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σ^S Controls Multiple Pathways Associated with Intracellular Multiplication of *Legionella pneumophila*▽†

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***Legionella pneumophila* is the causative agent of the severe and potentially fatal pneumonia Legionnaires' disease. *L. pneumophila* is able to replicate within macrophages and protozoa by establishing a replicative compartment in a process that requires the Icm/Dot type IVB secretion system. The signals and regulatory pathways required for *Legionella* infection and intracellular replication are poorly understood. Mutation of the *rpoS* gene, which encodes σ^S, does not affect growth in rich medium but severely decreases *L. pneumophila* intracellular multiplication within protozoan hosts. To gain insight into the intracellular multiplication defect of an *rpoS* mutant, we examined its pattern of gene expression during exponential and postexponential growth. We found that σ^S affects distinct groups of genes that contribute to *Legionella* intracellular multiplication. We demonstrate that *rpoS* mutants have a functional Icm/Dot system yet are defective for the expression of many genes encoding Icm/Dot-translocated substrates. We also show that σ^S affects the transcription of the *cpxR* and *pmrA* genes, which encode two-component response regulators that directly affect the transcription of Icm/Dot substrates. Our characterization of the *L. pneumophila* small RNA *csrB* homologs, *rsmY* and *rsmZ*, introduces a link between σ^S and the posttranscriptional regulator CsrA. We analyzed the network of σ^S-controlled genes by mutational analysis of transcriptional regulators affected by σ^S. One of these, encoding the *L. pneumophila* arginine repressor homolog gene, *argR*, is required for maximal intracellular growth in amoebae. These data show that σ^S is a key regulator of multiple pathways required for *L. pneumophila* intracellular multiplication.**

Legionella pneumophila is a gram-negative opportunistic human pathogen that causes the severe and potentially fatal pneumonia Legionnaires' disease (30, 47, 67, 83). *L. pneumophila*'s ability to replicate within human alveolar macrophages is essential for its capacity to cause disease (44–46). Transmission of *L. pneumophila* to the human lung occurs as a result of the inhalation of aerosolized contaminated water droplets (74), often from exposure to showers or whirlpool baths (96). Legionella species are ubiquitous in most naturally occurring and man-made aquatic systems, where the organism replicates within a variety of unicellular protozoan hosts (28, 38, 96). It has been suggested that the interaction of *Legionella* species with environmental protozoa has selected for the bacterium's evolutionary adaptation to intracellular life in mammalian cells (99).

Intracellular multiplication of *L. pneumophila* requires a series of ordered events that disrupt normal endocytic trafficking in both macrophage and protozoan host cells. These include preventing phagolysosome fusion and the acidification of the *Legionella*-containing vacuole (LCV), followed by the acquisition of membrane material derived from the Golgi and endoplasmic reticulum compartments of the host (71, 85, 93, 95). These events are dependent upon the Icm/Dot type IVB secretion system (TFBSS) (84, 90, 91). The Icm/Dot system is homologous to type IV conjugation systems and is able to translocate DNA (90, 105) and protein between bacteria as well as to translocate proteins from the bacteria into eukaryotic hosts (15, 18, 24, 25, 75). More than 150 putative Icm/Dot-translocated substrates have been identified (8, 14, 15, 18, 23, 25, 37, 52, 54, 61, 75, 78, 94, 103, 108), many based on the presence of eukaryotic-like protein domains (25), and these are predicted to modify endocytic trafficking and other host cell functions for the benefit of the bacterium (18, 48, 64, 75). However, the functions of the majority of Icm/Dot-translocated substrates are unknown, and most strains containing null mutations in the genes encoding Icm/Dot substrates are not defective for intracellular replication (77). The apparent functional redundancy of Icm/Dot-translocated substrates has resulted in the hypothesis that subsets of Icm/Dot substrates may target distinct protozoan hosts (77).

Legionella intracellular multiplication likely requires the sensing of, and response to, a myriad of signals, which suggests the need for a complex regulatory network (3, 31, 38–40, 62, 71, 72, 108). However, few regulators of intracellular multiplication have been identified, and their organization and interconnectivity are only partially understood. The two-component systems CpxRA and PmrAB directly regulate the transcription of subsets of Icm/Dot-translocated substrates (3, 108). An additional two-component system, LetAS (homologous to the GacAS system of pseudomonads [9, 32]), is proposed to re-

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spond to ppGpp by positively affecting the expression of postexponential growth phenotypes and is required for the efficient intracellular replication of *L. pneumophila* (5, 40, 71). The effect of LetAS has been linked to the global carbon storage regulator CsrA through shared phenotypes (72). Based on the GacAS system in *Pseudomonas aeruginosa*, LetAS is predicted to regulate the transcription of the small RNA *csrB*, which represses the CsrA protein (71). However, prior to this investigation no *L. pneumophila csrB* homolog had been characterized.

Null mutations in the *rpoS* gene, encoding the sigma factor σ^S, do not affect growth in rich medium but exhibit two interesting phenotypes: *rpoS* mutants retain significant stress resistance in postexponential phase, and they are not able to replicate in amoebae (38). The stress resistance phenotype of *L. pneumophila* is in contrast to *Escherichia coli*, in which *rpoS* mutants are much more sensitive to stress during postexponential growth than the wild type (43). In *E. coli*, σ^S is considered the general stress sigma factor because it senses and responds broadly to a variety of stress signals by regulating the transcription of a large number of target genes (42). In bacterial pathogens including *Salmonella enterica* serovar Typhimurium, *Yersinia enterocolitica*, and *Vibrio cholerae*, the σ^S regulon is involved not only in stress resistance but also in the regulation of virulence genes (49, 69, 76, 86). *L. pneumophila rpoS* mutants are unable to replicate in protozoa and primary macrophages (1) but are not defective for replication in human macrophage cell lines such as THP-1 and HL-60 (38). Because of its central role as a sigma factor and its altered host range compared to *icm*/*dot* TFBSS mutants, σ^S may be a key toward unlocking the regulatory requirements for *Legionella* intracellular multiplication.

In this study we show that although *rpoS* and *icm*/*dot* mutants share similar intracellular multiplication and trafficking defects, *rpoS* mutants are not defective in Icm/Dot-dependent protein translocation. To develop a comprehensive view of σ^S regulation in *L. pneumophila* and identify σ^S-regulated genes that might be required for intracellular multiplication, we compared the patterns of global gene expression of an *rpoS* mutant and wild-type *L. pneumophila* during exponential and postexponential growth in rich medium. The expression data were analyzed for global transcriptional effects of σ^S and interrogated for σ^S effects on the expression of genes known to be required for intracellular multiplication. We show that σ^S affects the expression of many genes encoding Icm/Dot substrates as well as genes encoding regulators required for intracellular multiplication. Through the mutational analysis of σ^S-affected transcription factor genes, we discovered that the *L. pneumophila* arginine repressor homolog, *argR*, is required for efficient intracellular multiplication. These data expand our understanding of the σ^S regulon and the requirements for *L. pneumophila* intracellular multiplication.

MATERIALS AND METHODS

Bacterial strains and mutants. The bacterial strains used in this study are listed in Table 1. Media and antibiotics were used as previously described (18). *L. pneumophila* strains used in this study were *L. pneumophila* JR32, a streptomycin-resistant, restriction-negative mutant of *L. pneumophila* Philadelphia-1 (87); LM1376 is an isogenic *rpoS*-null (lpg1284) derivative of JR32 (38);

TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Genotype or description	Reference or source
L. pneumophila strains		
JR32	Am511 salt-sensitive isolate	87
KS79	JR32 Δ*comR*	24
LELA3118	JR32 *dotA*::Tn*903*dIII*lacZ*	87
LM1376	JR32 *rpoS*::Tn*903*dGent	38
GAH199	KS79 *dotA*::Tn*903*dIII*lacZ*	This study
GAH235	KS79 Δ*rpoS*::Km^r	This study
GAH280	KS79 Δ*argR*::Km^r	This study
GAH338	KS79 Δ*letS*::KmR	This study
GAH026	JR32, pXDC31	This study
GAH028	LELA3118, pXDC31	This study
GAH147	LM1376, pXDC31	This study
GAH281	KS79, pXDC31	This study
GAH282	GAH199, pXDC31	This study
GAH286	GA280, pXDC31	This study
GAH228	KS79, pXDC61-FabI	This study
GAH214	KS79, pXDC61-LepA	This study
GAH215	GAH199, pXDC61-LepA	This study
GAH238	GAH235, pXDC61-LepA	This study
SPF38	JR32, pSF21	This study
SPF39	GAH338, pSF21	This study
SPF40	LM1376, pSF21	This study
GAH301	GAH199, pGAH123	This study
GAH305	KS79, pMMB207c, pGAH123	This study
GAH306	KS79, pGAH125, pGAH123	This study
GAH312	GAH280, pMMB207c, pGAH123	This study
GAH314	GAH280, pGAH125, pGAH123	This study
Plasmids		
pMMB207c	pMMB207 *mobA*	91
pBBR1MCS-2	pBBR1MCS Km^r	51
pXDC31	pMMB207c *Ptac*-GFP	This study
pXDC61-FabI	pMMB207c *Ptac*-TEM1-FabI	24
pXDC61-LepA	pMMB207c *Ptac*-TEM1-LepA	24
pSF21	pMMB270c *Ptac-letS*	This study
pGAH125	pMMB207c *Ptac-argR*	This study
pGAH123	pBBR1MCS2 *mobA*, two copies of *Ptac*-GFP; Gent^r	This study

