A Regulatory Feedback Loop between RpoS and SpoT Supports the Survival of *Legionella pneumophila* in Water

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Legionella pneumophila is a waterborne pathogen, and survival in the aquatic environment is central to its transmission to humans. Therefore, identifying genes required for its survival in water could help prevent Legionnaires' disease outbreaks. In the present study, we investigate the role of the sigma factor RpoS in promoting survival in water, where *L. pneumophila* experiences severe nutrient deprivation. The *rpoS* mutant showed a strong survival defect compared to the wild-type strain in defined water medium. The transcriptome of the *rpoS* mutant during exposure to water revealed that RpoS represses genes associated with replication, translation, and transcription, suggesting that the mutant fails to shut down major metabolic programs. In addition, the *rpoS* mutant is transcriptionally more active than the wild-type strain after water exposure. This could be explained by a misregulation of the stringent response in the *rpoS* mutant. Indeed, the *rpoS* mutant shows an increased expression of *spoT* and a corresponding decrease in the level of (p)ppGpp, which is due to the presence of a negative feedback loop between RpoS and SpoT. Therefore, the lack of RpoS causes an aberrant regulation of the stringent response, which prevents the induction of a successful response to starvation.

Legionella pneumophila is the causative agent of Legionnaires' disease. It is widely distributed in natural freshwater systems (1) and readily colonizes man-made water systems such as cooling towers and water distribution systems (2). *L. pneumophila* persists in aquatic environments thanks to its ability to adapt to a variety of different ecological niches, either as an intracellular parasite of amoebae or ciliate protozoans, as a free-living member of complex biofilm communities, or as planktonic cells (3, 4). Amoebae support intracellular multiplication of *L. pneumophila*, and protect against suboptimal growth conditions and exposure to chlorine (5–7). Infection of HeLa cells and *Tetrahymena tropicalis* leads to the differentiation of *L. pneumophila* into mature infectious forms (MIFs), characterized by ultrastructural changes and accumulation of poly- β -hydroxybutyrate (8–10). MIFs are highly infectious and are more resistant to antibiotics and detergent than other forms (9, 11). Increased resistance to antibiotics was also described after incubation of *L. pneumophila* in *Acanthamoeba castellanii* buffer for 16 h (12). MIFs are able to resist starvation better than stationary-phase forms in encystment buffer (11), but both forms show similar levels of resistance in distilled water (8).

In the free-living state outside the amoebal host, *L. pneumophila* encounters stressful conditions, such as limited nutrient availability in aquatic systems (1, 13). Nevertheless, previous studies have shown that *L. pneumophila* is able to survive for long periods (up to 1.5 years) in sterilized tap, surface and estuarine waters (14–17). The genetic determinants underlying the ability of *L. pneumophila* to survive in its natural habitat of low-nutrient water for a long period are currently not well understood. Nevertheless, it was shown that the type II secretion system is required for survival in cold water (15).

In general, one important adaptive strategy to deal with changing conditions is the reorganization of the transcriptome in order to express genes necessary to cope with a new condition and to repress genes that are no longer required or whose expression would be deleterious (18). The sigma factor (σ) is a subunit of RNA polymerase that confers promoter specificity to the core en-

zyme for the initiation of transcription (19). Thus, the pool of σ factors within the cell is critical to modulate transcription patterns in response to a particular cell state and condition (18, 20, 21). The *rpoS* gene encodes the alternative σ factor RpoS, which is the master regulator of the general stress response in *Escherichia coli* (22). RpoS is involved in resistance to high osmolarity, acid, heat, and oxidative stress and starvation and in the expression of virulence factors (22). Activation of genes associated with survival under adverse conditions is often required for pathogenesis. Indeed, RpoS homologs have been identified in several pathogens, and yet their roles are variable among organisms (23, 24). Hales and Shuman (25) reported that RpoS expression increases in stationary phase in *L. pneumophila* similar to *E. coli*; however, they found that RpoS is not required for stationary phase-dependent resistance to different exogenous stresses in *L. pneumophila* (acid, oxidative stress, and high osmolarity). Nevertheless, RpoS is required for efficient intracellular multiplication in the protozoan hosts *Acanthamoeba castellanii* (25) and *Acanthamoeba polyphaga* (26), in murine bone marrow-derived macrophages (27), and in human monocyte-derived macrophages (26). RpoS is not required for replication in cultured human macrophage-like HL-60 and THP-1-derived cells, probably because of increased permis-

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TABLE 1 Strains used in this study

Strain or plasmid	Relevant genotype ^a	Source or reference
Strains		
<i>Legionella pneumophila</i>		
JR32 (WT)	Philadelphia-1; Sm ^r ; r ⁻ m ⁺	31
LM1376 (<i>rpoS</i>)	JR32 <i>rpoS</i> ::Tn903dGent; Gm ^r	25
SPF176 (<i>prpoS</i>)	LM1376(pSF49)	This study
KS79	JR32 Δ <i>comR</i>	78
SPF197 (<i>relA</i>)	KS79 <i>relA</i> :: <i>aacC1</i> ; Gm ^r	This study
SPF204 (<i>relA spoT</i>)	SPF197 <i>spoT</i> :: <i>aptII</i> ; Gm ^r Km ^r	This study
<i>Escherichia coli</i>		
CF1648 (MG1655)	F ⁻ λ^- Δ <i>ilvG rfb-50 rph-1</i>	34
CF1693	Δ <i>relA251</i> Δ <i>spoT207</i>	34
(MG1655 <i>relA spoT</i>)		
Plasmids		
pMMB207c	Cm ^r ; Δ <i>mobA</i>	79
pSF49	<i>rpoS</i> in pMMB207c	This study

