Cyclic Diguanylate Signaling Proteins Control Intracellular Growth of *Legionella pneumophila*

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ABSTRACT **Proteins that metabolize or bind the nucleotide second messenger cyclic diguanylate regulate a wide variety of important processes in bacteria. These processes include motility, biofilm formation, cell division, differentiation, and virulence. The role of cyclic diguanylate signaling in the lifestyle of *Legionella pneumophila*, the causative agent of Legionnaires' disease, has not previously been examined. The *L. pneumophila* genome encodes 22 predicted proteins containing domains related to cyclic diguanylate synthesis, hydrolysis, and recognition. We refer to these genes as *cdgS* (*c*yclic *d*iguanylate *s*ignaling) genes. Strains of *L. pneumophila* containing deletions of all individual *cdgS* genes were created and did not exhibit any observable growth defect in growth medium or inside host cells. However, when overexpressed, several *cdgS* genes strongly decreased the ability of *L. pneumophila* to grow inside host cells. Expression of these *cdgS* genes did not affect the Dot/Icm type IVB secretion system, the major determinant of intracellular growth in *L. pneumophila*. *L. pneumophila* strains overexpressing these *cdgS* genes were less cytotoxic to THP-1 macrophages than wild-type *L. pneumophila* but retained the ability to resist grazing by amoebae. In many cases, the intracellular-growth inhibition caused by *cdgS* gene overexpression was independent of diguanylate cyclase or phosphodiesterase activities. Expression of the *cdgS* genes in a *Salmonella enterica* serovar Enteritidis strain that lacks all diguanylate cyclase activity indicated that several *cdgS* genes encode potential cyclases. These results indicate that components of the cyclic diguanylate signaling pathway play an important role in regulating the ability of *L. pneumophila* to grow in host cells.**

IMPORTANCE **All bacteria must sense and respond to environmental cues. Intracellular bacterial pathogens must detect and respond to host functions that limit their ability to carry out a successful infection. Small-molecule second messengers play key roles in transmitting signals from environmental receptors to the proteins and other components that respond to signals. Cyclic diguanylate is a ubiquitous bacterial second messenger known to play an important role in many sensing and signaling systems in bacteria. The causative agent of Legionnaires' disease, *Legionella pneumophila*, is an intracellular pathogen that grows inside environmental protists and human macrophages by subverting the normal processes that these cells use to capture and destroy bacteria. We show that the several cyclic diguanylate signaling components in *Legionella* play a role in the ability to grow inside both kinds of host cells. This work highlights the role of cyclic diguanylate signaling during intracellular growth.**

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Legionella pneumophila is a Gram-negative gammaproteobacterial species that is a common inhabitant of aqueous environments and is frequently associated with complex communities, including protists, which serve as hosts for replication (1, 2). Inhalation of aerosols containing *L. pneumophila* can result in a severe pneumonia called Legionnaires' disease, or legionellosis (3). The organism causes disease by infecting alveolar macrophages in which it can survive and replicate profusely (4). The abilities to evade the antimicrobial defenses of the macrophages and to replicate intracellularly require a complex protein translocation machine called the Icm/Dot type IVB secretion system (TFBSS) (5–7). The Icm/Dot TFBSS delivers a large repertoire of "effector" proteins to host cells, and presumably, it is the effectors that mediate the intracellular events by targeting a variety of host functions related to organelle trafficking (reviewed in references 8 to 10).

Much attention has been focused on identifying the effectors and studying how they interact with and control host cell functions (reviewed in references 8, 10, and 11). However, a major unanswered question involves the identification of the environmental conditions experienced by the bacterium inside its host. Indirect approaches, such as studying the global patterns of gene expression, may provide useful information about nutrient availability and environmental stresses based on the types of *L. pneumophila* genes that are preferentially expressed during infection (12, 13). An alternative approach to understanding the environment during *L. pneumophila* intracellular growth may be to focus

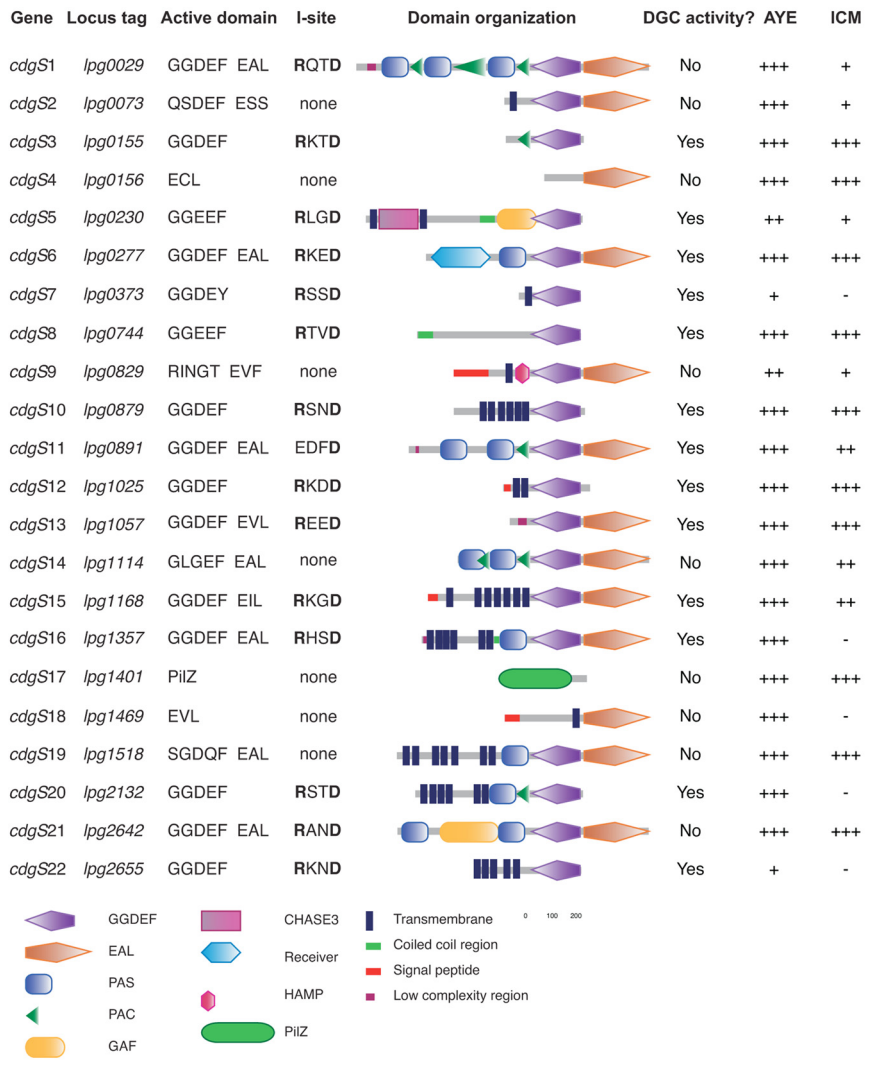


FIG 1 Domain organization of predicted GGDEF/EAL/PilZ domain proteins in the *Legionella pneumophila* Philadelphia-1 genome. Graphic representation of the domain arrangement of *Legionella pneumophila* Philadelphia-1 GGDEF, EAL, or PilZ domain-containing proteins (domains are not drawn to scale). See the legend at the bottom of the figure for the annotations of the domains and other elements. The "Active domain" column indicates the signature motifs for the GGDEF and EAL conserved regions. The "I-site" column indicates the presence of the RXXD allosteric binding site; the conserved arginine (R) and aspartate (D) residues are shown in bold. The domain annotation was performed using the SMART web tool (70). DGC activity was determined using the S. Enteritidis ΔXII heterologous expression system as described in Results and in the legend to Fig. 3. The abilities of the different *L. pneumophila* strains overexpressing the indicated genes to grow in axenic growth medium are indicated in the AYE column, while the abilities to grow in a eukaryotic host are shown in the ICM column ("+++" represents WT-like levels; "−" represents no detectable growth).

attention on genes that are known to play a role in adaptations to different environmental signals.