LELA3118 is an isogenic *dotA*-null (lpg2686) derivative of JR32 (87); KS79 is an isogenic Δ*comR* (lpg2717) (24) derivative of JR32 that renders the bacteria genetically competent for DNA uptake (24, 92). Mutants made in this study were produced by creating allelic exchange fragments using long-flanking homology PCR as described previously (106), which consisted of approximately 1 kb of homology to each end of the target gene flanking a 1-kb kanamycin resistance cassette, based on previous results for *Legionella* natural transformation (92). Purified allelic exchange fragments were introduced into competent KS79 cells by natural transformation as described previously (92), selected on kanamycin, screened for gene replacement by PCR, and sequenced for verification of correct insertion as described previously (38). Mutants made by this method are listed in Table 2 and include GAH280 Δ*argR* (lpg0490) and GAH338 Δ*letS* (lpg1912), both of which are isogenic derivatives of KS79. The primers for long-flanking homology PCR are listed in Table S6 in the supplemental material. GAH199, a *dotA*-null strain, was made by the transformation of KS79 with LELA3118 genomic DNA prepared according to the manufacturer's protocol (Wizard Genomic DNA Purification Kit; Promega) and selection on kanamycin.

Growth of bacterial strains and medium preparation. Media and antibiotics for the growth of *L. pneumophila* were used as described previously (18). Isolation of exponentially and postexponentially growing *L. pneumophila* was performed in the complex medium AYE [*N*-(2-acetamido)-2-aminoethanesulfonic acid-buffered yeast extract] in which cultures were started with an inoculum with an optical density (OD) of approximately 0.05 and grown with agitation at 37°C. Exponential phase cultures were collected at an OD of 0.70 to 0.80. Postexponential phase cultures were collected approximately 4 h following the cessation of growth, which occurred at an approximate OD of 3.0 to 3.5. For most exper-

TABLE 2. Transcriptional regulators affected by σ^S

ORF	Protein description	Mutation[a]	Gene	Transcription level ($\log_2$ ratio of *rpoS*/Wt)[b]	
				Exponential phase	Postexponential phase
lpg0490	Arginine repressor	Y	*argR*	3.80	−1.80
lpg0586	Transcriptional regulator	Y		−0.39	−4.10
lpg0853	Transcriptional regulator FleQ	N	*fleQ*	−1.75	−2.81
lpg1260	Putative repressor protein of prophage	Y	*prpA*	1.77	3.64
lpg1292	DNA-binding response regulator	N	*pmrA*	−0.94	−2.14
lpg1438	DNA-binding response regulator	N	*cpxR*	−2.43	−2.57
lpg1446	Transcriptional regulator	Y		−2.10	−1.27
lpg1577	RNA polymerase σ^E	Y	*rpoE*	−1.50	−4.93
lpg1782	Flagellar biosynthesis sigma factor	N	*fliA*	−0.27	−4.95
lpg1796	Transcriptional regulator, LysR family	Y		−1.86	−3.19
lpg2138	Transcriptional regulator, LysR family	Y		−0.41	−2.75
lpg2376	Transcriptional regulator, LysR family	Y		−0.76	−3.02
lpg2723	Transcriptional regulator, ArsR family	Y		−2.45	−0.71

[a] Y, the indicated gene was deleted by allelic exchange in this study; N, the indicated gene was not deleted.
[b] Wt, wild type.

iments bacteria were grown in triplicate; however, six independent bacterial cultures for each of the strains under any growth condition were tested for gene expression profiling in this study.

Plasmid construction. Plasmids pXDC31, pGAH125, and pSF21 were constructed from the restriction digest of green fluorescent protein (GFP), ArgR, and LetS PCR products, respectively, bearing the restriction sites EcoRI/HindIII (GFP) or KpnI/XbaI (ArgR and LetS) cloned into digested pMMB207c vector. Construction of pGAH123 consisted of the mutational inactivation of *mobA*, addition of two copies of p*tac*-GFP derived from pXDC31, and the addition of a gentamicin resistance cassette to pBBR1MCS2 in successive cloning steps.

TEM translocation assays. Measurement of Icm/Dot-dependent substrate translocation was performed as previously described using published TEM fusion plasmids (24). Translocation experiments were performed three times, and the data shown are from one representative experiment.

Fluorescence microscopy of *D. discoideum*. *Dictyostelium discoideum* AX2 cells were prepared and infected as described previously (18, 19) using bacteria expressing cytoplasmic DsRed and *D. discoideum* amoebae expressing a GFP-tagged subunit of the V-ATPase, VatM-GFP (22). The cells were observed by microscopy at 1 h and 4 h following infection, as previously described (18).

RNA isolation, cDNA preparation, and real-time qPCR experiments. RNA for microarray and real-time quantitative PCR (qPCR) was prepared using an RNeasy Mini Kit following manufacturer's protocols (Qiagen). In qPCR experiments, RNA was treated with DNase I according to the manufacturer's instructions (Invitrogen) prior to cDNA preparation. For microarray and qPCR samples, cDNA was prepared using Superscript II reverse transcriptase as described by the manufacturer (Invitrogen). Real-time qPCR experiments were performed using an Applied Biosystems StepOne Plus 96-well reverse transcription-PCR system with Power SYBR Green PCR Master Mix following the manufacturer's instructions (Applied Biosystems). 16S RNA was used as the reference sample in all comparative threshold cycle ($\Delta\Delta C_T$) experiments. All qPCR primers were tested for amplification efficiency (see Table S7 in the supplemental material). Real-time qPCR data were analyzed using StepOne System software and Microsoft Excel. Primers used in qPCR experiments are shown in Table S7 in the supplemental material.

Microarray analysis. The *L. pneumophila* strain Philadelphia-1 microarray consisting of 2,997 unique 70-mer oligonucleotides representing all of the *L. pneumophila* predicted open reading frames (ORFs) presented in duplicate was previously published (16). Oligonucleotides were resuspended to a concentration of 30 μM in 50% dimethyl sulfoxide and printed on UltraGaps aminosilane-coated slides (Corning Life Sciences) using a SpotArray 72 Microarray Printing System spotter (Perkin Elmer). After being spotted, the slides were stored in a vacuum at room temperature until further use.

To prepare the samples for microarray hybridization, 20 μg of total RNA from each of the samples was converted to cDNA by reverse transcription in the presence of aminoallyl-dUTP and fluorescently labeled by coupling the resulting cDNA with the succinimidyl ester fluorescent dyes Alexa Fluor 546 and Alexa Fluor 647 (Invitrogen), following the manufacturer's protocols. Labeled cDNA was hybridized to Corning UltraGAPs coated slides and washed following Corning, Inc., protocols. Hybridized arrays were scanned using a ScanArray Express (PerkinElmer) instrument at 5-μm resolution, and the resulting hybridization intensities for all probes from both channels on each array were exported for further analysis. Raw signal intensities were corrected for dye labeling effects within and between all slides using the normalize.lowess R-function implemented in the Bioconductor affy microarray analysis package (33). Genes with a P value of ≤0.005 and for which the ratio of the $\log_2$ spot intensity of the mutant (LM1376) over the wild type (JR32) was less than or equal to −2 or greater than or equal to +2 were considered for further analysis. The resulting sets of differentially expressed genes were further analyzed using hierarchical clustering algorithms implemented within the Spotfire DecisionSite software suite (Tibco Spotfire, Inc.).

