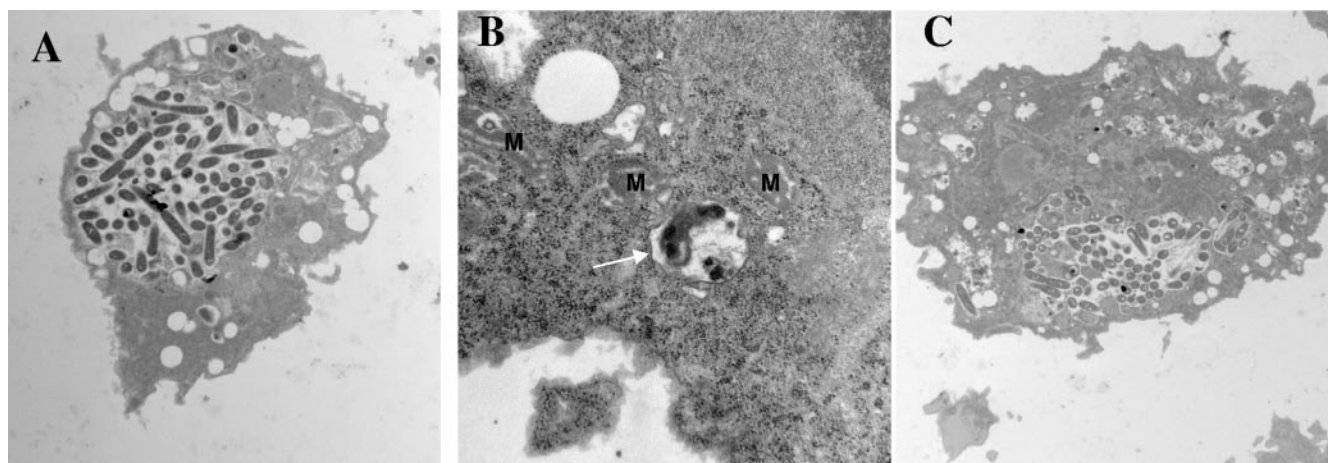


FIG. 2. *L. pneumophila rpoS* is essential for the biogenesis of replicative phagosomes. Shown are representative transmission electron microscopy images of *A. polyphaga* cells 24 h after infection by the *L. pneumophila* parental strain AA100 (A), the *rpoS* mutant Lex100 (B), or the complemented strain Lex100/pBRO (C). The white arrow in panel B indicates a phagosome that contains a partially degraded *rpoS* mutant. M, mitochondria.

replication, like the *dotA* mutant control (Fig. 1A). However, by 48 h after infection, the number of CFU of the *rpoS* mutant reached a level similar to those of the parental strain and the complemented strain Lex100/pBRO (Fig. 1A).

A similar defect in early intracellular survival and replication of the *rpoS* mutant was also observed in *A. polyphaga*, and the defect was complemented by wild-type *rpoS* (Fig. 1C), consistent with previous observations (7, 15). However, compared to hMDMs, the defect of the *rpoS* mutant was more severe in this protozoan host, since intracellular replication was not detectable up to 48 h postinfection (Fig. 1). This prompted us to investigate the viability of the *L. pneumophila rpoS* mutant within the first 2 h after infection. Our data showed that the levels of intracellular survival of the *rpoS* mutant strain Lex100 in hMDMs and *A. polyphaga* were 10 to 20% of those of the parental strain AA100. Transmission electron microscopy studies revealed that by 24 h after infection, only 3% of the

phagosomes harboring the *rpoS* mutant contained a few bacteria (Fig. 2). The majority of the Lex100-infected amoebae showed phagosomes with structures that are most likely remnants of degraded bacterial cells (Fig. 2). Although the intracellular bacterial numbers for the *rpoS* mutant were similar to those of the parental strain by 48 h, the mutant never recovered within amoebae. The phagosomes of the *rpoS* mutant that replicated after 24 h were not different from the wild-type strain phagosomes at 2 to 4 h (data not shown). Taking these findings together, we conclude that *L. pneumophila rpoS* is required for early survival and intracellular replication of *L. pneumophila* in hMDMs and amoebae. In addition, *rpoS* is essential for intracellular replication throughout the intracellular infection of *A. polyphaga*.