Bis-(3′-5′)-cyclic GMP (cyclic di-GMP) is a ubiquitous small-molecule second messenger in bacteria (reviewed in references 14 and 15). Diguanylate cyclases (DGCs) and cyclic di-GMP phosphodiesterases (PDEs), the enzymes that synthesize and hydrolyze cyclic di-GMP, respectively, regulate numerous processes in which bacteria alter their lifestyle in response to environmental cues. The ability of the cyclic di-GMP signaling network to control different levels of gene expression and function (transcriptional activity, posttranscriptional activity, enzymatic activity, and protein-protein interactions) is partially explained by the diversity of cyclic di-GMP binding modules. For example, cyclic di-GMP controls the production of *Pseudomonas aeruginosa* Pel extracellular polysaccharide (EPS) by altering DNA binding of the FleQ transcriptional regulator (16, 17). Cyclic di-GMP also regulates gene expression posttranscriptionally by binding to riboswitches and affecting mRNA translation (18, 19). In addition to its role in transcriptional and posttranscriptional gene regulation, cyclic di-GMP allosterically controls enzyme activity; binding of cyclic di-GMP to the PilZ domain of cellulose synthase, BcsA, is required for optimal activity of this enzyme (20, 21). In *Escherichia coli*, binding of cyclic di-GMP to the YcgR receptor protein results in altered flagellar motor output (22–24). Recent reviews (14, 15, 25, 26) and a comprehensive book published by the American Society for Microbiology (ASM) and edited by Alan J. Wolfe and Karen L. Visick (27) summarize a wealth of information concerning the role of cyclic di-GMP signaling in determining biofilm formation, motility, and pathogenesis in a wide variety of bacterial species.

There is very little information about the potential role of cyclic di-GMP signaling in regulating *L. pneumophila* physiology or intracellular growth. We examined the hypothesis that the cyclic di-GMP signaling network plays an important role in the interactions between *L. pneumophila* and host cells. We studied the *L. pneumophila* genes that encode enzymes that synthesize and hydrolyze cyclic di-GMP. We show that several *L. pneumophila* genes encoding domains associated with potential diguanylate cyclases and phosphodiesterases have strong negative effects on the ability of *L. pneumophila* to grow in both protist and mammalian hosts. Although most of these genes do not affect *L. pneumophila* growth in axenic growth medium and do not block the function of the Icm/Dot TFBSS, they are able to influence the outcome of the infection. In some cases, these genes influence the trafficking of the *Legionella*-containing vacuole (LCV), while in other cases they affect *L. pneumophila* intracellular multiplication without altering the behavior of the LCV. These observations open up an unexplored area of a previously unrecognized mechanism for controlling intracellular growth.

RESULTS

Identification of *L. pneumophila* genes encoding domains related to cyclic di-GMP metabolism. In order to identify *L. pneu-*

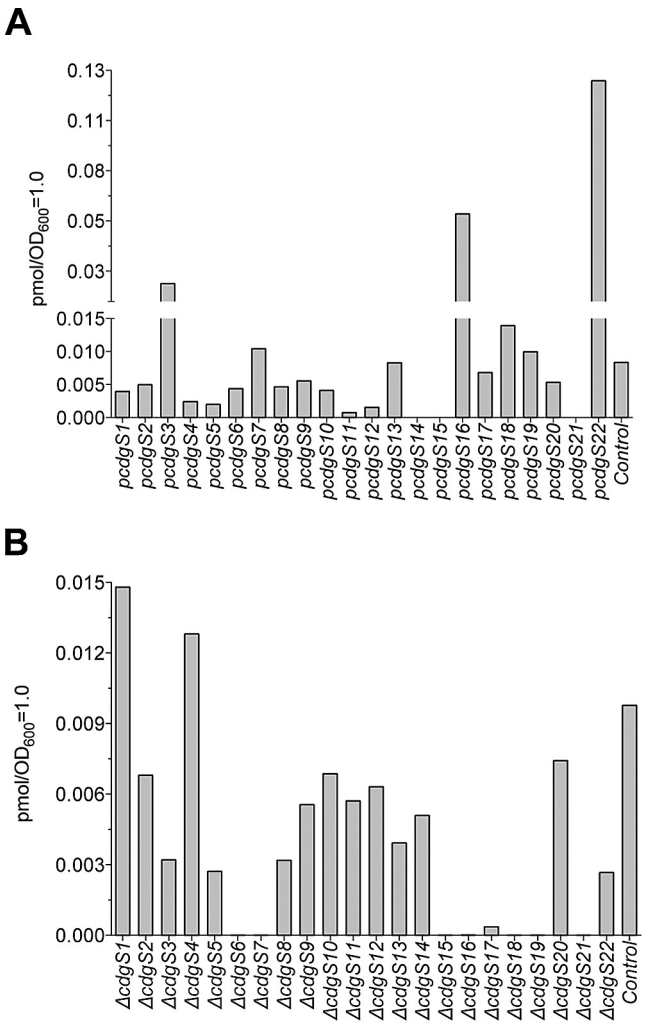


FIG 2 Total intracellular cyclic diguanylate concentrations in whole-cell nucleotide extracts of *L. pneumophila cdgS* strains. *L. pneumophila* strains overexpressing (A) or lacking (B) *cdgS* genes were analyzed by reverse-phase HPLC, and intracellular levels of cyclic di-GMP were determined. The presented bar graphs in this figure represent a typical analysis profile. The *y* axis shows the amount of cyclic di-GMP in pmol extracted from 1 ml of culture at an OD_{600} of 1.0.

mophila genes that may encode diguanylate cyclases (DGCs) and/or phosphodiesterases (PDEs), we searched the *L. pneumophila* genome sequence for open reading frames (ORFs) that encode products with either GGDEF or EAL domains. We found a total of 21 genes that encode one or both domains and only a single ORF, lpg1401, that encodes a PilZ domain. We refer to these 22 genes as *cdgS* genes (*c*yclic *d*iguanylate *s*ignaling). As shown in Fig. 1, several of these genes are predicted to encode products with both GGDEF and EAL domains as well as other domains, such as receiver domains, PAS domains, and GAF domains. Structural and biochemical studies have determined highly conserved residues that are essential for the GGDEF and/or EAL domain catalytic activities (15, 28, 29). We performed domain alignments to test whether the putative *L. pneumophila* CdgS proteins contain these highly conserved residues and used this knowledge to predict their catalytic activity. Based on these alignments, we hypothesize that the composite-domain proteins CdgS13 and CdgS21 are active only as DGCs, not as PDEs (see Fig. S4 in the supplemental material), since these proteins lack several conserved residues required for cyclic diguanylate cleavage (28–30). We also infer that the composite-domain protein CdgS14 likely acts only as a PDE (Fig. S4), since this protein lacks several essential residues that are required for DGC activity. However, several CdgS proteins include residues that are associated with functional active sites for both DGCs and PDEs (CdgS1, CdgS11, CdgS15, and CdgS16) (Fig. S4). Although *cdgS17* encodes a PilZ homolog, this gene product lacks all the residues previously shown to be essential for cyclic di-GMP binding (15, 21, 31, 32). The ability of PilZ domain proteins to bind cyclic di-GMP could be demonstrated in an *in vivo* cyclic di-GMP sequestration assay (32). Heterologous overexpression of *cdgS17* in other bacterial species did not result in the *in vivo* sequestration of cyclic di-GMP (data not shown), and therefore, we conclude that CdgS17 does not directly bind cyclic di-GMP.