Eukaryotic cell line maintenance and kinetic measurement of intracellular multiplication. *Acanthamoeba castellanii* was maintained in peptone-yeast-glucose medium as described previously (38). Preparation for measurement of *L. pneumophila* intracellular multiplication was similar to previously described methods (38) with minor adaptations for 96-well plates. Monolayers of *A. castellanii* cells were formed in 96-well plates at 1×10^5 cells per well. *L. pneumophila* strains harboring GFP-positive plasmids (pXDC31 or pGAH123) were induced overnight on charcoal-yeast extract plates containing 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and then resuspended and diluted to the desired multiplicity of infection (MOI) in the appropriate medium for infection, which included an antibiotic for plasmid maintenance and 0.5 mM IPTG for constitutive expression of GFP. Infection of *A. castellanii* was carried out in Ac buffer as described previously (38) containing 0.5 mM IPTG and 5 μM chloramphenicol (Gibco). Prior to infection, cell attachment medium was removed and replaced with infection medium containing *L. pneumophila* strains at the desired MOIs. Plates were centrifuged for 10 min at 2,000 rpm. Intracellular multiplication was monitored automatically by measuring GFP fluorescence at an excitation of 485 nm and emission of 520 nm in a Tecan Infinite M200 plate reader every hour for 72 h. Fluorescence data, expressed in relative fluorescence units, was collected using Magellan software and exported to Microsoft Excel for background subtractions, time x/time zero calculations (to produce normalized relative fluorescence values), and graphical analysis. All intracellular multiplication experiments were performed at least three times, and the data shown are from one representative experiment performed in triplicate and averaged.

Northern blot analysis. RNA was isolated by using TRIzol reagents as described by the manufacturer (Invitrogen). One microgram of RNA was separated on a 6% Tris-borate-EDTA–urea polyacrylamide gel (Invitrogen) and transferred to a positively charged nylon membrane (Micropore) using a semidry gel blotting system (Bio-Rad) for 20 min at 200 mA. Blots were prehybridized in Ultrahyb-Oligo (Ambion) for at least 1 h before overnight hybridization with 5′ biotin-labeled oligonucleotide probes (*rsmY* probe, 5′-biotin-GCAGCGAAGT ACATCCTTTGTACTGGTCCCTTAGTTGACTTCCTGTCAGACATATCC; *rsmZ* probe, 5′-biotin-CGCAGTCATCCGTATAAGAACTTGCGTTCTTATT GTCATCCTGACAAATC). Blots were then washed two times with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 0.5% sodium dodecyl sulfate, and the biotin probes were detected by using a Chemiluminescent Nucleic Acid Detection Module (Pierce) as directed by the manufacturer.

Microarray data accession number. The microarray data developed in this study have been deposited in the NCBI Gene Expression Omnibus database under accession number GSE14830.

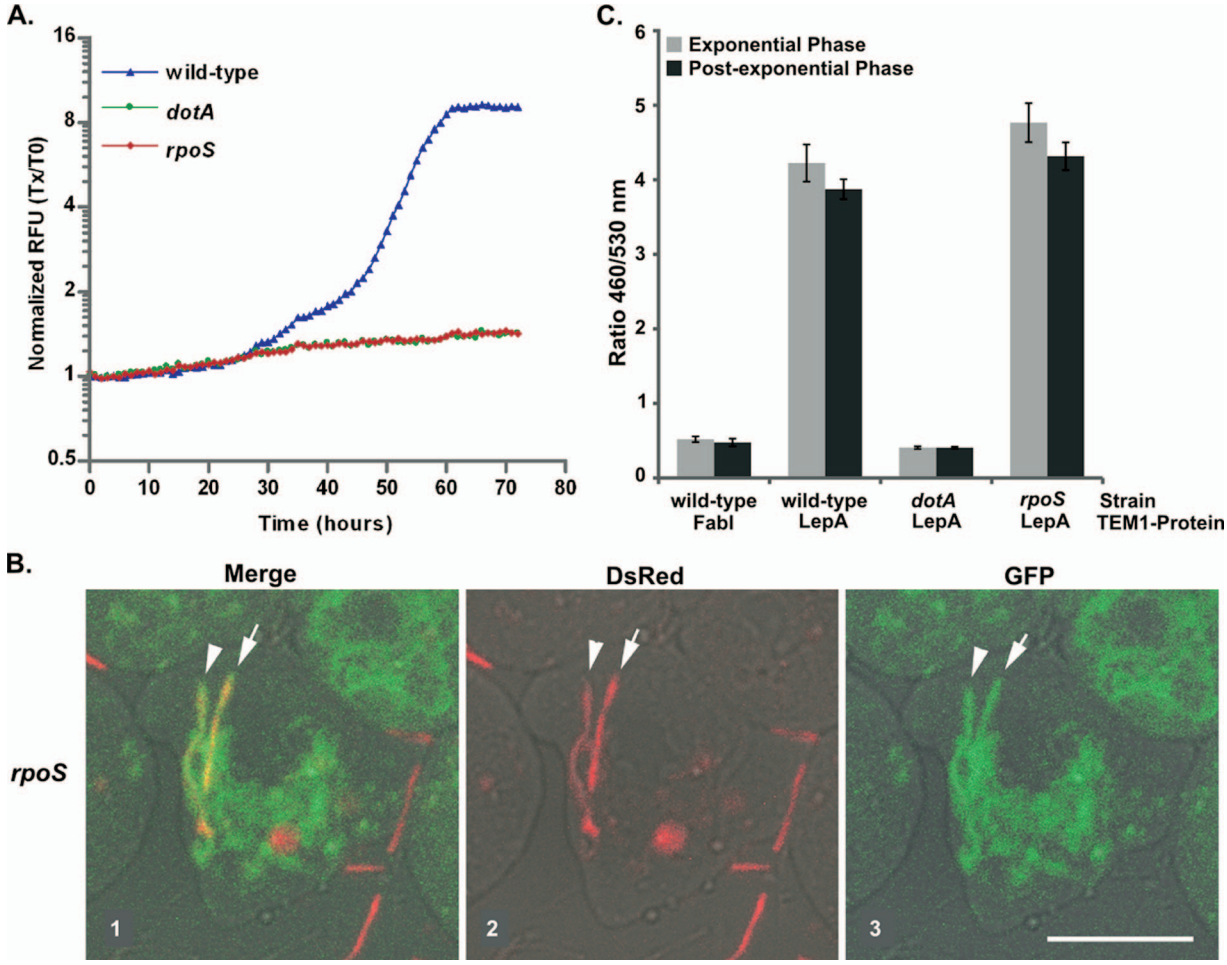

FIG. 1. Phenotypes of *rpoS* mutants. (A) Monolayers of *A. castellanii* were infected at an MOI of 1.0 with *L. pneumophila* harboring GFP on the plasmid pXDC31. Fluorescence was measured every 1 h for 72 h in a Tecan Infinite M200. Relative fluorescence units (RFU) were normalized to fluorescence at time zero for the wild type (JR32), the *rpoS*-null strain (blue line; LM1376), and the *dotA*-null strain (green line; LELA3118) T_x/T_0, time *x*/time zero. (B) *Dictyostelium* amoeba expressing VatM-GFP were combined with *the rpoS*-null strain (LM1376) expressing cytoplasmic DsRed and analyzed after 4 h as described previously (18, 22). The left panel shows an overlay of GFP, monomeric red fluorescent protein, and a bright-field image; the middle panel shows monomeric red fluorescent protein and a bright-field overlay; and the right panel shows GFP and a bright-field overlay. Arrowheads indicate VatM-GFP-positive phagosomes containing bacteria. Scale marker, 10 μm. (C) Translocation of the Icm/Dot protein substrate LepA or the negative control FabI by KS79 (wild type), the *dotA*-null strain (GAH199), or Δ*rpoS* (GAH235) harboring TEM-1 fusion plasmids was measured by infecting J774 cells at an MOI of 50. Cells were loaded with CCF4-AM, and translocation was determined by measuring the ratio of cleaved (460 nm) to uncleaved (530 nm) CCF4-AM-labeled cells as described previously (17, 24). Error bars represent the standard deviation of triplicate samples from one experiment.