**RelA is dispensable for intracellular replication of *L. pneumophila* in hMDMs.** In response to amino acid starvation upon entry into the PE phase, *L. pneumophila* accumulates the strin-

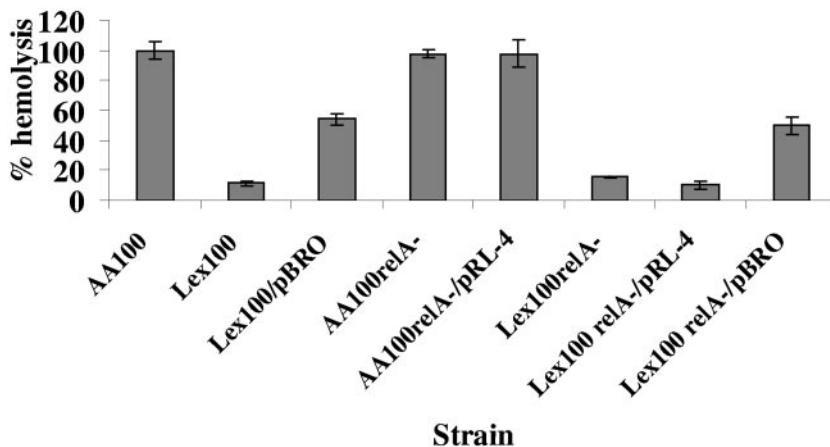


FIG. 3. *L. pneumophila rpoS* is required for contact-dependent pore-forming activity. Contact-dependent hemolysis of sheep erythrocytes was performed using an MOI of 25 for 2 h. The level of contact-dependent pore-forming activity for each strain was determined as a function of the optical density of the released hemoglobin measured at a wavelength of 415 nm. The pore-forming activity of each strain is presented as a percentage of that of the parental strain AA100. The data are representative of three independent experiments. Error bars, standard deviations.