The *L. pneumophila* cyclic di-GMP intracellular pool is affected by the overexpression or deletion of *cdgS* genes. Alterations in the intracellular pool of cyclic di-GMP due to the overexpression or deletion of each of the *cdgS* genes were tested using reverse-phase high-performance liquid chromatography (HPLC) to quantify cyclic di-GMP in whole-cell nucleotide extracts (Fig. 2). Overexpression of the predicted DGC-encoding genes *cdgS3* and *cdgS22*, as well as of the composite-domain-protein-encoding gene *cdgS16*, resulted in a significant increase in the amount of this signaling molecule, consistent with their suggested role as DGC-encoding genes. The deletion of predicted DGC-encoding genes, such as *cdgS3*, *cdgS5*, *cdgS6*, *cdgS7*, *cdgS16*, and

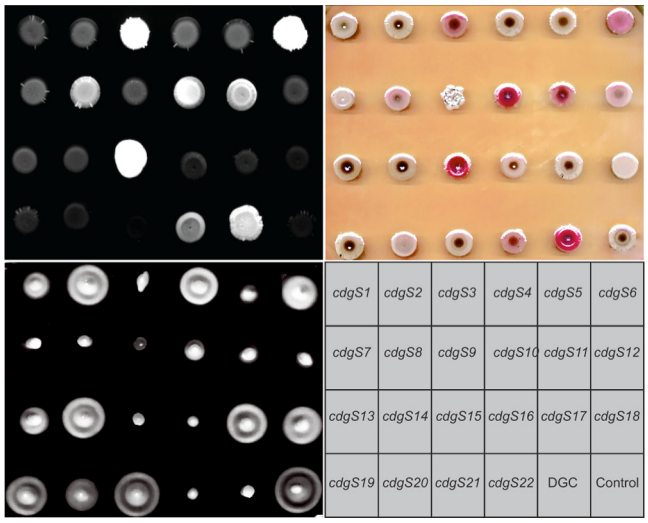


FIG 3 Heterologous expression system for the in *vivo* detection of diguanylate cyclase activity. Phenotypes of *L. pneumophila cdgS* genes ectopically expressed in the background of the GGDEF-less strain *Salmonella enterica* serovar Enteritidis ΔXII. Each *L. pneumophila cdgS* gene was expressed in *Salmonella* strain ΔXII, and samples were spotted on LB plates containing calcofluor (upper left) or Congo red (upper right) or were spotted on motility agar (lower left). These three bioassays test for diguanylate cyclase activity. Cyclic di-GMP accumulation induces cellulose synthesis and appears as white on the LB calcofluor plates, promotes Congo red binding and appears red on Congo red plates, and decreases the swarm size on motility agar plates. The key at the lower right shows the identity of each sample, where DGC is a well-characterized *C. crescentus* protein that has strong diguanylate cyclase activity (DgcA). The control sample corresponds to the empty-vector plasmid.

cdgS22, caused a significant reduction in the amount of the intracellular levels of cyclic di-GMP, while deletion of putative PDE-encoding genes, such as *cdgS1* and *cdgS4*, resulted in minor increases in the amounts of this molecule (Fig. 2B). The overexpression of *cdgS14*, encoding a predicted PDE, resulted, as expected, in undetectable levels of cyclic di-GMP, while the deletion of this gene had almost no effect on the intracellular pool of this molecule (Fig. 2). Although the single-GGDEF-domain-protein-encoding genes *cdgS5*, *cdgS8*, *cdgS10*, *cdg12*, and *cdgS20* were predicted to encode active DGCs, the overexpression of these genes had no significant effect on the intracellular pool of cyclic di-GMP (Fig. 2A); moreover, the overexpression of a predicted PDE-encoding gene, *cdgS18* (which carries a single EAL domain), unexpectedly resulted in increased levels of cyclic di-GMP, while the deletion of this gene resulted in undetected amounts of this molecule (Fig. 2). The apparent discrepancies from the expected results of overexpressing or deleting DGC- or PDE-encoding genes may be explained by indirect effects on other DGCs or PDEs (33).

Heterologous expression of *Legionella cdgS* genes reveals potential DGC activities. For some of the cloned *cdgS* genes, those that encode only a single GGDEF domain (*cdgS3*, *cdgS5*, *cdgS7*, *cdgS8*, *cdgS10*, *cdgS12*, *cdgS20*, and *cdgS22*) or a single EAL domain (*cdgS4* and *cdgS18*), it was possible to infer that their products function potentially as cyclases or phosphodiesterases, respectively. However, for the remaining genes (*cdgS1*, *cdgS2*, *cdgS6*, *cdgS9*, *cdgS11*, *cdgS13*, *cdgS14*, *cdgS15*, *cdgS16*, *cdgS19*, and *cdgS21*), which encode composite-domain proteins, these assignments were more complicated. The presence of both domains in some of the cloned *cdgS* genes suggested that these proteins might possess DGC activity, PDE activity, or both. To evaluate the potential DGC activities of the *L. pneumophila* CdgS proteins and avoid the effects of endogenous DGCs or of unpredicted protein-protein interactions, we expressed the *cdgS* genes ectopically in the *Salmonella enterica* serovar Enteritidis 3934 ΔXII strain background. This strain contains deletions of all of its endogenous GGDEF domain protein-encoding genes and produces no detectable cyclic di-GMP (34). As a positive control, we included the *dgcA* gene from *Caulobacter crescentus*, which encodes the well-characterized diguanylate cyclase DgcA (33). We examined the resulting strains for phenotypes that are normally associated with DGC activity, such as increased rugosity/Congo red binding, cellulose synthesis, and inhibition of motility (35–37). As expected for single-GGDEF-domain proteins, CdgS3, CdgS8, CdgS10, CdgS22, and the DgcA-positive control strain showed phenotypes associated with increased amounts of intracellular cyclic di-GMP in all three bioassays, while CdgS5, CdgS7, CdgS12, and CdgS20 exhibited a DGC-associated phenotype only in the motility assay (Fig. 3). In addition, based on the phenotypes exhibited for at least one of the three assays, we conclude that composite-domain proteins CdgS6, CdgS11, CdgS13, CdgS15, and CdgS16 possess DGC activity. We observed that CdgS13 did not result in strong calcofluor and Congo red binding, although it did partially block motility. A recent paper by Carlson et al. demonstrated *in vitro* that CdgS13 (lpg1057) acts as a weak DGC (38). This is consistent with our observations for the ΔXII strain. Although the use of the *S.* Enteritidis 3934ΔXII strain for heterologous expression is simple, the observed phenotypes could be influenced by several factors, such as the stability of the gene product or the presence or absence of signals needed to activate the enzymatic activity of the cloned gene product.

Several *cdgS* genes decrease the ability of *L. pneumophila* to grow in host cells. To find out if any of the *cdgS* genes plays a role in the ability of *L. pneumophila* to grow in host cells, we examined *L. pneumophila cdgS* deletion strains and strains containing the corresponding overexpression plasmids for their abilities to grow axenically in rich medium and intracellularly in protist and mammalian host cells (Fig. 4; see also Fig. S1 in the supplemental material). All 22 strains lacking individual *cdgS* genes listed in Fig. 1 were found to grow in rich medium, in *Acanthamoeba castellanii*, and in THP-1 macrophage-like cells in way that was similar to what was observed for the parent strain, JR32 (Fig. 1). However, when we examined strain JR32 containing plasmids encoding the same genes under the control of the *tac* promoter, we found that two of these genes, *cdgS5* and *cdgS9*, had a moderately negative effect on growth in rich broth, and two others, *cdgS7* and *cdgS22*, had more-severe growth defects (Fig. 4A). Seven of the strains that express the cloned genes under the control of P*tac* (*cdgS1*, *cdgS11*, *cdgS14*, *cdgS15*, *cdgS16*, *cdgS18*, and *cdgS20*) exhibited no severe growth defects in rich medium but significantly decreased abilities to grow in *Acanthamoeba castellanii* (Fig. S3) and/or THP-1 macrophage-like cells (Fig. 4B).