RESULTS

***rpoS* mutant intracellular multiplication phenotypes are not due to a defective Icm/Dot TFBSS.** The Icm/Dot TFBSS is essential for the formation of the *Legionella* replicative vacuole and intracellular replication in all host cells (91). Mutations in the *rpoS* gene exhibit some, but not all, *icm*/*dot* mutant intracellular multiplication phenotypes (1, 38). Characterization of the phenotypes of the *rpoS* mutant required the use of multiple host cell types: *A. castellanii* was used to measure intracellular multiplication, *D. discoideum* was used for endocytic trafficking microscopy studies, and the J774 macrophage cell line was used to assay Icm/Dot-dependent translocation. To analyze the intracellular multiplication defect of an *rpoS* mutant, we measured intracellular growth by bacterial strains expressing GFP over 72 h using an automated fluorescence microplate spectrofluorometer. The data show that in *A. castellanii*, *rpoS* mutants have an intracellular multiplication defect that is indistinguishable from the Icm/Dot TFBSS mutant *dotA* (Fig. 1A; for supporting viable count data see Fig. S1A in the supplemental material). We tested whether *rpoS* mutants could block trafficking of the vacuolar ATPase to the LCV in the protozoan host *D. discoideum*. Using bacteria expressing cytoplasmic DsRed and *D. discoideum* expressing VatM-GFP, we observed for the *icmT* mutant and in contrast to the wild type (JR32) (18) that VatM-GFP association could be detected for some *rpoS*-containing phagosomes about 1 h after the cells and bacteria were mixed. By 4 h, most *rpoS*-containing phagosomes were surrounded by VatM-GFP, and many contained bacteria whose cytoplasmic DsRed marker had been largely destroyed, presumably because the bacteria were being digested by 4 h

after infection (Fig. 1B), a behavior similar to the Icm/Dot TFBSS mutant *icmT*, a negative control (data not shown). If σ^S were required for the expression of a functional Icm/Dot system, it then would provide an explanation for the observed intracellular multiplication defects of *rpoS* mutants. Icm/Dot-dependent substrate translocation was tested using a previously described assay (24), in which *Legionella* strains harboring a TEM-1 (β-lactamase) fusion to an Icm/Dot substrate on a plasmid were used to infect host cells loaded with a fluorescent dye (CCF4-AM) containing a β-lactam ring, which, when cleaved by TEM-1, is converted from green (520 nm) to blue (460) fluorescence. We found that *rpoS* mutations did not result in reduced translocation of the Icm/Dot substrates LepA (Fig. 1C) and RalF (data not shown) into to J774 macrophages and that growth phase did not have a significant effect on Icm/Dot substrate translocation (Fig. 1C). This shows that the phenotypic similarities of *rpoS* and *icm/dot* mutants are not the result of an *rpoS* mutant Icm/Dot secretion defect. However, an alternative explanation is that an unknown *rpoS*-dependent factor may be required for Icm/Dot-dependent substrate translocation into amoebae but not macrophages.

Mutation of the *rpoS* gene has widespread effects on *L. pneumophila* gene expression. To investigate the regulatory networks associated with the intracellular multiplication defect of an *rpoS* mutant, we employed global gene expression analysis. RNA was isolated from six replicate cultures of both the wild type (JR32) and an isogenic *rpoS* mutant (LM1376) during exponential and postexponential growth in rich medium and used for microarray analysis (89). The $\log_2$ ratio of mutant/wild-type bacteria during exponential and postexponential growth for all the annotated ORFs in the *L. pneumophila* Philadelphia-1 genome is shown in Table S1 in the supplemental material. Genes whose steady-state transcript levels were significantly affected by the mutation of *rpoS* were defined as having a $\log_2$ ratio of mutant/wild-type bacteria greater than or equal to +2 or less than or equal to −2, i.e., a minimum of a fourfold change, with a *P* value of ≤0.005 (see Table S2 in the supplemental material). Positive values for the *rpoS* mutant/wild-type ratio indicate genes whose steady-state transcript level is negatively affected by σ^S, and negative values for the *rpoS* mutant/wild-type ratio indicate genes whose steady-state transcript level is positively affected by σ^S. This resulted in the identification of 739 *rpoS*-affected genes within the *L. pneumophila* genome (Fig. 2), which suggests that σ^S affects the transcription of approximately one-fourth of the 3,007 annotated genes (73). Sigma factors are generally associated with gene activation through transcription initiation (11); however, the data show that the global effects of σ^S are fairly balanced between positive and negative with 386 and 360 genes, respectively. This suggests that many of the observed effects might not occur through direct interaction of σ^S with the promoter of the affected gene but, rather, indirectly through additional regulators. As is true in other organisms, σ^S affects the expression of many more genes during the postexponential phase of growth (571) than during exponential phase (218) (42). This could be due to the postexponential phase accumulation of σ^S protein (38).

A global view of σ^S regulation was acquired by determining the percentage of σ^S-affected genes within each major category assigned to the annotated genome sequence of the *L. pneumophila* Philadelphia-1 (73) during exponential and postexponential growth (Fig. 3). An additional category was created to include the 89 proteins for which Icm/Dot-dependent translocation has been shown (3, 19, 23, 24, 52, 58, 61, 65, 75, 78, 94, 103, 108). In exponential phase, the steady-state transcript level of amino acid metabolism genes is negatively affected, whereas genes associated with DNA/RNA degradation, transcription, and tRNA synthesis are positively affected (Fig. 3A). In postexponential phase σ^S negatively affects the steady-state transcript level of genes in multiple categories associated with translation and metabolism while positively affecting genes categorized in pathogenic functions, motility, signal transduction, transcription factors, and substrates of the Icm/Dot system (Fig. 3B). Transcription of *L. pneumophila* secretion system genes (55, 66, 87), including *icm/dot* genes, was not significantly affected by mutation of *rpoS* in either growth phase (see Table S3 in the supplemental material). The absence of a defect in *icm/dot* gene expression is supported by the finding, shown above, that *rpoS* mutants are not defective in Icm/Dot-dependent substrate translocation (Fig. 1C).

Thus, during postexponential phase, σ^S does not affect *L.*

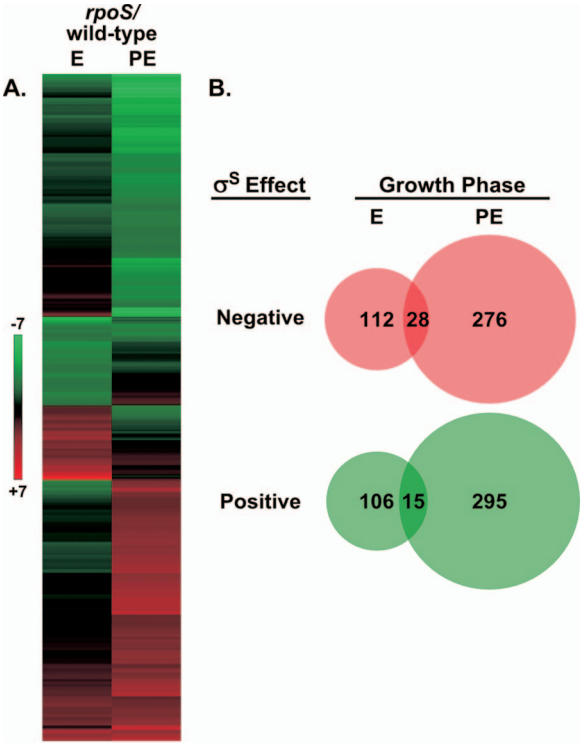

FIG. 2. σ^S is a global regulator of *L. pneumophila* gene expression. The global gene expression profile of an *rpoS*-null mutant (LM1376) was compared to a wild-type strain (JR32) during exponential (E) and postexponential (PE) growth phases. Genes for which the ratio of the *rpoS*-null strain to the wild-type strain is $-2 \geq \log_2 \geq +2$ with a *P* value of ≤0.005 were clustered using Tibco Spotfire DecisionSite default settings (A). Green indicates reduced expression in the mutant, and red indicates increased expression in the mutant. The number of positively (green) and negatively (red) σ^S-affected genes in both growth phases (E and PE) are represented in a Venn diagram (B), which depicts all major types of σ^S regulation but does not illustrate the seven genes that are positively regulated in one phase of growth and negatively regulated in the other.