^a Cm^r, chloramphenicol resistance; Gm^r, gentamicin resistance; Sm^r, streptomycin resistance; Km^r, kanamycin resistance.

siveness of cultured mammalian host cells (25). RpoS, together with LetA/S and integration host factor, regulate the differentiation of *L. pneumophila* into MIFs (10, 28, 29). Comparison of the global transcriptional pattern between the wild type (WT) and the *rpoS* mutant in rich broth showed that RpoS controls multiple pathways associated with intracellular multiplication of *L. pneumophila* such as pathogenic functions, motility, transcriptional regulators, and Icm/Dot effectors (30). Interestingly, RpoS has a notable negative effect on genes associated with translation and metabolism during the postexponential phase (30).

Here, we examine the role played by the σ factor RpoS in promoting the survival of *L. pneumophila* in water in a free-living, planktonic state.

MATERIALS AND METHODS

Bacterial strains. Media and antibiotics were used as previously described (31). The *L. pneumophila* strains used are derivatives of JR32, a streptomycin-resistant variant of the *L. pneumophila* strain Philadelphia-1 (31). All of the bacterial strains and plasmids are described in Table 1. The construction of the *rpoS* mutant strain was previously described in detail (25). The JR32 strain was used as the wild-type (WT) control. In experiments testing the *relA spoT* double mutant, the KS79 strain was used as the WT control, together with the JR32 strain.

Construction of complemented strains and the *relA spoT* double mutant. The plasmid used for complementation of the *rpoS* mutation was constructed by cloning the *rpoS* gene in pMMB207c (32). The *rpoS* gene was first amplified by PCR using *Taq* polymerase (Invitrogen) and primers *rpoS* F and *rpoS* R that contain XbaI and EcoRI restriction sites, respectively. The *rpoS* amplicon and pMMB207c were digested with XbaI and EcoRI. The fragments were ligated using T4 DNA ligase (NEB) and transformed into *E. coli* DH5 α . The resulting plasmid was transformed into the *rpoS* mutant strain by electroporation as previously described (33).

For the construction of the *relA spoT* double-mutant strain, the *relA* gene was deleted first, since a single *spoT* mutant is unviable due to its inability to degrade (p)ppGpp (34, 35). Deletion of *relA* was performed using primers described in Table 2. Briefly, primers *relA5* and *relA2* (containing EcoRI), and primers *relA4* (containing PvuII) and *relA6* were used to amplify a 1-kb fragment homologous to the upstream and downstream regions of the target gene, respectively. Primers *relA1* and *relA3* that con-

TABLE 2 Primers used in this study

Primer (restriction enzyme)	Sequence (5'-3') ^a
<i>rpoS</i> F (EcoRI)	ACAGAATT <u>CGCTT</u> GTTAAGAAGTAAAAAACTA
<i>rpoS</i> R (XbaI)	CGCTCTAGATCAAAAACAAATCCTTGTGT
16s_QF	AGAGATGCATTAGTGCCTTCGGG
16s_QR	ACTAAGGATAAGGGTTGCGCTCGT
<i>qrpoA</i> F	AGAATCACGTGCTGACAATAA
<i>qrpoA</i> R	TCGAACAGTCAACTCCAAATC
qDNA polymerase F	CTCAGCAGGATGTTCTGATATT
qDNA polymerase R	CATTCTGTGCCCATCAGTAG
q50S F	GCCATCCCTACAAAACCAATC
q50S R	GAACAATACGCCAGCTTTA
<i>qrelA</i> F	GATGGCGATGTGTTGGATTAC
<i>qrelA</i> R	GGCAACGATGTCCCAAATTAC
<i>qspoT</i> F	GCAGCGCACTGGATCTATAA
<i>qspoT</i> R	CCTGTGCTCCTTTGCATTTTC
<i>relA1</i> (EcoRI)	CGCGAATTCACGGCATGATGAACCT
<i>relA2</i> (EcoRI)	CACGAATTCACATATTATCCTTTTTGA
<i>relA3</i> (PvuII)	CACCAGCTGTTAGGTGGCGGTACTTG
<i>relA4</i> (PvuII)	CGCCAGCTGCTTTGGTATATTTAGCCA
<i>relA5</i>	CTGAACCTCAATCAATGTTGTGCGC
<i>relA6</i>	GCCGAAAAATTCAAAACCAAGGC
<i>spoT1</i> (EcoRI)	CACGAATTCACGCCAGAAACAGGCCCA
<i>spoT2</i> (EcoRI)	CACGAATTCGGCTATTATACCTCACA
<i>spoT3</i> (XbaI)	CGCTCTAGATCAGAAGAAGCTCGTCAAGA
<i>spoT4</i> (XbaI)	CGCTCTAGATGCAACCAATCAATA
<i>spoT5</i>	TACATTGTTGCAGCACCTC
<i>spoT6</i>	GTCCAGAATACAATTGCC

^a Restriction sites are indicated by underlining.

tain EcoRI and PvuII, respectively, were used to amplify a gentamicin resistance cassette from the plasmid pBBR1MCS-5 (36). The three fragments were ligated by T4 DNA ligase (NEB) and the ligation mix (3 kb) was amplified by PCR using Phusion high-fidelity DNA polymerase (NEB). The 3-kb amplified fragment was then introduced into KS79 as described previously (30). The *spoT* mutation was constructed as described in the protocol above with three modifications: (i) primers *spoT4* and *spoT3* both contain XbaI restriction sites, (ii) primers *spoT1* and *spoT3* that contain EcoRI and XbaI restriction sites, respectively, were used to amplify a kanamycin resistance cassette from the plasmid pBBR1MCS-2 (36), and (iii) the resulting 3-kb fragment was introduced into the *relA* mutant background, resulting in the *relA spoT* double mutant. Allelic exchange was confirmed by PCR. The restriction sites described above are underlined in Table 2.