In order to find out if the effects on growth inside cells are related to the presumed activities of the cloned genes, we constructed mutations that alter key residues in the active sites of the putative DGCs and PDEs. Overexpression of the putative DGC-encoding genes *cdgS11* and *cdgS20* resulted in different degrees of inhibition of intracellular growth; however, when active-site mutant variants (GGDEF → GGAAF) of these genes, *cdgS11*-A or *cdgS20*-A, were overexpressed, the growth rates of these strains were similar to those of the wild-type (WT) control strain (see Fig. S2 in the supplemental material). This suggests that the DGC activity of these proteins is required for the intracellular growth defect. Similarly, overexpression of the putative-PDE-encoding gene *cdgS14* also caused an intermediate intracellular growth defect, and in this case as well, overexpression of an active-site mutant variant, *cdgS14*-A (EAL → AAL), resulted in better intracellular growth of this strain than of the strain carrying the wild-type gene (Fig. S2), suggesting that misregulated PDE activity can also cause a growth defect. Interestingly, however, the growth defects were identical in strains expressing active-site mutants of other DGC or PDE proteins. As shown in Fig. S2, overexpression of active-site variants of CdgS1, CdgS5, CdgS7, CdgS16, CdgS18, and CdgS22 resulted in growth defects similar to those caused by the intact proteins, suggesting that in these cases, the effects on *L. pneumophila* intracellular growth are independent of catalytic activity. We cannot rule out the possibilities that the mutant proteins retain the ability to bind cyclic di-GMP and that their effects on *L. pneumophila* are dependent on this binding.

Several *cdgS* genes interfere with Icm/Dot-related functions but do not block translocation. In order to understand the mechanisms underlying the CdgS-induced growth defects, we first asked if expression of the *cdgS* genes resulted in other phenotypes associated with the overall functions of the Icm/Dot TFBSS, the major determinant of *Legionella*'s ability to grow in host cells. *Legionella* Icm/Dot mutants are (i) unable to resist grazing by amoebae, (ii) defective in killing of human monocyte cells (cytotoxicity), and (iii) unable to lyse red blood cells (RBC). The ability of *L. pneumophila* strains containing the cloned *cdgS* genes to resist grazing was measured by counting the surviving bacterial cells following coincubation with *A. castellanii* trophozoites for differ-

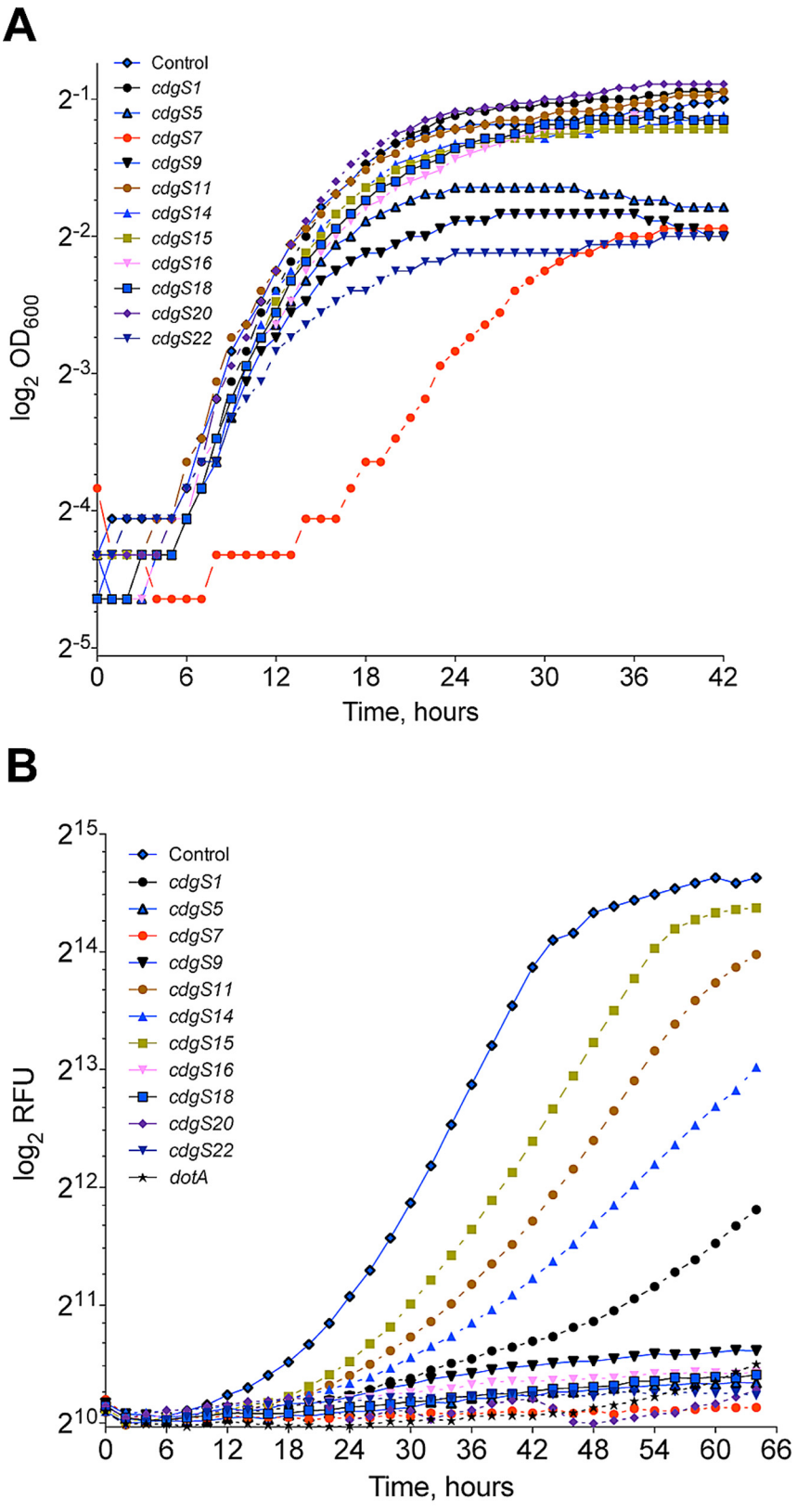
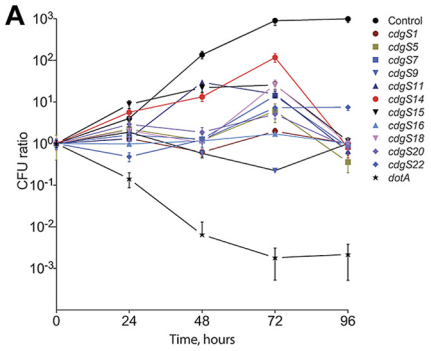


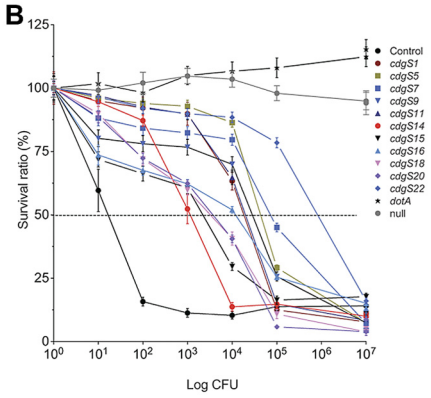
FIG 4 Growth profiles of strains containing *cdgS* plasmids. (A) Growth in rich broth measured by OD_{600}. (B) Intracellular growth in THP-1 cells as measured by accumulation of GFP fluorescence. RFU, relative fluorescence units.

ent time periods. The WT *L. pneumophila* control strain not only resisted *A. castellanii* grazing but also grew inside the amoebae, resulting in a 1,000-fold increase in CFU (the amoeba/bacterium ratio at time zero [t_0] was 1:100) (Fig. 5A). In contrast, the amoebae consumed the *dotA* negative-control strain, as shown by the 1,000-fold reduction of CFU for this strain (Fig. 5A). *L. pneumophila* strains expressing the different *cdgS* genes exhibited only limited growth (*cdgS11*, *cdgS14*, and *cdgS15*) or no increase in bacterial cell number whatsoever (*cdgS1*, *cdgS5*, *cdgS7*, *cdgS16*, *cdgS20*, and *cdgS22*) (Fig. 5A). Although none of the *cdgS*-overexpressing strains exhibited considerable growth in the presence of the amoebae, they showed no significant reduction in CFU either (Fig. 5A). The resistance demonstrated by these strains to amoebal grazing suggests that the defect exhibited by these strains is different from that exhibited by the *dotA* control strain.