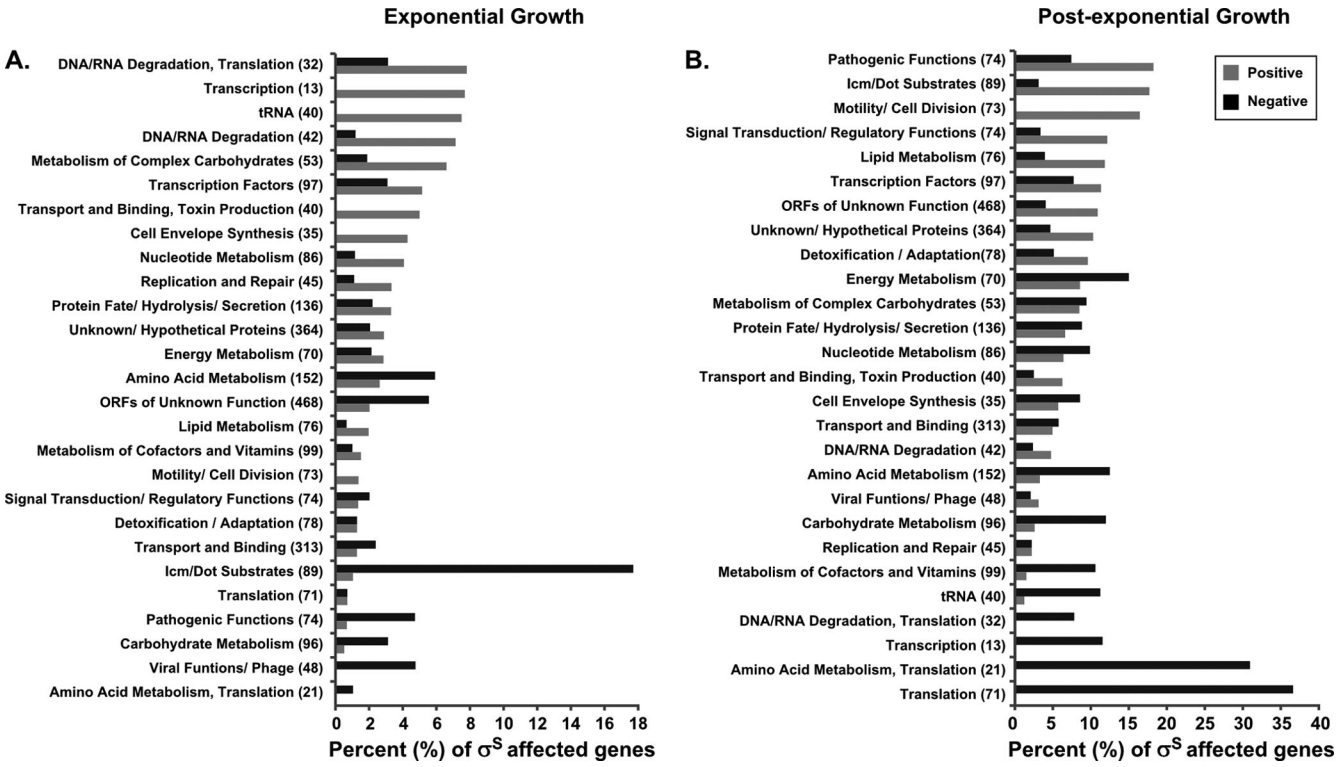

FIG. 3. σ^S affects the expression of genes in diverse functional categories. The percentage of σ^S-affected genes (*x* axis) within each functional category was identified from the *rpoS* mutant microarray based on the functional categories (*y* axis) assigned to the *L. pneumophila* Philadelphia-1 genome (20). The values are sorted from the highest percentage of positively affected genes (gray bars versus black bars for negatively affected) per category in both exponential and postexponential growth phases. The total number of genes present in each category is shown in parentheses; the σ^S-affected genes are shown as a percentage of this total. The category Icm/Dot substrates was created for this study. The difference in scale between panels A and B reflects σ^S regulatory effects.

pneumophila secretion system genes but participates in modulating the expression of genes associated with actively growing cells while enhancing the expression of motility genes, which are known to be induced during postexponential phase, and genes categorized with pathogenic functions. Many genes encoding Icm/Dot-translocated substrates were affected by σ^S during both growth phases.

σ^S affects the transcription of many genes encoding Icm/Dot-translocated substrates. The Icm/Dot-dependent translocation of proteins from the bacterium to the host cell interior is proposed to determine the outcome of a *Legionella* infection (18, 24, 25). Some Icm/Dot-translocated proteins have established effects on host cell processes that are proposed to contribute to the establishment of the LCV (18, 48, 64, 75). Previous studies have implicated σ^S in the regulation of Icm/Dot substrate expression (25). In order to obtain a comprehensive understanding of the effects of σ^S on Icm/Dot substrate gene expression, a list of 89 proteins for which Icm/Dot-dependent translocation has been demonstrated was compiled (see Table S4 in the supplemental material). Analysis of this list showed that out of 89 Icm/Dot substrates, the transcription of 38 genes (43%) is significantly affected by mutation of *rpoS* (see Table S2 in the supplemental material). The major effects of σ^S are equally balanced between genes whose expression is negatively affected in exponential phase and those positively affected in postexponential phase, with 17 genes in each category (Fig. 4A). Transcription of many of the *ceg* genes is modulated by σ^S during exponential phase, whereas most *sid* and *sde* gene transcription is enhanced by σ^S in postexponential phase (Fig. 4B; see also Table S4 in the supplemental material). The transcription of only one Icm/Dot-translocated substrate gene, *vpdC*, was positively affected during exponential phase, and there were three negatively affected during postexponential phase, *legC7*, *legS2*, and *lepB* (Fig. 4; see also Table S4 in the supplemental material). Microarray results were validated by quantitative real-time qPCR for a subset of Icm/Dot-translocated substrate genes (see Table S5 in the supplemental material). The observed effect of σ^S on Icm/Dot substrate gene expression may contribute to the intracellular multiplication defects of *rpoS* mutants.

Transcription of the *L. pneumophila csrB* homologs, *rsmY* and *rsmZ*, requires σ^S. Efficient intracellular multiplication of *L. pneumophila* requires a functional LetAS two-component system (32, 62) as well as the regulator CsrA, which is responsible for the posttranscriptional repression of genes that promote postexponential phenotypes (5, 7, 71, 72). In other bacterial species, LetAS homologs (e.g., GacAS in *P. aeruginosa* [56, 104]) activate the postexponential transcription of homologs of the small RNA *csrB*, which sequesters CsrA proteins, thus relieving the repression of postexponential genes (4, 9, 41, 56, 71, 104). In *L. pneumophila*, a phenotypic link between LetAS and CsrA has been established and proposed to