Survival in water. The strains used in the present study were tested for survival in water. Since the composition of tap water is variable (14–17, 37, 38), experiments were carried out in the chemically defined water medium (DFM [NaCl, 50 mg/liter; KH₂PO₄, 20 mg/liter; KCl, 50 mg/liter]; pH 6.9) to ensure reproducibility. Survival was also tested in Fraquil [0.004% CaCl₂, 0.004% MgSO₄, 0.001% NaHCO₃, 0.0002% K₂HPO₄, 0.004% NaNO₃, 10 nM FeCl₃, 1 nM CuSO₄, 0.22 nM (NH₄)₆Mo₇O₂₄, 2.5 nM CoCl₂, 23 nM MnCl₂, 4 nM ZnSO₄] and in tap water. Bacteria were suspended in DFM at an optical density at 600 nm (OD₆₀₀) of 0.1, diluted 1:5 further with DFM, and then incubated for 1 month at 25°C. The CFU were determined every 7 days unless noted otherwise.

Live/Dead assay. Bacteria were suspended in DFM at an OD₆₀₀ of 0.1 and then incubated for 1 month at 25°C. Every 7 days, unless noted otherwise, bacterial viability was determined by using a Live/Dead BacLight kit in combination with flow cytometry. Cells were stained with 6 μ M red fluorescing propidium iodide (PI) and 1 μ M green fluorescing SYTO9. Stained cells were incubated for 15 min at room temperature in the dark. The fluorescence of each strain was then analyzed by flow cytometry (Guava easyCyte; 488-nm laser).

Transcriptomic study by microarray: samples and labeling. The WT and the *rpoS* mutant strains were grown at 37°C in AYE to exponential phase ($OD_{600} = 1$), washed three times with DFM and suspended in 100 ml of DFM at a final OD_{600} of 0.1 in a tissue culture flask. After 24 h at 25°C, 100 ml of cells was pelleted by centrifugation, resuspended in 40 μ l of Tris-EDTA, and lysed by the addition of 1 ml of TRIzol reagent. RNA extraction was performed with TRIzol reagent according to the manufacturer's protocol. The RNA was subsequently treated with Turbo DNase (Ambion) and purified by acid phenol extraction. The purity and concentration of RNA were determined by UV spectrophotometry and its integrity was confirmed on a formaldehyde-agarose gel. RNA from the replicates was pooled, and 15 μ g of RNA was labeled with amino-allyl dUTP (Sigma) during reverse transcription (Superscript II; Invitrogen) using random hexamers (Invitrogen) as previously described (30, 39). Genomic DNA was used as a reference channel and labeled by random priming using Klenow fragments, amino-allyl dUTP, and random primers as described previously (39). DNA was subsequently coupled to the succinimidyl ester fluorescent dye (Invitrogen) Alexa Fluor 546 (for cDNA) or Alexa Fluor 647 (for gDNA) according to the manufacturer's protocols.

DNA microarray design. One 50-mer oligonucleotide for each *L. pneumophila* gene was designed using the OligoWiz software version 2.2.0 (40, 41). The prokaryotic setting was used with default parameters. The microarray was then produced by photolithography by Mycarray (Ann Arbor, MI). Each probe is replicated six times on the array, and negative and positive probes designed by Mycarray were also included. The platform is available under GEO accession number [GPL18472](#).

Hybridization, washing, and data analysis. Hybridization was performed as previously described (39), and data were acquired using an InnoScan 710 microarray scanner. Statistical analysis between the *rpoS* mutant strain and the wild-type control was performed using an unpaired one-tailed Student *t* test. Genes were considered differentially expressed if they demonstrated a ratio-to-control value of ± 2 -fold with a *P* value of < 0.05 .

qPCR. For analysis of gene expression by reverse transcription-quantitative PCR (RT-qPCR), RNA was extracted from strains exposed to DFM as described above. One microgram of RNA was then converted to cDNA by using random primers and Superscript II (Invitrogen) according to the manufacturer's instructions. For each sample, a negative control without reverse transcriptase was used. qPCRs were then performed with 1 μ l of cDNA using iTaq Universal SYBR green Supermix (Bio-Rad). Primer sets used for real-time PCR analysis are listed in Table 2. A relative quantification strategy was used to perform analysis of the qPCR data. Transcript levels were normalized to 16S rRNA in each sample, and the fold change was calculated as previously described (42).

Rifampin sensitivity. Different concentrations (0, 1, 12, and 25 μ g ml^{-1}) of rifampin were added to 24-h-old DFM-exposed bacteria, followed by incubation at 25°C. After 24 h, the CFU counts were determined.