The ability of *L. pneumophila* to kill host cells (cytotoxicity) is also dependent on a functional Icm/Dot TFBSS (39). In order to examine the cytotoxicity of the different *cdgS* strains, we measured the number of surviving THP-1 cells following infection with increasing numbers of bacteria after a period of 6 days. The surviving THP-1 cells were quantified by using a tetrazolium dye {MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]} that is reduced to an insoluble blue formazan by the respiratory chain of adherent living cells. We found that infection of THP-1 cells with the different *cdgS* strains resulted in severely reduced killing of the host cells compared to infection with the vector control strain (Fig. 5B), indicating that expression of the *cdgS* genes decreased the ability of *L. pneumophila* to kill the THP-1 host cells. The number of bacteria required to kill 50% of the target cells (CT_{50}) of a WT control strain was 9 CFU, while the CT_{50}s of the *cdgS* clones ranged from 850 CFU for *cdgS14* to 3.1×10^5 CFU for *cdgS22* (Fig. 5B). As many as 1×10^7 CFU of the *dotA* mutant strain did not decrease the viability of the THP-1 cells (Fig. 5B).

We considered a variety of explanations for these defects. The most obvious explanation would be that the overproduction of the cloned gene products in-





Strain	CT_{50}
cdgS1	$1.6x10^4$
cdgS5	$4.5x10^4$
cdgS7	$9.0x10^4$
cdgS9	$4.0x10^4$
cdgS11	$1.6x10^4$
cdgS14	$8.5x10^2$
cdgS15	$2.1x10^3$
cdgS16	$1.3x10^4$
cdgS18	$4.6x10^3$
cdgS20	$5.0x10^3$
cdgS22	$3.1x10^5$
Control	$0.9x10^1$
dotA	ND

FIG 5 (A) Grazing activity of *A. castellanii* on strains overexpressing *cdgS* genes. *A. castellanii* monolayers were infected with different *L. pneumophila cdgS*-overexpressing strains at an MOI of 100. At each indicated time point, the extracellularly and intracellularly grown bacteria were pooled together, and aliquots were plated on a charcoal-yeast extract (CYE) plate. CFU were counted and calculated as the ratio of t_x/t_0. Error bars represent standard deviation (SD) values from three independent experiments. (B) Cytotoxicity of strains overexpressing *cdgS* genes for THP-1 cells. The MTT assay measures the number of viable macrophages following infection with *L. pneumophila*. Monolayers of THP-1 monocytes were infected with *L. pneumophila cdgS*-overexpressing strains for 6 days. The absorbance at 570 nm was measured and the CT_{50} was calculated for each strain. THP-1 cell monolayers incubated with RPMI medium alone (null in graph) was used as a baseline for CT_{50} calculations. THP-1 cell survival rate versus number of infecting CFU is plotted in this graph; error bars represent the SD of results from six independent replicates. CT_{50} values are presented in the table. The *dotA* strain showed no cytotoxic effect in these experimental settings.

terfered with the expression or function of the Icm/Dot TFBSS. In order to test this idea, we used two measures of Icm/Dot TFBSS function: (i) the ability of wild-type *L. pneumophila* to lyse sheep RBC (40) and (ii) the ability to translocate TFBSS effectors to host cells (41, 42). As shown in Fig. 6, we measured both properties and we found that the strains expressing the *cdgS* genes did not exhibit defects in either assay that would account for the observed intracellular-growth defect. Thus, we conclude that the defects in intracellular multiplication and host cell killing are not due to a defect in the activity of the Icm/Dot TFBSS. Strains overexpressing *cdgS7* and *cdgS22* produced significantly less TEM-LepA and TEM-SdeA fusion proteins than the wild-type strain, based on Western immunoblots (data not shown); therefore, we were unable to accurately measure translocation in these strains.

Expression of some *cdgS* genes interferes with prevention of phagosome-lysosome fusion. In order to understand how the GGDEF/EAL domain proteins may be interfering with the intracellular life cycle of *Legionella*, we focused on the interaction of the different strains with host cells. Infection of host cells by *Legionella* includes (i) binding, (ii) phagocytosis, (iii) prevention of phagosome-lysosome fusion, and (iv) intracellular multiplication. Several lines of evidence suggest that the cloned *cdgS* genes did not affect binding or internalization by host cells. Binding assays, in which the different *L. pneumophila cdgS* strains were incubated with THP-1 cells under conditions that prevent uptake by the host (at 4°C or in the presence of the phagocytosis inhibitors, such as cytochalasin D), did not reveal a significant difference in the number of bacteria bound to the host cells (data not shown). In addition, internalization assays based on gentamicin

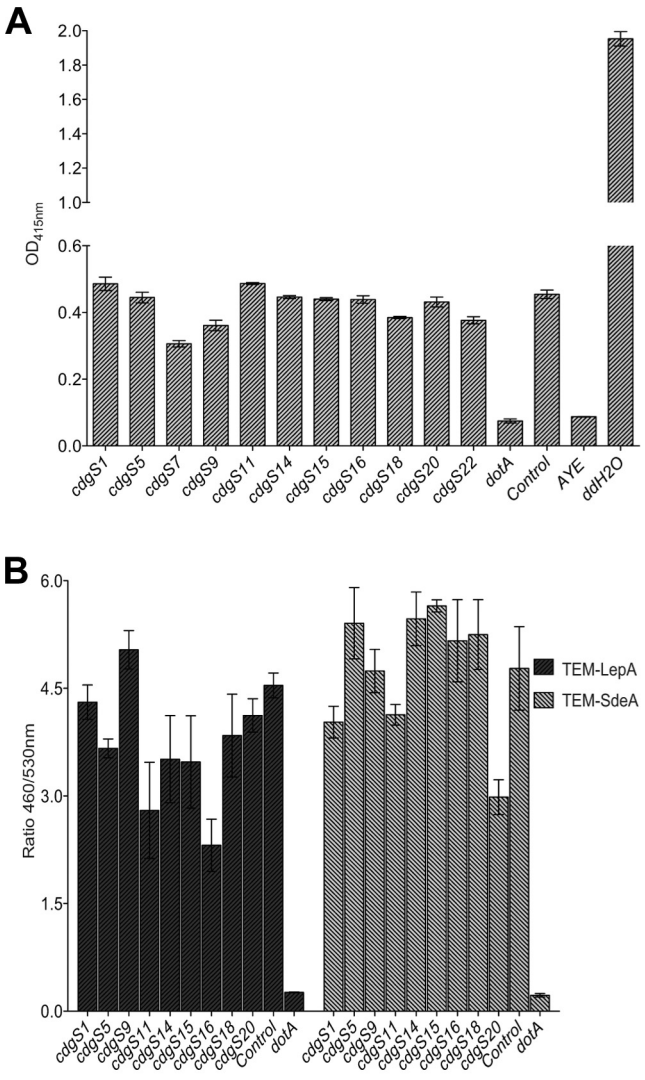


FIG 6 Dot/Icm-related activities of strains overexpressing *cdgS* genes. (A) Icm/Dot-dependent red blood cell (RBC) lysis by *L. pneumophila* overexpressing *cdgS* genes. (B) Detection of translocation of the LepA effector protein (LepA translocation is independent of the IcmS chaperone) and the SdeA effector protein (IcmS-dependent translocation). TEM-LepA or TEM-SdeA fusions were expressed in *L. pneumophila*, and the bacteria were used to infect THP-1 cell monolayers at an MOI of 40. The fluorescence intensity was measured, detecting emission wavelengths of 460 and 530 nm following excitation with UV at 405 nm. The ratios of the two emission intensities are plotted; error bars represent the SD of results from five independent infections.