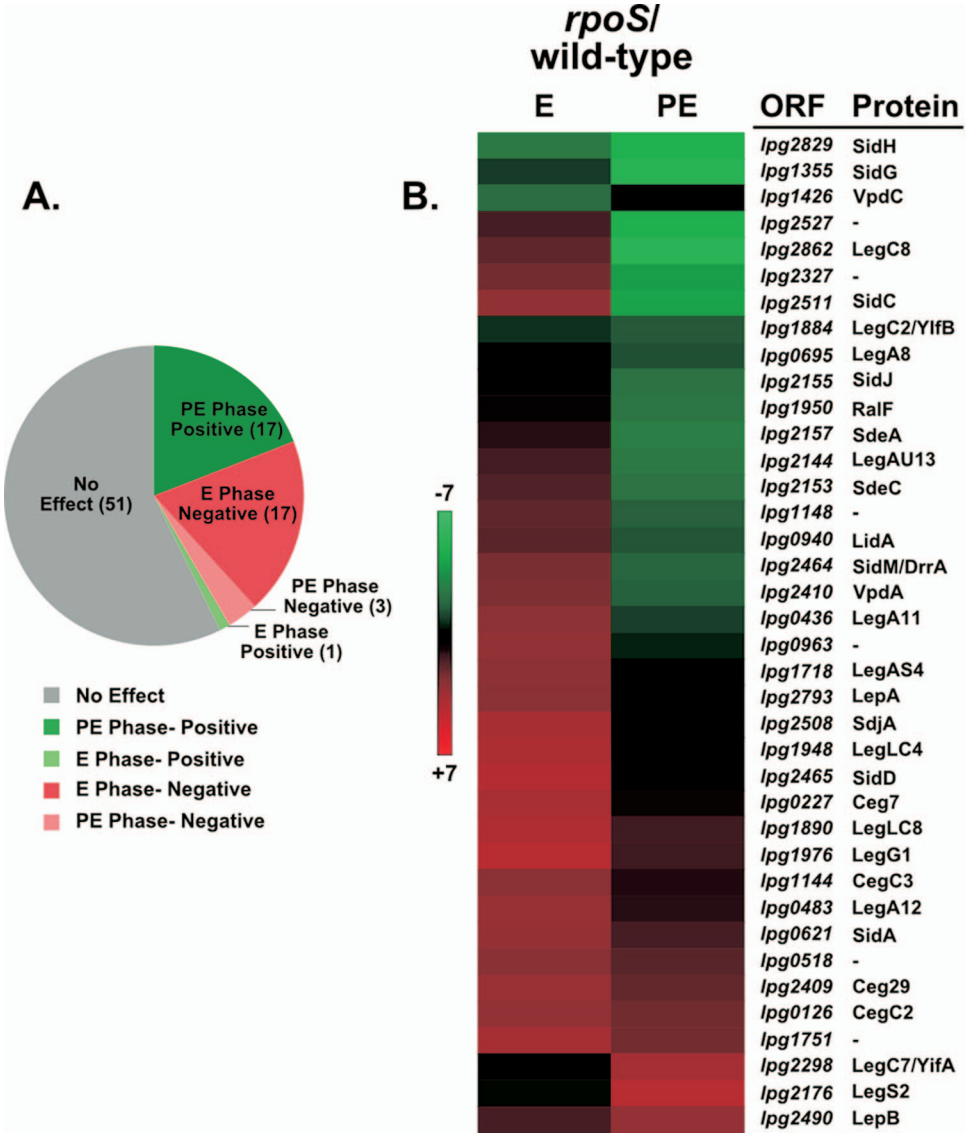

FIG. 4. σ^S affects the expression of many Icm/Dot-translocated substrate genes. The expression of 89 genes whose protein products were previously shown to be translocated in an Icm/Dot-dependent manner (for references, see Table S4 in the supplemental material) were analyzed from the *rpoS* mutant microarray. The effects of σ^S on the 89 genes analyzed is depicted in a pie chart (A), and the 38 σ^S-affected Icm/Dot substrate genes are shown in a hierarchical clustering diagram along with ORF designations and protein names (if assigned) (B). E, exponential phase; PE, postexponential phase.

occur by the LetAS regulation of a *csrB* homolog (5, 7, 68, 71, 72). Recently, two *L. pneumophila csrB* homologs were predicted using a bioinformatics approach; these were named *rsmY* and *rsmZ* based on their structural similarity to the *P. aeruginosa* small RNAs (53). The microarray data showed a twofold reduction in *letS* transcripts in the *rpoS* mutant during postexponential phase (see Table S1 in the supplemental material). Although this suggests a possible role for σ^S in a pathway that controls CsrA, it is not considered significant based on the statistical criteria established in this study. To determine if σ^S affects the regulatory pathway that controls CsrA function in *L. pneumophila*, we investigated the expression of the *crsB* homologs.

To determine if the predicted small RNAs *rsmY* and *rsmZ* are transcribed in *L. pneumophila*, we performed Northern blot analysis on wild-type bacteria (JR32). These results demonstrated the postexponential production of *rsmY* and *rsmZ* transcripts near their predicted sizes of 106 nucleotides and 132 nucleotides, respectively (53) (Fig. 5). The *L. pneumophila rsmY* and *rsmZ* genes were further validated as homologs of *csrB* by demonstrating that their transcription is reduced in an *letS* mutant (GAH338) (Fig. 5). This is the expected result from studies of *gacAS* mutants in *P. aeruginosa* (56). We also found that overexpression of *rsmY* results in increased pigmentation (data not shown), consistent with the phenotype of an *L. pneumophila csrA* mutant (72). To determine if σ^S has a role in this pathway, we analyzed the amounts of *rsmY* and *rsmZ* transcripts in an *rpoS* mutant background (LM1376) and found

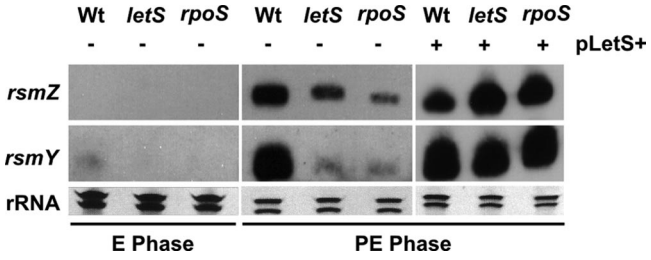

FIG. 5. σ^S and LetS positively affect the transcription of the *L. pneumophila csrB* homologs *rsmY* and *rsmZ*. Transcripts of *rsmY* and *rsmZ* were detected by Northern blot analysis during exponential (E) and postexponential (PE) growth in wild-type (Wt; JR32), Δ*letS* (GAH338), and *rpoS*-null (LM1376) mutants. Wild-type (SPF38), Δ*letS* (SPF39), and *rpoS*-null (SPF40) strains harboring an inducible *letS* gene on the plasmid pSF21 (pLetS+) were induced with IPTG (1.0 mM), and transcripts were measured during the PE phase. Levels of RNA loaded are demonstrated by the amount of 23S and 16S rRNA observed from the ethidium bromide-stained gel.

that the levels of both transcripts were reduced in the mutant compared to the wild type during postexponential growth (Fig. 5). Interestingly, the levels of the *rsmY* and *rsmZ* transcripts were similarly reduced in both the *letS* and *rpoS* mutant backgrounds (Fig. 5). This suggests that LetS and σ^S might function in the same pathway. To analyze the relationship between σ^S and LetS in the *csrB* regulatory pathway, we overexpressed LetS in both the *rpoS* and *letS* mutant backgrounds. We found that this resulted in the recovery of wild-type *rsmY* and *rsmZ* transcript levels during postexponential phase in both mutants (Fig. 5). These data suggest that σ^S acts upstream of LetS in a regulatory circuit that results in increased postexponential transcription of *L. pneumophila rsmY* and *rsmZ* (see Fig. 7).

Identification of σ^S-affected transcription factors required for intracellular multiplication. The widespread effects of σ^S can occur through its direct interaction with the promoter of an affected gene or indirectly by way of its effect on additional regulators. We mined the *rpoS* mutant microarray to identify σ^S-affected regulatory genes that may be required for intracellular multiplication. Genes encoding transcription factors whose expression is affected by σ^S were identified from the *rpoS* mutant global gene expression profile (see Table S2 in the supplemental material). Initially, 25 genes annotated as encoding transcription factors from the *L. pneumophila* genome (20) were identified. Of these, the 13 encoding putative DNA-binding domains were considered further (Table 2). Of the 13 putative transcription factors, 9 had not been studied previously and were deleted by allelic exchange (see Table S6 in the supplemental material). The four previously described transcription factors whose expression is affected by σ^S are *fleQ*, *fliA*, *cpxR*, and *pmrA*. It has been previously demonstrated that the postexponential transcription of the flagellar regulator genes *fleQ* and *fliA* is σ^S dependent and is not critical for *L. pneumophila* intracellular multiplication (50, 71). A new finding from this study is that the steady-state transcript levels of the response regulator genes of the two-component systems *cpxRA* and *pmrAB* are decreased by more than fourfold in an *rpoS* mutant compared to the wild type (Table 2; see also Table S5 in the supplemental material). CpxRA and PmrAB directly regulate the transcription of specific Icm/Dot-translocated substrate genes (3, 108), some of which were also shown to be affected by σ^S in this study. Although *L. pneumophila cpxR* mutants are not defective for intracellular replication, *pmrA* mutants are unable to replicate in *A. castellanii* (3, 108). Thus, reduced *pmrA* expression may contribute to the inability of the *rpoS* mutant to replicate in *A. castellanii*.

During the course of this investigation we developed a fluorescence-based method for the measurement of intracellular multiplication in which bacteria constitutively expressing a fluorescent marker (e.g., GFP) are used to infect host cells in a multiwell microplate. The level of fluorescence in each well can be monitored automatically at programmed intervals in a microplate fluorimeter (Fig. 1A). We found that the data obtained by this method agreed with data obtained by the traditional method of determining the number of CFU over time (see Fig. S1 in the supplemental material). The primary advantage of the fluorescence-based method, in contrast to viable-count assays, is that it permits the detailed side-by-side comparison of a large number of strains or conditions. We used this method to analyze the ability of the nine putative transcription factor mutants to replicate within the protozoan host *A. castellanii*. We found that mutation of the *L. pneumophila* arginine repressor homolog, *argR* (lpg0490), resulted in a reduction in intracellular multiplication in *A. castellanii* (Fig. 6A). None of the other mutants constructed in this study exhibited defects in intracellular multiplication (data not shown). The *rpoS* microarray data show that the steady-state transcript level of *argR* is up more than 12-fold during exponential phase and down by more than twofold during postexponential phase in the mutant (Table 2; see also Table S5 in the supplemental material). The intracellular multiplication defect of the *argR* mutant in *A. castellanii* was complemented in strains harboring an inducible *argR* gene (Fig. 6B). It is interesting that when the *argR* gene is expressed under p*tac* promoter control in the Δ*argR* mutant, there is an increase in intracellular multiplication compared to wild-type *L. pneumophila* (Fig. 6B). In contrast, when the same construct is present in the wild-type background, it results in a small but consistent reduction in intracellular multiplication (Fig. 6B). Although the mechanism for these phenotypes requires further investigation, it is possible that native transcriptional regulation of *argR* is required to accurately recapitulate the wild-type phenotypes. We conclude that derepression of the arginine regulon may contribute to the intracellular multiplication defect of the *rpoS* mutant. The role of the *L. pneumophila* arginine repressor and the *argR* regulon in intracellular multiplication is unclear at the present time and will require further investigation.