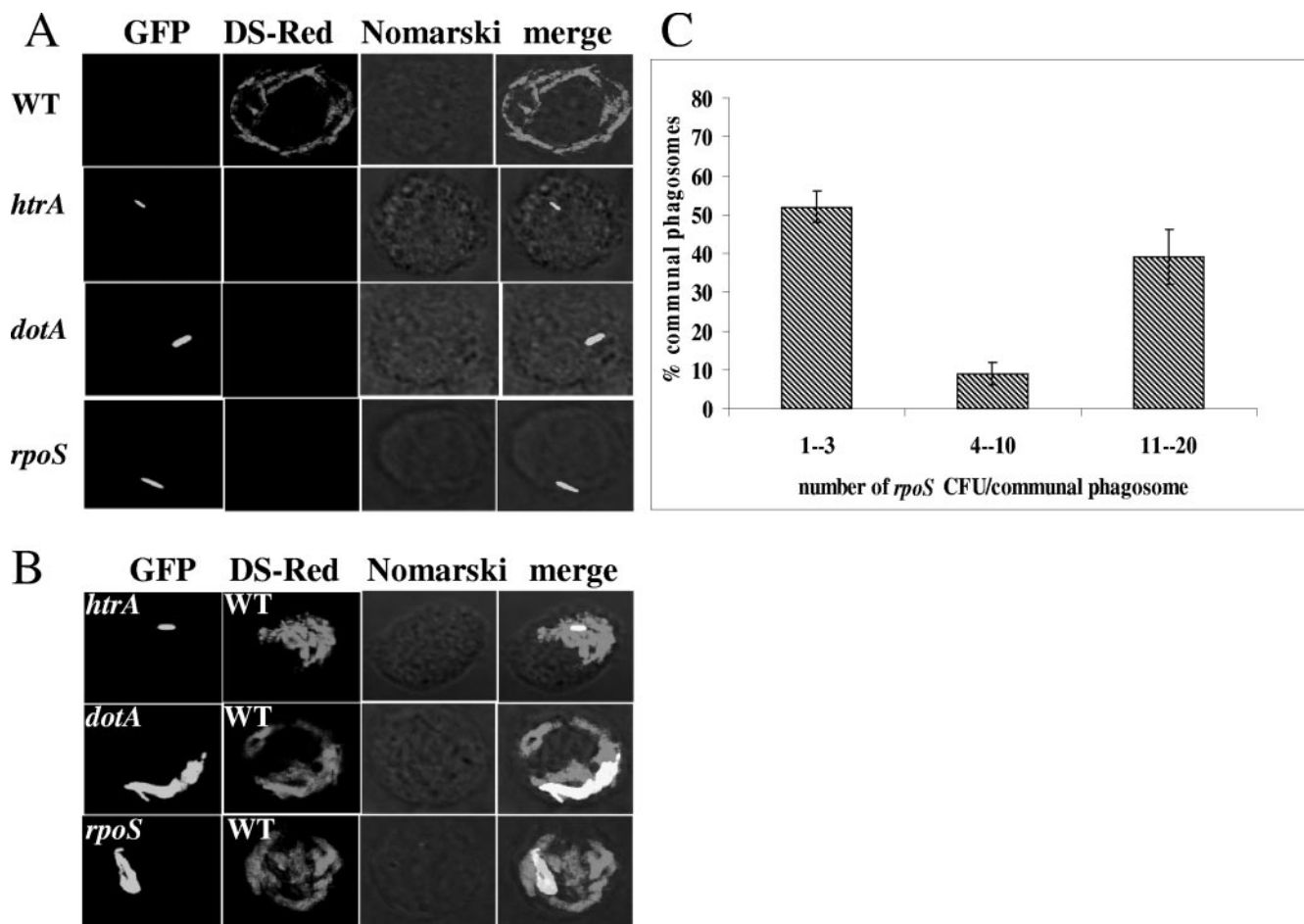


FIG. 4. Rescue of intracellular replication of the *rpoS* mutant of *L. pneumophila* within communal phagosomes remodeled by the parental strain. (A and B) Human monocyte-derived macrophages were either singly infected with the DsRed-expressing parental strain AA100 or one of the GFP-expressing mutants *htrA*, *dotA*, and *rpoS* (A) or coinfected with the parental strain and one of the mutants (B) for 1 h, followed by incubation for 11 h prior to analysis by confocal microscopy for formation of large replicative phagosomes. (C) Quantitation of the number of *rpoS* mutant bacteria per communal phagosome at 11 h postinfection. For each infection, ~100 single or communal phagosomes were analyzed from three coverslips for each experiment, and the results are representative of two independent experiments.

gent response alarmone ppGpp (16, 47), which is thought to be the main signal that activates the sigma factor RpoS (7, 17, 29). The proposed role of the stringent response in the induction of *L. pneumophila* virulence traits is based on gratuitous expression of RelA of *E. coli* in *L. pneumophila* and not on *relA* mutagenesis (7, 16). Zusman et al. have shown that a *relA* mutant of *L. pneumophila* does not accumulate ppGpp upon entering into the PE phase, but the *relA* mutant is not affected in intracellular replication in amoebae and human HL-60 macrophages (47). Thus, there is disagreement on the involvement of the stringent response in triggering *L. pneumophila* virulence traits and on the proposed functional link between the stringent response and the RpoS signaling cascade. Therefore, we reevaluated the role of *relA* in intracellular replication of *L. pneumophila* in hMDMs. We generated a *relA* mutant and an *rpoS relA* double mutant and examined their intracellular growth kinetics in hMDMs. The data showed that both the AA100*relA*<sup>-</sup> strain and its complemented counterpart AA100*relA*<sup>-</sup>/pRL-4 exhibited intracellular replication patterns in hMDMs that were indistinguishable from that of

parental strain AA100 (Fig. 1B). In addition, the pattern of intracellular replication of the *rpoS relA* double-mutant strain Lex100*relA*<sup>-</sup> was indistinguishable from that of the *rpoS* mutant strain Lex100 (Fig. 1A and B). This indicated that *L. pneumophila* RpoS functions independently of the RelA product in the ability of *L. pneumophila* to replicate intracellularly. Taking these findings together, we conclude that *L. pneumophila* *rpoS* but not *relA* is required for early survival and intracellular replication in hMDMs.

***L. pneumophila* *rpoS* but not *relA* is required for of the pore-forming activity.** Upon entering the PE phase, *L. pneumophila* expresses a pore-forming activity that facilitates the egress of *L. pneumophila* from the spent host cell (5, 22–24). To examine whether *L. pneumophila* *rpoS* and/or *relA* is required for the induction of the pore-forming activity, we carried out contact-dependent hemolysis of erythrocytes, as described previously (5, 20). The data showed that the *rpoS* mutant Lex100 was severely defective in its pore-forming activity, which was complemented by the wild-type *rpoS* gene (Fig. 3A). In contrast, both the AA100*relA*<sup>-</sup> mutant strain and



its complemented strain AA100*relA*<sup>-</sup>/pRL-4 exhibited a pore-forming activity level similar to that of the parental strain AA100 (Fig. 3A). The *rpoS relA* double mutant Lex100*relA*<sup>-</sup> showed a pore-forming activity similar to that of the *rpoS* mutant strain Lex100. The pore-forming activity of the double mutant was complemented by the wild-type *rpoS* but not by *relA* (Fig. 3A). Our results indicated that *L. pneumophila rpoS* but not *relA* was required for the induction of the pore-forming activity upon entry into the PE phase. This further confirms the lack of a functional link between the stringent response and the RpoS-regulated signaling cascade in *L. pneumophila* pathogenesis.