De novo RNA synthesis. To quantify newly transcribed RNA in each strain, cells exposed to DFM for 24 h were fed with 1 mM the uridine analog 5-ethynyl uridine (EU) for 1 h. Cells were fixed with 3.7% formaldehyde for 15 min at room temperature and permeabilized with 0.5% Triton X-100 for 15 min at room temperature. The modified RNA was then detected *in situ* with the Alexa Fluor 488 dye according to the manufacturer's instructions for the Click-iT RNA imaging assay (Invitrogen) (43). Cells fed with EU but not labeled with Alexa Fluor 488 served as a negative control to measure the autofluorescence of *L. pneumophila* cells. Stained cells were then analyzed by flow cytometry (FACSCanto II; 488-nm laser) by comparing the amounts of fluorescently labeled cells for each strain. The data were collected and analyzed using the FlowJo software. A total of 50,000 cells were analyzed for each sample.

(p)ppGpp quantification. The presence of (p)ppGpp was detected with a fluorescent chemosensor, PyDPA, selective for (p)ppGpp (44). To ensure that PyDPA can be used with *L. pneumophila*, the WT and the *relA spoT* double mutant were grown to exponential phase in rich medium (AYE). The cultures were then split in two, and the stringent response was

induced in half of the cultures by the addition of 500 μ M serine hydroxamate (SHX), followed by incubation for 1 h. *L. pneumophila* strains, as well as the *E. coli* control strains (WT and *relA spoT* double mutant), were also exposed to DFM for 24 h ($OD_{600} = 1$). Next, 2 ml of the cells from AYE (with or without the addition of SHX) or cells from DFM was harvested by centrifugation at $8,000 \times g$ for 5 min at 4°C and resuspended in 0.5 ml of ice-cold 13 M formic acid (45). After incubation for 30 min at 4°C, cellular debris was pelleted by centrifugation at $8,000 \times g$ for 15 min at 4°C. An equal volume of phenol-chloroform-isoamyl alcohol (50:49:1 [vol/vol/vol]), saturated with 1 M Tris-HCl (pH 6.6), was added to the resulting supernatant, and the mixture was agitated and then centrifuged at $8,000 \times g$ for 15 min at 4°C (46). This supernatant was concentrated by freeze-drying (47). The dried extracts were resuspended in 200 μ l of 1 mM HEPES buffer (pH 7.4) containing 16% (vol/vol) dimethyl sulfoxide (47). PyDPA was added to a final concentration of 20 μ M to 100 μ l of supernatant. In the presence of (p)ppGpp, PyDPA emits a strong pyrene-excitimer fluorescence (excitation, 344 nm; emission, 470 nm), while in the presence of nucleotides (ATP, GTP, CTP, TTP, UTP, cAMP, or cGMP) and inorganic pyrophosphate (PP_i), PyDPA forms a pyrene monomer fluorescence (excitation, 344 nm; emission, 380 nm). The ratio of fluorescence emissions ($I_{470\text{ nm}}/I_{380\text{ nm}}$) was detected by using a Tecan microplate reader. For each sample, a negative control without PyDPA was carried out to determine the background fluorescence emissions at 470 and 380 nm.

Nucleotide sequence accession number. The sequence data are available from the GEO database ([GSE56258](#)).

RESULTS

Survival of *L. pneumophila* in water. Numerous studies have shown that *L. pneumophila* is able to survive in tap water for extended periods of time; however, differences in the survival times were reported, presumably because of local differences in the composition of tap water (14–17, 37, 38). One of the projects conducted in our group consisted in developing an artificial freshwater medium to increase the reproducibility of the survival assay. One such medium, here called DFM, is based on previous publications reporting the use of defined freshwater recipes and on the salt and buffer content of the chemically defined medium developed for *L. pneumophila* (48, 49). As part of the project, we tried to identify genetic determinants underlying the ability of *L. pneumophila* to survive in water by exposing several mutant strains from our collection to DFM and determined survivability by CFU counts. A strain carrying a transposon insertion in *rpoS* showed reduced survival in DFM compared to the WT strain (Fig. 1). To confirm that the observed phenotype was due to inactivation of RpoS, the *rpoS* gene was cloned downstream of the isopropyl- β -D-thiogalactopyranoside (IPTG)-inducible promoter (*P_{tac}*) of the pMMB207c vector (prpoS). Partial recovery of the WT phenotype was observed in the complemented strain induced with 0.1 mM IPTG (prpoS ON). Survival of the complemented strain not exposed to IPTG (prpoS OFF) was significantly lower than that of the WT and approximated that of the *rpoS* mutant (Fig. 1). prpoS OFF exhibited a slightly higher survival rate than the *rpoS* mutant, probably because of partial complementation due to leakage of the *P_{tac}* promoter (32). These results confirm that the survival defect observed is *rpoS* dependent. The reduction in CFU counts could be due to the death of the *rpoS* mutant cells or their premature entry into a viable but nonculturable state (reviewed in reference 50). Therefore, we assessed bacterial viability by using a Live/Dead BacLight kit in combination with flow cytometry (51). The viability of the *rpoS* mutant strain decreases over time proportionally to the reduction in CFU (Fig. 1B), showing that in the absence of

FIG 1 RpoS is necessary for survival in water. (A) *L. pneumophila* wild-type (WT), *rpoS* mutant (*rpoS*), and the complemented strains induced with 0.1 mM IPTG (*prpoS* ON) and not induced with IPTG (*prpoS* OFF) were tested for survival in defined freshwater medium (DFM) for 28 days at 25°C. CFU were counted every week on CYE plates. (B) Cells retrieved from DFM were stained with 6 μ M PI and 1 μ M green fluorescent SYTO9 and then analyzed for cell viability with flow cytometry. (C) The wild-type (WT) and the *rpoS* mutant (*rpoS*) were tested for survival in Fraquil and in tap water as described in panel A. DL, detection limit. Error bars represent the standard deviations from three independent biological replicates. We used an unpaired Student *t* test to assess statistical significance for each time point. *, $P \leq 0.05$; **, $P \leq 0.005$; ***, $P \leq 0.0005$ (versus WT).

rpoS, *L. pneumophila* cells die in DFM. To determine whether this survival defect could be seen in other types of water or if it is specific for DFM, the survival of the WT and the *rpoS* mutant strain was tested in Fraquil, a defined freshwater medium that approximates the composition of freshwater of North America (52), and tap water. The *rpoS* mutant was defective for survival in both Fraquil and tap water (Fig. 1C), although the reduction in tap water was slower than that in Fraquil and DFM. Therefore, it seems that the *rpoS* mutant is defective for survival in water in general.

rpoS deficiency leads to overexpression of numerous genes.