DISCUSSION

The σ^S factor has been widely studied in multiple species of gram-negative bacteria and has been shown to be important for the expression of virulence-related genes in several pathogens (49, 69, 76, 86). Although loss of the *L. pneumophila rpoS* gene has no noticeable effect on growth in complex medium, it results in severe defects in intracellular replication (5, 6, 38, 109) and endocytic trafficking that are not the result of impaired Icm/Dot TFBSS function (Fig. 1) or *icm*/*dot* gene expression (see Table S3 in the supplemental material). This study demonstrates that, in addition to genome-wide transcrip-

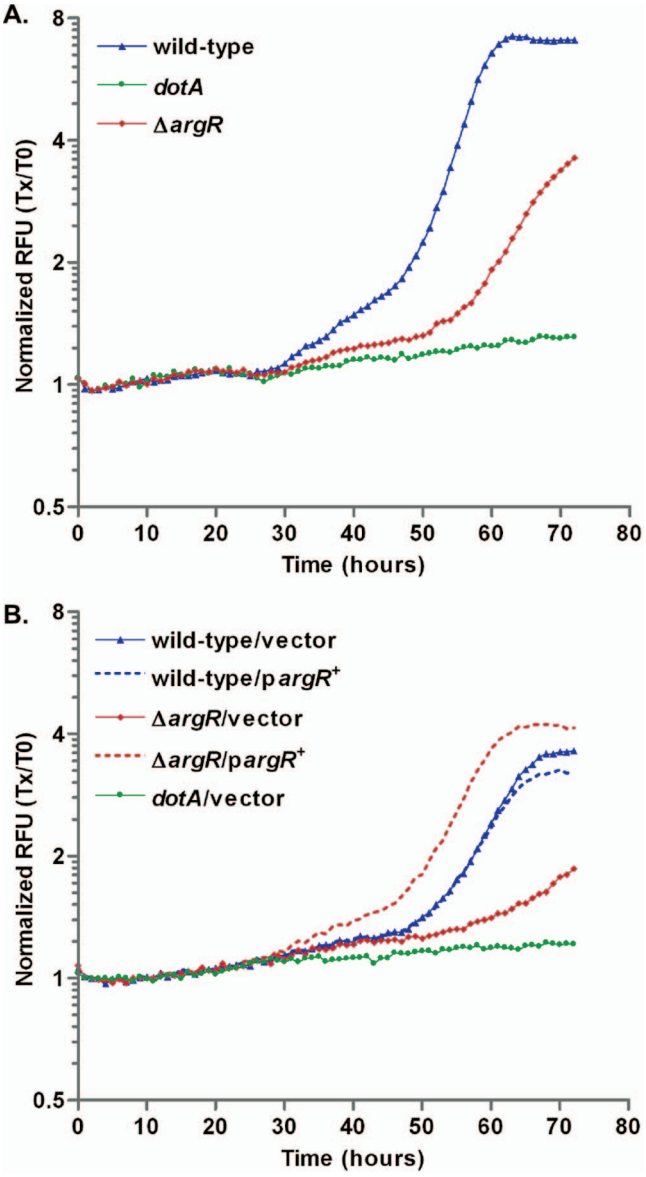

FIG. 6. The *L. pneumophila* arginine repressor homolog, *argR*, is required for efficient intracellular replication in *A. castellanii*. Intracellular multiplication of *L. pneumophila* strains harboring GFP on plasmids was measured by monitoring fluorescence in a Tecan Infinite M200 plate reader every 2 h for 72 h. Monolayers of *A. castellanii* were infected at an MOI of 1.0 with wild-type (KS79), *dotA*-null strain, or Δ*argR* bearing pXDC31 (GFP$^+$) (A). For complementation studies (B), GFP was produced from pGA123 (GFP$^+$), and strains harbored either empty vector (vector; pMMB207c) or an *argR* overexpression vector (p*argR*$^+$; pGAH125). All strains were induced with 0.5 mM IPTG. RFU, relative fluorescence units; T_x/T_0, time *x*/time zero.

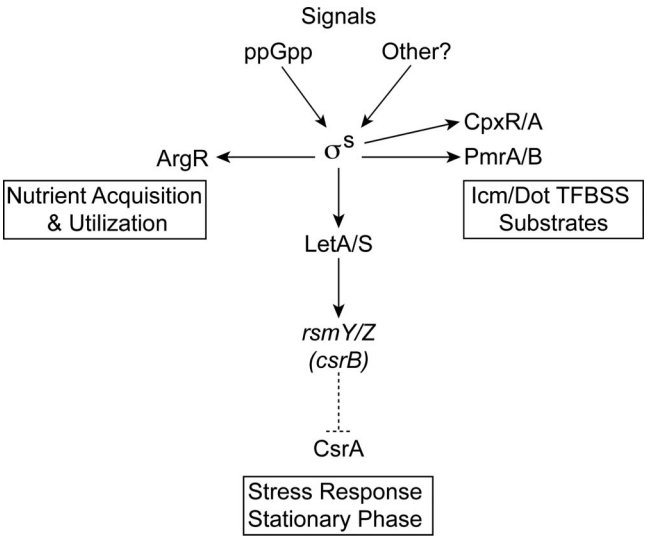

FIG. 7. Model of σ^S effects on multiple pathways associated with intracellular multiplication. In the model, solid arrows extending from σ^S illustrate transcriptional effects, which may occur directly, indirectly, or through feed-forward control, demonstrated in this study by microarray, qPCR, or Northern blot analysis. The dashed line to CsrA indicates the predicted posttranscriptional effect of the *csrB* homologs, *rsmY and rsmZ*, identified in this study. The boxes suggest the predicted outcomes of the σ^S-affected pathway.

tional effects on basic cellular processes (Fig. 2 and 3), σ^S affects the expression of genes associated with multiple pathways (Fig. 7) required for *L. pneumophila* intracellular multiplication including the following: (i) Icm/Dot-translocated substrates (Fig. 3), (ii) the two-component systems CpxRA and PmrAB (Table 2; see also Table S5 in the supplemental material), (iii) the small RNA *csrB* homologs *rsmY* and *rsmZ* (Fig. 5), and (iv) the *L. pneumophila* arginine repressor gene, *argR*, whose importance in intracellular multiplication was discovered in this study (Fig. 6).

The fact that the expression of more than one-fourth of all of the *L. pneumophila* predicted ORFs are affected in the *rpoS* mutant indicates that σ^S is a major regulator of *L. pneumophila* gene expression (Fig. 2). In *E. coli*, σ^S can partially replace the vegetative sigma factor σ^{70} under many stress conditions, resulting in the transcriptional activation of numerous σ^S-dependent genes (42). Expression of σ^S in *E. coli* is regulated by a complex array of signals including cell density, temperature, osmolarity, pH, and limiting concentrations of multiple nutrients (36, 42). However, unlike *E. coli*'s survival, *L. pneumophila's* ability to survive stress in postexponential phase is largely σ^S independent (38), a phenotype similar to that observed in *P. aeruginosa* (98, 104), which suggests that the function of σ^S varies among bacterial genera. Despite this difference, the global gene expression patterns of *E. coli* and *L. pneumophila* *rpoS* mutants are comparable with respect to the large number of genes that are both positively and negatively regulated in both phases of growth (27, 80, 107). The extent of σ^S negative regulation may be due, in part, to the activation of transcriptional repressors such as the *argR* homolog identified in this study. Although the mechanism of σ^S action is likely conserved among bacterial species, the signals that control σ^S activity and the outcomes of that response vary depending on the lifestyle of the bacterium (2, 12, 38, 86, 104).