***L. pneumophila rpoS* is not required for the induction of caspase-3 activation in macrophages.** Activation of caspase-3 by *L. pneumophila* is essential for evasion of the endosomal-lysosomal pathway and intracellular replication (28). To determine whether *L. pneumophila* RpoS is required for caspase-3 activation, we compared the levels of caspase-3 activation in macrophages infected by the parental strain AA100, the *rpoS* mutant Lex100, and its complemented strain Lex100/pBRO. Our data showed that unlike the *dotA* mutant, which is completely defective in activating caspase-3 (46), both Lex100 and Lex100/pBRO activate caspase-3 similarly to the parental strain AA100 (data not shown). Since RelA was dispensable for intracellular replication in hMDMs, the role of the *relA* gene in caspase-3 activation was not examined.

**Rescue of the *rpoS* mutant within a communal phagosome harboring the parental strain.** The phagosome harboring the replication-defective *L. pneumophila rpoS*, *dotA*, and *htrA* mutants are trafficked through the endosomal-lysosomal degradation pathway (7, 32, 34). The defect of the *dot/icm* mutants in the modulation of the biogenesis of the LCP is rescued within communal phagosomes remodeled by the parental strain (10). In contrast, the *htrA* mutant is not rescued within communal phagosomes remodeled by the parental strain, which is due to its defect in adaptation to the phagosomal microenvironment (32). We examined whether the *rpoS* mutant can be rescued for its defect in intracellular replication within communal phagosomes remodeled by the parental strain. Monolayers of hMDMs were coinfecting for 1 h by a green fluorescent protein (GFP)-expressing (21) mutant (*rpoS*, *dotA*, or *htrA*) and/or the DsRed-expressing (31) parental strain AA100 exactly as we described previously (32). Eleven hours after infection, the infected cells were fixed and examined by confocal microscopy. In control single infections, the parental strain AA100 showed replicative phagosomes with 20 to 40 bacteria/phagosome in 75% of infected hMDMs. Both the *dotA* and *htrA* mutants were found as single bacteria in most of the hMDMs infected with either mutant. In 65% of communal phagosomes harboring the parental strain and the *dotA* mutant, the *dotA* mutant replicated as efficiently as the wild type strain (Fig. 4). In 80% of communal phagosomes harboring the *htrA* mutant and the parental strain, the mutant failed to replicate (Fig. 4), while in the other 20% of communal phagosomes, the *htrA* mutant exhibited very weak replication (2 to 6 *htrA* bacteria/communal phagosome) (data not shown), consistent with previous observations (32). In contrast, the *rpoS* mutant replicated in ~40% of communal phagosomes harboring the parental strain (Fig. 4). The number of *rpoS* mutant bacteria per communal phagosome was 10 to 20 organisms,

which is significantly less ( $P < 0.01$  by the Student *t* test) than that of the parental strain AA100 (20 to 40 CFU/communal phagosome) (Fig. 4). Our data indicate that *L. pneumophila* RpoS is involved in Dot/Icm-mediated remodeling of the LCP into a replicative niche and also in adaptation of *L. pneumophila* to the phagosomal microenvironment, which has been shown to be stressful (3).

It has been proposed that the stringent response is the primary signal that triggers the expression of virulence traits of *L. pneumophila* at the PE phase, and some of these traits are regulated by RpoS (6, 29, 30). Expression of these virulence traits at the PE phase prior to egress of intracellular bacteria is essential in order for the progeny released to establish an intracellular proliferative niche within new susceptible host cells, and one of the traits essential for this step is evasion of lysosomal fusion and intracellular replication (7). Here we show that *L. pneumophila rpoS* but not *relA* is essential for early survival and intracellular replication in primary human macrophages and amoebae. This indicates that there is no functional link between RpoS and the stringent response in intracellular replication of *L. pneumophila*.

Within mouse primary macrophages, the *L. pneumophila rpoS* mutant resides in a phagosome that acquires the late endosomal-lysosomal marker LAMP-1 (7, 34). Our data indicate that RpoS is involved in Dot/Icm-mediated remodeling of the LCP in hMDMs, as evidenced by rescue of the RpoS mutant for intracellular replication in communal phagosomes remodeled by the parental strain. However, within communal phagosomes, replication of the *rpoS* mutant is less robust than that of the parental strain or the *dotA* mutant. Our data suggest a modest role for RpoS in bacterial adaptation to the harsh phagosomal microenvironment (3).

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