We hypothesized that the lack of *rpoS* could lead to a misregulation of genetic factors involved in survival in water, causing the observed defect. DNA microarray technology was used to analyze the regulatory network of the *rpoS* mutant in water. RNA samples used for microarray analysis were isolated from three replicates of both the WT and the *rpoS* mutant after exposure to DFM for 24 h. Since the mutant strain dies in DFM, we chose this early time point to ensure that enough RNA could be extracted (Fig. 1). The normalized signal intensity of differentially expressed genes is shown in Fig. 2A (see also Table S1 in the supplemental material). Microarray analysis showed that, compared to the WT, the lack of RpoS significantly affects 668 genes, 506 positively (induced in the mutant) and 162 negatively (repressed in the mutant).

An analysis of the Clusters of Orthologous Groups (COG) protein database revealed that, in most categories, more genes were induced in the *rpoS* mutant than repressed, such as in the “icm/dot effector,” “toxin production/other pathogen function,” “energy metabolism,” “transcription,” “translation,” and “replication and repair” categories shown in Fig. 2B.

The expression of three genes was tested by RT-qPCR, including the RNA polymerase gene *rpoA* (*lpg0354*), the DNA polymerase III beta chain gene (*lpg0002*), and the 50S ribosomal protein L25 gene (*lpg2652*) involved in transcription, replication, and translation, respectively. Consistent with the microarray data, qPCR analysis showed that the expression of these genes increased in the *rpoS* mutant strain compared to the WT (Fig. 2C). Their expression in the complemented strain (*prpoS* ON) was similar to that of the WT. The increased expression of genes associated with transcription, replication, and translation, in the absence of RpoS, indicates that the expression of these genes is negatively controlled by RpoS, possibly through gene products that it regulates, such as transcriptional regulators or small regulatory RNAs (53).

Lack of RpoS results in a higher level of transcription. The overexpression of several genes in the *rpoS* mutant, together with the overexpression of many genes involved in transcription, led us to hypothesize that the *rpoS* mutant suffers from an increase in the overall level of transcription, wasting metabolites and energy, and leading to the premature reduction in CFU counts observed in Fig. 1. In addition, the total RNA quantification revealed that an ~2-fold increase was observed in the amount of RNA extracted by TRIzol from the *rpoS* mutant over that from the WT after exposure to DFM for 24 h (Fig. 3A). The sensitivity of the WT strain and the *rpoS* mutant to rifampin, which blocks RNA synthesis (54), was then tested. Since most antibiotics exert their effects on processes that are required for cell growth, their effect is usually proportional to the level of activity of their target (55), and thus rifampin is more potent against transcriptionally active bacteria. After 24 h of exposure to DFM, the *rpoS* mutant was significantly more sensitive to rifampin compared to the WT (Fig. 3B). The complemented strain showed a WT phenotype (Fig. 3B). These results are consistent with the hypothesis that the *rpoS* mutant undergoes more transcription than the WT. Nevertheless, there is a possibility that the lack of *rpoS* may affect cell wall synthesis or resistance mechanisms leading to increased lysis by TRIzol and increased sensitivity to antibiotics and therefore to the apparent increase in RNA level and rifampin sensitivity.

To confirm our hypothesis, we quantified the RNA synthesized *de novo* in the WT, the *rpoS* mutant, and the complemented strains by using the Click-iT RNA Alexa Fluor 488 imaging kit

FIG 2 Transcriptional response of the *rpoS* mutant after 24 h in water. The global gene expression profile of the *rpoS* mutant was compared to the WT strain after exposure to DFM for 24 h. (A) Hierarchical clustering of genes showing a ratio to control value of ± 2 -fold and a P value of ≤ 0.005 . The normalized signal intensity is shown. The cluster of genes induced in the *rpoS* mutant is shown by a red line; the cluster of genes repressed is shown by a green line. (B) Cluster of orthologous group (COG) analysis of the microarray data. (C) RT-qPCR was used to confirm the expression of genes related to translation (50S ribosomal protein L25 gene, *lpg0002*), replication (DNA polymerase III gene, *lpg2652*), and transcription (*rpoA*, *lpg0354*). Strains are described in Fig. 1. The data are presented as means of three biological replicates, and the error bars represent the standard deviations. Since the data represent a ratio against the WT strain, we used the nonparametric Mann-Whitney test to assess statistical significance. *, $P \leq 0.05$.