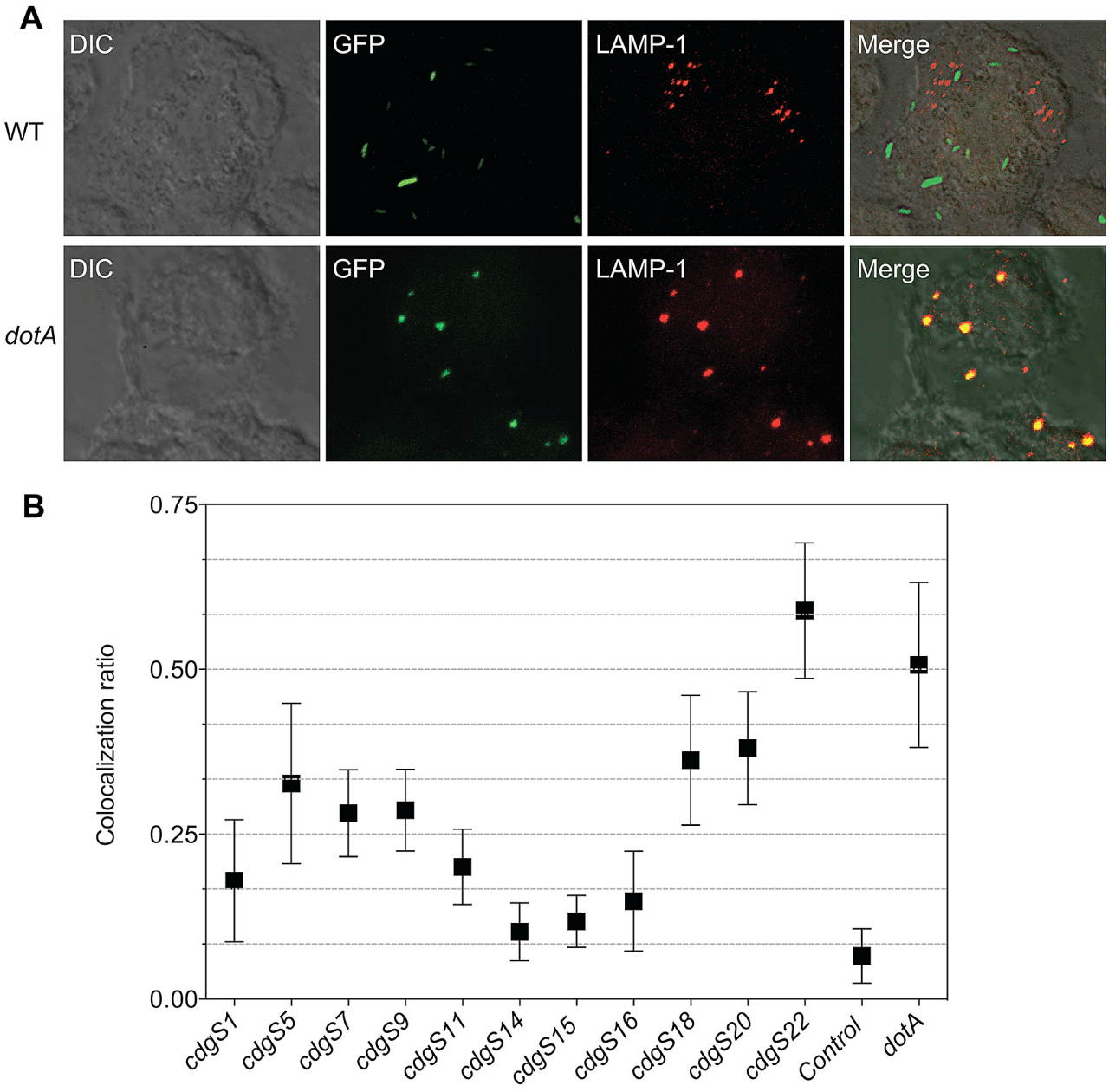


FIG 7 Colocalization of LAMP-1 and *L. pneumophila* strains overexpressing *cdgS* genes. THP-1 cells were infected with GFP-labeled *L. pneumophila cdgS*-overexpressing strains, a JR32-negative control, and a *dotA*-positive control. Infected cells were fixed, and lysosomes were labeled using anti-LAMP-1 (α-LAMP-1) antibodies (red). (A) Representative confocal images demonstrating the locations of GFP-labeled WT (top panels) and *dotA* (bottom panels) bacteria and LAMP-1-labeled lysosomes. (B) Colocalization events were scored and calculated as ratios of LAMP-1 colocalized bacteria (yellow) to total bacterial number (yellow plus green) for all *cdgS* expressing strains. The graph represents average count of several fields. Error bars represent the 95% confidence interval of the mean. DIC, differential interface contrast.

protection showed that there was no significant difference between the uptake of the *cdgS* strains and the control by either *A. castellanii* or THP-1 cells (data not shown). Because binding and entry of the *cdgS*-overexpressing strains were not affected, we hypothesized that the same defects in intracellular growth would be observed if *cdgS* gene expression was induced after the *Legionella* infection of the host cells was initiated and the early events of infection, i.e., binding and uptake, had already occurred. Figure S3 in the supplemental material shows that the defects in intracellular multiplication of the *cdgS*-overexpressing *L. pneumophila* strains are still observed when IPTG (isopropyl-β-D-thiogalactopyranoside) is added at 6 h postinfection. We conclude that the growth defect exhibited by the *cdgS* strains is not due to an inability of these strains to be recognized by, to bind to, or to be internalized by host cells.

The abilities of *L. pneumophila* to alter the nature of the newly formed phagosome and to prevent it from fusing with lysosomes following infection are strictly dependent on the Icm/Dot TFBSS

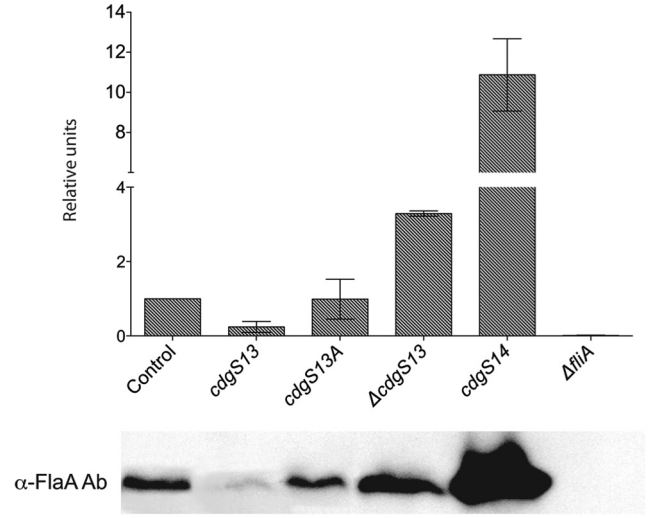


FIG 8 Levels of flagellin in strains with different *cdgS13* alleles. Whole-cell lysates of the indicated strains were loaded on a 12% SDS-PAGE gel and blotted on a polyvinylidene difluoride (PVDF) membrane. Western blot analysis using *L. pneumophila* α-FlaA polyclonal antibodies (Ab) was performed, and band intensities of three independent replicate blots were quantified and normalized to the level for the control sample using the ImageJ software package. Error bars represent standard deviation values from three independent blots. The results for a typical blot are presented under the corresponding strain name on the graph.