Although the focus of this study was the *rpoS* mutant intracellular replication defect, we found that σ^S also has notable effects on the transcription of genes associated with translation and metabolism. The transcription of more than 50 ribosomal protein, tRNA synthesis, and tRNA genes is negatively affected by σ^S during postexponential phase (Fig. 3B; see also

Table S2 in the supplemental material). Reduced transcription of ribosomal protein genes and tRNA genes has been linked to the postexponential accumulation of the signaling molecule ppGpp and the stringent response to limiting amino acid availability (26, 79). In both *L. pneumophila* and *E. coli*, ppGpp results in σ^S accumulation; however, the stringent response and σ^S regulatory networks appear to be independent (1, 39, 81). It is interesting that with respect to amino acid biosynthetic gene transcription, the roles of ppGpp, which functions as a direct activator (81), and σ^S, whose effect is predominantly negative (Fig. 3B; see also Table S2 in the supplemental material), are opposite. The importance of amino acid metabolism regulation may be especially critical in *Legionella* because amino acids are its sole source of carbon and nitrogen and meet most of the bacterium's energy needs (34, 95). The transcription of more than 70 genes required for cellular metabolism is negatively affected during postexponential phase by σ^S (see Table S2 in the supplemental material), and approximately 40 of these genes are associated with amino acid metabolism (see Table S2 in the supplemental material). Understanding the biological consequences of σ^S effects on metabolism and translation will require further investigation.

The positive effects of σ^S during postexponential phase may determine the fate of *L. pneumophila* infection. During postexponential phase, σ^S positively affects the transcription of many genes categorized as "pathogenic function," such as the enhanced entry system, as well as many genes encoding Icm/Dot-translocated substrates of *L. pneumophila* (Fig. 3B). Expression of the operon containing *enhC*, a Sel1-like tricopeptide repeat-containing protein that promotes the entry of *L. pneumophila* into host cells (21, 57), is reduced on the *rpoS* mutant microarray eightfold compared to the wild type during postexponential phase (see Table S2 in the supplemental material). Mutants of *enhC* also demonstrate increased stress sensitivity (57). Although *rpoS* mutants have a functional Icm/Dot TFBSS (Fig. 1B), they exhibit extensive alterations in the transcription of genes encoding Icm/Dot-translocated substrates (Fig. 4). This study shows that there are two major divisions of σ^S transcriptional effects on Icm/Dot-translocated substrates: genes negatively affected in exponential phase and those positively affected in postexponential phase (Fig. 5). It has been proposed that the large number of Icm/Dot substrates encoded in the *L. pneumophila* genome correlates with the broad host range of *Legionella* species and that subsets of the translocated proteins may influence vesicular trafficking in different hosts (77). In contrast to *icm*/*dot* mutants, *rpoS* mutants are able to replicate in stabilized macrophage cell lines (38). One possible explanation for this observation is that the *rpoS* mutant is defective for expression of genes encoding specific Icm/Dot substrates required for replication in amoebae and primary macrophages that are not required for replication in stabilized macrophage cell lines.

Previously, the response regulators CpxR and PmrA have been shown to directly regulate the transcription of genes encoding subsets of Icm/Dot substrates in *L. pneumophila* (3, 31, 108). This study links σ^S to both *cpxR* and *pmrA* (Table 2 and Fig. 7; see also Table S5 in the supplemental material) by showing that their transcription and the transcription of some of their Icm/Dot substrate target genes are affected by the mutation of *rpoS*. In *L. pneumophila* PmrA enhances the transcription of a number of genes encoding Icm/Dot-translocated substrates including *sdeA*, *sdeC*, *sidG*, and *ceg23* (108), which we found are also positively affected by σ^S (Fig. 5B; see also Table S4 in the supplemental material). Transcripts of *pmrA* are reduced significantly in an *rpoS* mutant during postexponential growth (Table 2; see also Table S5 in the supplemental material), and both *rpoS* and *pmrA* mutants are defective for intracellular multiplication in unicellular protozoa (38, 108). The *cpxR* gene, whose transcription is also positively affected by σ^S (Table 2; see also Table S5 in the supplemental material) but is reported to not play a role in intracellular multiplication (3, 31), has the ability to activate or repress the transcription of its targets (3). Transcripts of the Icm/Dot-translocated substrate genes *cegC2*, *ceg7*, and *legA11* are increased in both *cpxR* (3) and *rpoS* mutant backgrounds (Fig. 4B; see also Table S4 in the supplemental material), whereas transcription of the gene that encodes *sidM-drrA*, an Icm/Dot substrate that recruits Rab1 to *Legionella*-containing vacuoles (48, 64), is decreased in both *cpxR* and *rpoS* null mutants (3) (Fig. 4B; see also Table S4 in the supplemental material). Further mapping of the regulatory networks identified here will require comparative microarray analysis of *cpxR*, *pmrA*, and *rpoS* mutants.

It has been proposed that the postexponential accumulation of ppGpp observed in *L. pneumophila* activates two parallel pathways (1): one controlled by σ^S (1, 38, 68, 109) and another in which activation of the two-component system LetAS results in a reduction in functional CsrA protein (5, 6, 10, 39, 40, 62, 71, 72). However, previous studies have shown an overlap in the LetAS and σ^S regulatory outcomes that suggest that the pathways might be interconnected (5, 10, 68, 70, 71). In other bacteria the global regulator CsrA inhibits the translation of its targets by binding their mRNAs (82). CsrA repression is released following the transcription of the small RNA *csrB*, which can bind and inactivate multiple copies of CsrA protein (4, 82). Studies of CsrA in *L. pneumophila* suggest that it functions by a similar mechanism (5, 7, 29, 71, 72); however, a *csrB* homolog of the system had not been characterized. In this study we showed that the postexponential transcription of the two predicted *L. pneumophila csrB* (53) homologs, *rsmY* and *rsmZ*, is dependent upon both of the *letS* and *rpoS* genes and that overexpression of the *letS* gene can restore the transcription of *rsmYZ* in *letS* and *rpoS* mutant backgrounds (Fig. 5). These data are consistent with a unified pathway in which σ^S acts upstream of LetS in the postexponential induction of *csrB* homolog transcription, which is predicted to result in CsrA repression (5, 29, 41, 71, 72, 82) (Fig. 7).

To identify previously undiscovered pathways required for intracellular multiplication, putative transcription factor genes affected by σ^S (Table 2) were deleted and analyzed for their intracellular growth phenotypes. We discovered that the *L. pneumophila* arginine repressor homolog, encoded by the *argR* gene, is required for efficient intracellular replication in *A. castellanii* (Fig. 6). In other bacterial species the arginine repressor *argR* oligomerizes into a hexamer that binds DNA in the presence of arginine, resulting primarily in the repression of genes required for arginine biosynthesis (35, 63, 101, 102). Additional reported functions of *argR* include the positive regulation of arginine catabolism (59) and the resolution of ColE1 plasmid multimers (97). *Legionella* species are arginine auxo-

trophs that do not possess the *N*-acetylglutamate synthetase enzyme required to complete the first step of arginine biosynthesis from glutamic acid (34, 100). Subsequent steps of arginine biosynthesis can be accomplished (100), and the genes required for the conversion of ornithine to arginine, *argFGH*, are divergent from *argR* (lpg0490) in a region that also includes a putative ABC transporter (lpg0491-lpg0496). In *E. coli* and *P. aeruginosa*, ArgR regulates the expression of a number of transporters required for the uptake of amino acids and other compounds (13, 60), and ArgR has been predicted to regulate arginine uptake from the host cell in some members of the *Chlamydiaceae* (88). It is intriguing to speculate that ArgR regulates one or more *L. pneumophila* transporters required for the acquisition of critical nutrients from the host cell. Determining the role of ArgR in *Legionella* biology and intracellular multiplication is the subject of further investigation.

This investigation utilized *rpoS* mutant phenotypes and global gene expression data to identify and connect regulatory networks required for *L. pneumophila* intracellular multiplication. The data presented here allow us to construct a basic model (Fig. 7) in which σ^S responds to signals, which likely include ppGpp (5, 71, 104, 109), to control the expression of downstream regulators such as the two-component systems CpxRA and PmrAB, which affect Icm/Dot substrate gene expression (3, 108), the arginine repressor ArgR, which we hypothesize may be involved in nutrient acquisition in the LCV, and the small RNAs *rsmY* and *rsmZ*, which are predicted to inhibit the function of the regulator CsrA and result in postexponential phase gene induction (4, 71, 82).

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