(Molecular Probes). Cells were exposed to water and fed for 1 h with 5-ethynyl uridine (EU), which is incorporated into nascent RNA. The cells were then fixed and permeabilized before the modified RNA was labeled with Alexa Fluor 488. To ensure that the difference in signals would be due to a difference in transcription and not to a difference in permeability, the cells were also stained with PI, which only enters cells with damaged cytoplasmic membranes (56). There were no differences in PI staining between the strains tested, whether they were stained before or after the fixation and permeabilization step (see Fig. S1 in the supplemental

material). After 24 h in water, the *rpoS* mutant showed more Alexa Fluor 488 fluorescence than did the WT strain, which indicates a higher level of EU incorporation into new RNA molecules and, consequently, a higher level of transcription (Fig. 4A and B). The complemented strain showed a WT phenotype. Interestingly, the strains grown in rich broth to exponential phase showed no significant difference in staining, confirming that the increased level of transcription in the *rpoS* mutant strain is specific to water exposure and, consequently, to nutrient starvation (Fig. 4C and D). Taken together, our results clearly show that the *rpoS* mutant

FIG 3 The *rpoS* mutant shows a higher transcription level. (A) Total RNA was extracted from the WT and the *rpoS* mutant strain after 24 h in DFM and quantified by spectrophotometry. Error bars represent the standard deviations from three independent biological replicates. (B) Comparison of rifampin sensitivities of the *rpoS* mutant and the WT in DFM. Four concentrations of rifampin (0, 1, 12, and 25 µg/ml) were added to the DFM, which were then incubated at 25°C for 24 h. The data are presented as the means of three biological replicates, and the error bars represent the standard deviations. We used an unpaired Student *t* test to assess statistical significance. *, $P = 0.05$; **, $P \leq 0.005$ (versus WT). DL, detection limit.

strain displays an increased transcription level compared to the WT when exposed to nutrient-poor water.

(p)ppGpp is necessary for survival in water. Traxler et al. (57) showed that in response to amino acid starvation, an *E. coli* mutant strain lacking (p)ppGpp shared a similar phenotype as our *rpoS* mutant strain in producing significantly more RNA than the WT strain. Based on this result and knowing that RpoS is one of

the key effectors of the stringent response (58), we sought to further investigate the involvement of the stringent response in the RpoS regulatory network associated with survival in water. In most bacteria, the alarmone (p)ppGpp lies at the top of the network that governs global gene expression in response to nutrient limitation (59). In most gammaproteobacteria, including *L. pneumophila*, the (p)ppGpp synthetase RelA monitors amino acid

FIG 4 The *rpoS* mutant shows higher *de novo* RNA synthesis in DFM. Cells exposed to DFM for 24 h or grown in broth (AYE) to exponential phase were fed for 1 h with the uridine analog 5-ethynyl uridine (EU), which is actively incorporated into nascent RNA. The cells were then fixed and permeabilized and the modified RNA was labeled with Alexa Fluor 488 dye using a Click-iT RNA imaging assay (Invitrogen). Stained cells were analyzed by flow cytometry (FACSCanto II; 488-nm laser). A total of 50,000 cells were analyzed for each sample. The fluorescence histograms of typical experiments performed in DFM (A) and in broth AYE (C) are shown. The horizontal bar represents the signal of Alexa Fluor 488 considered positive (above autofluorescence noise). The geometric mean from three independent experiments performed in DFM (B) and in broth (AYE) (D) was calculated for Alexa Fluor 488 positive cells. Cells fed with EU but not labeled with Alexa Fluor 488 served as a negative control to assess autofluorescence noise (Autofluo). Error bars represent the standard deviations from three independent biological replicates. We used a paired Student *t* test to assess statistical significance. Strains are described in Fig. 1.

availability through its association with the ribosome, whereas the bifunctional synthetase and hydrolase SpoT responds to a variety of stimuli, including fatty acid starvation, which requires direct interaction with an acyl-carrier protein (26, 35, 45, 60, 61).

In the present study, the DFM water represents a nutrient-poor condition; thus, we expected that cells unable to produce (p)ppGpp would be unable to survive in water. Indeed, as shown in Fig. 5A, the *relA spoT* double-knockout mutant showed a quick decrease in survival in water compared to the WT strain. The viability of the *relA spoT* double-mutant strain decreases over time proportionally to the reduction in CFU (Fig. 5B), showing that in the absence of (p)ppGpp, *L. pneumophila* cells die in water.

Lack of RpoS results in higher expression of *spoT* and a reduction of the (p)ppGpp level. At this point, we hypothesized that the lack of RpoS in water somewhat influences the stringent response circuitry in *L. pneumophila* which would, in turn, disturb the level of (p)ppGpp. The transcriptome of the *rpoS* mutant during exposure to water showed an increased expression of *spoT*, whereas the levels of expression of *relA* were similar in both strains (Fig. 5C, black bar). We used RT-qPCR to monitor the expression of the *spoT* and *relA* genes in the *rpoS* mutant, the WT, and the complemented strain after 24 h of exposure to DFM. In agreement with the microarray analysis, *spoT* was overexpressed in the *rpoS* mutant, and complementation restored the WT expression level (Fig. 5C). No significant difference was observed for the *relA* gene. Taken together, these observations strongly support our hypothesis that the stringent response is affected when RpoS is absent. The upregulation of the *spoT* gene in the *rpoS* mutant suggests that the (p)ppGpp level would, in turn, be affected. An increased expression of *spoT* in the *rpoS* mutant strain might decrease the level of (p)ppGpp through its hydrolase activity. To test this hypothesis, we determined the level of (p)ppGpp in the *rpoS* mutant. The (p)ppGpp assay was performed using PyDPA, a selective fluorescent chemosensor for (p)ppGpp (44). Extraction of nucleotides from bacterial cells was carried out with 13 M formic acid as described previously (45, 46), further purified with phenol-chloroform (46), and concentrated by freeze-drying (47). The production of (p)ppGpp after exposure of exponential-phase *L. pneumophila* to SHX for 1 h was measured first to ensure that PyDPA can be used for *L. pneumophila*. SHX is a serine analog that causes amino acid starvation and induces (p)ppGpp synthesis (62). Exposure of the WT strain to SHX leads to an increase in the signal, but exposure of the *relA spoT* double mutant did not (Fig. 6A). This shows that production of (p)ppGpp from *L. pneumophila* can be successfully detected with PyDPA. Then, the production of (p)ppGpp was measured after exposure to DFM for 24 h, at which point the *rpoS* mutant strain showed increased transcription (Fig. 2, 3, and 4). The *E. coli* WT and *relA spoT* double mutant, as well as the *L. pneumophila* WT and *relA spoT* double mutant, were used as a control for this assay. As expected, the *relA spoT* double mutants showed significantly less (p)ppGpp than did the WT strain (Fig. 6B). As hypothesized, we found that the *rpoS* mutant strain has a significantly lower level of (p)ppGpp than the WT strain (Fig. 6B). The complemented strain (*prpoS* ON) was found to produce more (p)ppGpp than the WT (Fig. 6B). Taken together, our results clearly show a link between the repression of *spoT* by RpoS and a higher level of (p)ppGpp in the WT. Therefore, it seems likely that a lower level of (p)ppGpp accumulation