(5, 6, 39, 42). We examined the intracellular fate of the *Legionella* strains expressing the *cdgS* genes by fluorescence microscopy. We used *L. pneumophila* strains that express green fluorescent protein (GFP) and measured their colocalization with LAMP-1 protein, a marker for the lysosomal compartment. As illustrated in Fig. 7A, wild-type *Legionella* bacteria rarely colocalize with the LAMP-1 marker, indicating that the majority of phagosomes containing the bacteria do not fuse with lysosomes. In contrast, *dotA* mutant *Legionella* bacteria frequently colocalize with LAMP-1, indicating a defect in the ability to prevent phagosome-lysosome fusion (43). In order to find out if the *Legionella cdgS*-overexpressing strains retain the ability to prevent phagolysosome fusion, we measured their colocalization with the LAMP-1 marker. Statistical analysis of the confocal laser scanning microscopy (CLSM) images revealed that the *cdgS*-overexpressing strains could be sorted into three different groups based on their colocalization with LAMP-1 (Fig. 7B). Group I included strains that exhibit a WT-like ability to prevent phagosome-lysosome fusion (i.e., their colocalization ratio was not significantly different from that of the WT control strain), including the *cdgS1*, *cdgS11*, *cdgS14*, *cdgS15*, and *cdgS16* strains. Group II included strains that exhibit a colocalization ratio significantly different from that of either the WT or the *dotA* mutant, including the *cdgS5*, *cdgS7*, and *cdgS9* strains. Finally, group III included strains that exhibit a colocalization ratio that is not significantly different from that of the *dotA* mutant, including the *cdgS18*, *cdgS20*, and *cdgS22* strains (Fig. 7B). Some, but not all, of the members of the first group (the *cdgS11*, *cdgS14*, and *cdgS15* strains), which show WT-like phagosome-lysosome colocalization ratios, also exhibited less severe growth defects when grown in THP-1 host cells than strains of group II and III showed, suggesting that their only partial growth defect might be due to their ability to prevent the phagosome-lysosome fusion; however, this is probably not the only explanation, since other strains in group I still exhibited severe growth defects (the *cdgS1* and *cdgS16* strains).

***L. pneumophila* flagellin biosynthesis is regulated by cyclic di-GMP.** In numerous bacterial species, cyclic di-GMP has been shown to regulate the transition from a motile, planktonic lifestyle to a sessile lifestyle by controlling various cellular processes (25, 44–47). Flagellum-based motility is regulated by cyclic di-GMP in several species (reviewed in reference 48). In order to study the role of cyclic di-GMP in the regulation of *L. pneumophila* flagellar biosynthesis, we measured the levels of flagellin in all 22 *L. pneumophila cdgS*-overexpressing strains. We found only one *cdgS*-overexpressing strain that showed a strong decrease in flagellin production (the *cdgS13* strain) and one *cdgS*-overexpressing strain (the *cdgS14* strain) that showed a large increase in flagellin expression. The first gene, *cdgS13* (*lpg1057*), encodes a composite-domain protein, which seems to act as a DGC, based on the decreased motility of the *Salmonella* ΔXII strain expressing *cdgS13* (Fig. 3). Figure 8 shows a Western blot using anti-FlaA (α-FlaA) antibodies with whole-cell extracts of strains overexpressing *cdgS13*, the *cdgS13* A-site mutant (*cdgS13*-A), and the Δ*cdgS13* strain, and as a negative control, we included a strain that lacks the alternative sigma factor FliA (σ-28), known to be required for *flaA* expression (49). The amounts of flagellin were reduced almost 80% in a strain overexpressing *cdgS13* and markedly increased in a strain lacking *cdgS13*, suggesting that *cdgS13* in fact encodes a negative regulator of *flaA* expression. In addition, overexpression of the active-site-deficient variant of *cdgS13*, *cdgs13*-A (GGDEF → GGAAF), did not cause a reduction in the amount of the flagellin subunit protein, indicating that the DGC activity of *cdgS13* is required for the inhibitory effect of *cdgS13* of FlaA production. We also show that overexpression of *cdgS14*, a gene encoding a presumed PDE, results in a >10-fold increase in the amount of the FlaA protein (Fig. 8). These data provide strong evidence for cyclic di-GMP-dependent regulation of flagellar biosynthesis in *L. pneumophila*.

DISCUSSION

Cyclic di-GMP orchestrates a wide range of cellular processes in bacteria, including cell differentiation, transition between motile and sessile lifestyles, long-term survival, and persistence, as well as virulence (reviewed in references 25 and 46). We carried out a systematic analysis of *L. pneumophila* genes encoding GGDEF and EAL domain proteins in order to study the importance of this signaling network to the *L. pneumophila* life cycle. We identified 22 genes in the *Legionella pneumophila* Philadelphia-1 genome that are predicted to encode proteins that are involved in cyclic di-GMP metabolism; we refer to these genes as *cdgS* genes. Most of the CdgS proteins identified in this search were composite-domain proteins, comprising both GGDEF (DGC) and EAL (PDE) domains; about one-third are single-domain GGDEF proteins, and only two are single-domain EAL proteins. We have also identified one protein of the PilZ domain, a domain that was shown to bind the cyclic di-GMP molecule in other bacterial species (21). In most cases, *L. pneumophila* GGDEF and EAL domains are found in proteins fused to various sensory domains (in many cases PAS domains), suggesting that these proteins bind and/or metabolize cyclic di-GMP as a response to a specific signal or cue (50, 51).

The cyclic di-GMP signaling network was shown to be involved in regulation of virulence-related processes in several

pathogenic bacterial species (reviewed in reference 26). In *Salmonella enterica* serovar Typhimurium, a predicted PDE, a single EAL domain protein, CdgR, was shown to control several host-pathogen-related processes, such as resistance to H_2O_2 and macrophage toxicity (52). In *Vibrio cholerae*, cyclic di-GMP was shown to inhibit the expression of *toxT*, which is required for cholera toxin production; the activity of the VieA PDE relieves this inhibition (53). In a comprehensive analysis of *Pseudomonas aeruginosa* GGDEF and EAL domain proteins, Kulasakara et al. (54) found that several GGDEF and EAL domain proteins are involved in type III secretion system-mediated cytotoxicity; they also demonstrated the involvement of the cyclic di-GMP signaling network in the virulence of this bacterium in a burn-wound murine model (54). Here, we provide evidence that the cyclic di-GMP signaling network regulates functions associated with *L. pneumophila* virulence, such as intracellular growth and host cell killing. Surprisingly, we found no evidence for the involvement of any of the *cdgS* genes in controlling the ability of *L. pneumophila* to be recognized by, to bind to, or to become internalized by host cells.

We performed a broad analysis of phenotypes of *L. pneumophila* lacking or overexpressing each of the *cdgS* genes and tested the effects of these modifications on the ability of *L. pneumophila* to infect its host. We found that there was no simple correlation between the concentrations of cyclic diguanylate in whole-cell extracts and the abilities of the strains to grow intracellularly. Strains containing deletions of certain *cdgS* genes, such as the *cdgS1* or *cdgS4* strain, where intracellular levels of cyclic di-GMP are increased (Fig. 2), showed no less growth than the wild-type strain. Furthermore, overexpression of *cdgS3*, which resulted in >3-fold higher concentrations of cyclic di-GMP in comparison with those observed for the wild-type *L. pneumophila* strain, did not exhibit any growth defect under any of the tested conditions. We conclude that changes in the total intracellular concentration of cyclic di-GMP do not directly correlate with the observed effects on intracellular growth. Interestingly, in most cases the growth defect exhibited by the *cdgS*-overexpressing strains was independent of the cloned gene's presumed DGC or PDE activities, suggesting that GGDEF and EAL domain proteins not only have a role in cyclic di-GMP metabolism but may also interact with other regulatory proteins. It seems that certain DGCs and PDEs evolved from active enzymes, and although they may have lost or maintained their enzymatic activity, they have retained or gained additional biological functions. Although lacking enzymatic activity, the mutated proteins likely retain their nucleotide binding abilities and may still impact other regulatory processes yet to be uncovered. DGC-independent regulatory activity has been demonstrated in many microbial systems; the *E. coli* GGDEF-EAL domain protein CsrD controls biofilm formation by targeting two small regulatory RNAs for degradation (55). The authors could not detect CsrD DGC activity, and they showed that active GGDEF and the EAL domains are not required for CsrD activity (55). Holland et al. showed that the *Staphylococcus epidermidis* GGDEF protein GdpS positively controls biofilm formation by elevating the expression of the *icaADBC* operon carrying the PIA/PNAG biosynthesis genes independently of diguanylate cyclase activity (56). These two examples demonstrate that inactive GGDEF domain proteins can still regulate a "classical" DGC-dependent pathway such as a biofilm formation without actually synthesizing cyclic di-GMP. Furthermore, the recent identification of PopA, a cyclic di-GMP-dependent effector that regulates cell division in *C. crescentus*, showed that cyclic di-GMP binding proteins can exert regulatory control even in the absence of enzymatic activity (57). Solano et al. (34) show that the cyclic di-GMP signaling network in *S.* Enteritidis is involved in the regulation of multiple cellular processes, such as motility, fimbria production, EPS biosynthesis, and virulence. The authors deleted all the GGDEF domain proteins in this strain and were able to restore all cyclic di-GMP-dependent processes except for cellulose biosynthesis by ectopically expressing a single GGDEF domain protein, STM4551. Furthermore, the authors showed that an inactive mutant variant of the STM4551 DGC protein also restored these phenotypes (34).