FIG 5 The stringent response is required for survival in water and is affected by mutation of *rpoS*. (A) The stringent response mutant strain (*relA spoT*) and the wild-type strain (WT) were tested for survival in defined water (DFM) for 30 days at 25°C. CFU were counted every week on CYE plates. DL, detection limit of the CFU. (B) Cells retrieved from DFM were stained with 6 μ M PI and 1 μ M green fluorescent SYTO9 and then analyzed for cell viability with flow cytometry. We used an unpaired Student *t* test to assess statistical significance for each time point. **, $P \leq 0.005$ (versus WT). (C) RT-qPCR was used to determine the level of expression of *spoT* and *relA* compared to the WT strain. The microarray data for the expression of *spoT* and *relA* (*rpoS*/WT) are indicated by black bars. The data are presented as the means of three biological replicates, and the error bars represent the standard deviations. Since the data represent a ratio against the WT strain, we used the nonparametric Mann-Whitney test to assess statistical significance. *, $P \leq 0.05$ (versus WT).

upon starvation in water is causing the survival defect of the *rpoS* mutant strain.

DISCUSSION

L. pneumophila is commonly found in natural and man-made water systems. Our goal was to find genetic determinants involved in the survival of *L. pneumophila* in water. Hence, in the present

FIG 6 Production of ppGpp in the *rpoS* mutant is reduced. Quantification of the (p)ppGpp level was performed by using PyDPA, a selective fluorescent chemosensor for (p)ppGpp. (A) The WT and *relA spoT* double-mutant strains were grown to exponential phase in AYE broth in triplicate. The cultures were split in two, and SHX was added to one tube. Samples were collected after 1 h, and (p)ppGpp was quantified by using PyDPA. The data are presented as the ratio between SHX-treated cells (+SHX) and the untreated control (–SHX). (B) Cells were exposed to DFM for 24 h. The (p)ppGpp was then extracted and quantified with PyDPA. The strains are described in Fig. 1 and Fig. 5, except for the *E. coli* wild-type (*E. coli* WT) and the *E. coli* stringent response mutant (*E. coli relA spoT*). The data are presented as ratios against the wild-type strain of three replicates, and the error bars represent the standard deviations. Since the data represent a ratio, we used the nonparametric Mann-Whitney test to assess statistical significance. *, $P \leq 0.05$ (versus WT *L. pneumophila* [*L. pneumophila* mutants] or WT *E. coli* [*E. coli* mutant]). DL, detection limit.

study, we report the role that the σ factor RpoS plays in promoting survival in water, where *L. pneumophila* experiences severe nutrient deprivation. As expected, the lack of RpoS led to a decreased survival in water (Fig. 1), indicating that RpoS is a key regulator in *L. pneumophila* adaptation to the starving conditions found in water. It is possible that RpoS is also necessary for survival in rich broth after the postexponential phase has been reached, but this was not tested here since this possibility does not have any implication for the transmission of *L. pneumophila* from the water environment to the human host.

Several metabolic systems are induced in the *rpoS* mutant, such as transcription, translation, and replication (Fig. 2B and C). Our results are in agreement with those of Hovel-Miner et al. (30), who examined the expression profile of the *L. pneumophila rpoS* mutant during postexponential-phase growth in rich broth. Their study also showed that RpoS has notable negative effects on the transcription of genes associated with translation and metabolism such as those encoding ribosomal proteins, tRNA synthesis genes, and tRNA genes. In addition, we found that the *rpoS* mutant displays an accumulation of *de novo*-synthesized RNA (Fig. 4A and B), which means that upon exposure to water, *L. pneumophila* reduces transcription through negative regulation by RpoS. Consequently, the *rpoS* mutant showed reduced resistance to rifampin (Fig. 2B). Similarly, MIFs show an increased resistance to rifampin, presumably because of the lower transcription rate (8), and RpoS is necessary for the differentiation of *L. pneumophila* into MIFs (10).

For the model organism *E. coli*, RpoS is essential for the transcription of genes important for cell survival under conditions leading to the switch from the fully active to the slow metabolic state, as in the case of nutrient starvation (63). Among the many

genes in the RpoS regulon, some are directly regulated by RpoS and others might be indirectly regulated through transcription factors (activators and repressors) and other σ factors (64).

The signaling alarmone guanosine tetraphosphate [(p)ppGpp] is a key player in the reduction of the transcription level during the postexponential phase in *E. coli* (65, 66). In most bacteria, nutrient depletion prompts a restructuring of global transcription patterns known as the stringent response (60, 67). (p)ppGpp is the general indicator of the nutritional status of the cell, and it lies at the top of the regulatory network of the stringent response (59, 60). In Gram-negative bacteria, including *L. pneumophila*, (p)ppGpp is predominantly produced by RelA, and the balance of (p)ppGpp is regulated by SpoT (60, 68), which mediates (p)ppGpp turnover via its hydrolase activity and weak synthase activity (34, 45). In *E. coli*, a mutant lacking *relA* and *spoT* is completely devoid of (p)ppGpp (69), a state that disrupts repression of rRNA transcription (70) and results in the synthesis of stable RNA (69), similar to the phenotype observed here in the *rpoS* mutant of *L. pneumophila*. Therefore, we confirmed that (p)ppGpp is necessary for survival of *L. pneumophila* in water by testing the *relA spoT* double mutant, which lacks all (p)ppGpp synthetase activity (45) (Fig. 5A and B). This mutant also showed a survival defect after a 60 h of incubation in rich medium (45).

In *E. coli*, it was shown that RpoS is one of the key regulators activated in response to a high level of (p)ppGpp (58). Control exerted by (p)ppGpp on RpoS is observed at several levels: gene transcription (71), regulon induction (58), protein stabilization (72), and σ factor competition (73). As with *E. coli*, the transcription of *rpoS* in *L. pneumophila* is sensitive to the (p)ppGpp level, since *rpoS* is induced in response to (p)ppGpp accumulation (74). Although the stringent response and RpoS are major virulence regulators in *L. pneumophila*, the link between RelA, SpoT, and RpoS remains to be clarified. During infection of host cells, *L. pneumophila* goes through two distinct phases: the replicative phase, characterized by active replication, and the transmissive phase, characterized by inhibition of replication and activation of motility and cytotoxicity (75). RelA activity is essential in inducing the transmissive phase and is dispensable during the replicative phase (35). Two studies reported the role of RelA in *L. pneumophila* in the postexponential phase, which mimics the transmissive phase. Zusman et al. (35) showed that a *relA* mutant made in JR32, which is the WT strain used here, does not produce detectable levels of (p)ppGpp, whereas Dalebroux et al. (45) showed that a *relA* mutant made in Lp02 still produces an extremely small amount of (p)ppGpp. Genetic differences or experimental variation could explain this apparent discrepancy. It was established that, in *L. pneumophila*, RelA produces (p)ppGpp in response to limiting amino acids, while SpoT produces (p)ppGpp in response to perturbation in fatty acid biosynthesis (45, 76). SpoT also regulates (p)ppGpp degradation and thus, it is critical for transmissive cells to reenter the replicative phase (45). To our knowledge, the mechanism controlling the synthetase and hydrolase activity of SpoT is currently not understood but could involve interactions with acyl-carrier proteins or other proteins (26, 35, 45, 60).

In the present study, we showed a new connection between RpoS and the stringent response effector (p)ppGpp associated with survival of *L. pneumophila* in water. As shown in both microarray and RT-qPCR analyses (Fig. 5C), expression of the *spoT* gene is increased in the *rpoS* mutant strain. This phenotype is confirmed by complementation, which restored the WT expres-

sion level. As a result, we found that the *rpoS* mutant strain has a significantly lower level of (p)ppGpp compared to that of the WT, and complementation of the *rpoS* mutant restored the WT phenotype (Fig. 6B). Overexpression of *spoT* would presumably overcome the regulatory mechanism controlling the synthetase and hydrolase functions of SpoT, which, according to our data, will ultimately result in an increase in its hydrolase activity. This is supported by a recent study reporting that overexpression of *spoT* in *Salmonella enterica* serovar Typhimurium drastically reduces the amount of (p)ppGpp and rescues the growth defect of a *tufA499* mutant, which produces high levels of (p)ppGpp (77). These findings highlight the negative effect of RpoS on the expression level of *spoT* that helps maintain a high level of (p)ppGpp to efficiently cope with the nutritional limitations encountered upon exposure to water. The molecular mechanism behind this negative feedback loop is currently unknown, but could involve RpoS-dependent regulation of a negative regulator of *spoT*.

In light of our data, we propose the following model that might explain the survival defect of the *rpoS* mutant strain. Upon exposure to water, *L. pneumophila* would accumulate (p)ppGpp at a level sufficient to induce the RpoS regulatory network which would, in turn, adjust the gene expression program by reducing metabolic processes in favor of survival. Then, RpoS reduces the expression of *spoT*, probably through an indirect mechanism such as the induction of a negative regulator of *spoT*. This negative feedback loop ensures that (p)ppGpp is not degraded by SpoT and that the alarmone level is high enough to fully induce the stringent response. Nevertheless, the manner in which (p)ppGpp relays information to RpoS and the manner in which RpoS-mediated regulation is integrated into the stringent response in *L. pneumophila* remain unclear and will require further study.

To conclude, our study challenges the current paradigm that RpoS is merely a messenger of the stringent response and instead supports the hypothesis that RpoS is an active player in the induction and maintenance of the stringent response by regulating the expression of SpoT and, consequently, the level of (p)ppGpp itself. This proposed pathway could be present in other bacterial species as well.

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