L. pneumophila requires effector translocation for lysosomal evasion (43). We show that at least half of the strains in which *cdgS* gene overexpression caused an intracellular growth defect also cannot prevent phagosome-lysosome fusion to some extent but that others retained this ability. In cases where *cdgS* overexpression resulted in defects in both intracellular growth and phagosome-lysosome fusion, it will be interesting to elucidate the mechanism(s) that connects the cyclic di-GMP signaling network to regulation of organelle trafficking. In those cases where the intracellular growth defect is not accompanied by a failure to prevent phagosome-lysosome fusion, it will be equally interesting to discover the block to intracellular growth even though the LCV appears to traffic normally. Interestingly, even for those strains in which LCV trafficking was defective, we did not observe any general defect in effector translocation by the Dot/Icm TFBSS. It is conceivable that these *cdgS* genes might affect specific effector proteins either by downregulation of their expression or by their ability to be translocated. Recently, McWhirter et al. (58) described how cyclic di-GMP is sensed in the cytosol of mammalian cells and induces the production of type I interferons. The authors show that the sensing pathway of cyclic di-GMP appears to be different from the previously described nucleic acid-sensing pathways (58). It is possible that the phenotypes associated with increased cyclic di-GMP production in some of the *L. pneumophila* *cdgS* strains presented in this work result from the host cell response to the cyclic di-GMP molecule.

Recent published studies describe a link between cyclic di-GMP signaling pathways and the physiological state of the bacterium. Weber et al. (59) described a complex regulatory circuit where, upon entry into stationary phase, σ^s positively regulates multiple GGDEF/EAL domain proteins and causes the production of cellulose and curli fimbriae (59). Boehm et al. described a novel regulatory mechanism where, in response to ribosomal stress (caused by translational inhibitors), ppGpp and cyclic di-GMP signaling pathways coregulate poly-GlcNAc production and biofilm formation (60). Many *L. pneumophila* *cdgS* genes are known to be upregulated upon entry into stationary phase, and their expression requires σ^s (13). Mutation of the *rpoS* gene does not affect growth of *L. pneumophila* in rich medium but severely reduces growth in amoebae, without affecting the Dot/Icm machinery (61), similar to the phenotype caused by overexpression of some of the *cdgS* genes reported in this work. The link between the *L. pneumophila* cyclic di-GMP signaling network and its physiological state will be an additional important area for future work.

Cyclic di-GMP signaling has been shown to regulate flagellum-based motility in numerous bacterial species. In a recent review, Wolfe and Visick summarized the current knowledge about the different mechanisms of regulation (48). The cyclic di-GMP sig-

naling network was shown to affect motility by influencing flagellar gene transcription (62, 63), flagellar assembly (64, 65), flagellar activity (23, 24), and flagellar stability (66). In this work, we show that *L. pneumophila* flagellar biosynthesis is also a cyclic di-GMP-regulated process. Overexpression of a putative DGC-encoding gene, *cdgS13*, results in decreased levels of flagellin, while a deletion of *cdgS13* results in an increased amount of FlaA protein. In contrast, overexpression of CdgS14, which decreases the intracellular levels of this signaling molecule, resulted in a markedly increased amount of this flagellin subunit protein. Recently, Carlson et al. have shown that overexpression of *cdgS13* (*lpg1057*), which *in vitro* acts as a DGC-encoding gene, results in a hyperbiofilm phenotype in *L. pneumophila* (38). Taken together, the work that we present here and the work published by Carlson et al. demonstrate that in *L. pneumophila*, just as in many other bacterial species, motility and biofilm formation are counterregulated by the cyclic di-GMP signaling network.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains and plasmids used in this study are listed in Table S1 in the supplemental material.

Genome-wide search for *L. pneumophila* GGDEF, EAL, PilZ, and HD-GYP domain-encoding genes. To identify *L. pneumophila* genes encoding proteins of the cyclic di-GMP signaling network, we used the Pfam, SMART, and TIGR domain annotation tools.

Construction of mutants and overexpressions. Mutants were created by allelic exchange of the gene of interest (GOI); a 1-kb homologous region at each end of the GOI flanking a 2.5-kb gentamicin resistance cassette fused to *luxAB* genes was created using splicing by overlap extension by PCR (SOE PCR). Allelic exchange fragments were introduced into competent KS79 cells by natural transformation as described previously (67). Deletions were confirmed by PCR. Cloned genes were amplified from JR32 genomic DNA by use of the Phusion DNA polymerase system. Primers were designed to introduce restriction sites at both ends to allow ligation into the plasmid vector. The same ribosome binding site (RBS) (GAAGGAGATATACAT) was used for all the cloned genes to optimize the cloned-gene expression. PCR fragments were digested with restriction enzymes and cloned into the pMMB207c vector (45). Primers used for the construction of overexpression plasmids and deletion mutants are presented in Table S2 in the supplemental material.

Growth of bacterial strains and medium preparation. Media and antibiotics for the growth of *L. pneumophila* were used as described previously (68). Normally, unless otherwise stated, *L. pneumophila* cultures were started with a dilution of post-exponential (PE)-grown culture to an optical density (OD) of approximately 0.05 and grown with agitation at 37°C. Exponential-phase cultures were collected at an OD of 0.5. Post-exponential-phase cultures were collected at an OD of approximately 1.8 to 2.0. Induction of genes cloned in pMMB207c- or pBBR1MCS2-based plasmid was achieved with 1 mM IPTG (unless stated otherwise).

Phenotypic evaluation of *Salmonella* strains. The Congo red and calcofluor binding assay was modified from the method described in reference 36. Briefly, 10 μl of an overnight culture (OD at 600 nm [OD_{600}], ~5) was spotted onto LB agar plates without NaCl, supplemented either with Congo red (40 μg/ml) and Coomassie brilliant blue (20 μg/ml) or with calcofluor (50 mg/ml). Plates were incubated at room temperature (RT) for 72 hours. The LB soft agar plates (0.3% agar) that were used to evaluate the motility of *Salmonella* strains were documented after 8 h of incubation at 37°C.

Cytotoxicity assay. Ninety-six-well microplate dishes containing 1×10^5 differentiated THP-1 monocyte cells were infected with 10-fold serial dilutions of the tested strains in RPMI medium supplemented with 1 mM IPTG, starting with 1×10^7 CFU per well. After 6 days of incubation at 37°C under CO_2 (5%), 0.5 mg/ml MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] was added to each well. MTT-culture was incubated for an additional 2 h at 37°C under CO_2 (5%). The culture medium was removed, and the remaining reduced formazan dye was dissolved with 100 μl of acidified isopropanol (isopropanol with 0.04 M HCl and 1% SDS). The A_{570} values of results from six replicate wells were averaged. The number of bacterial cells required to kill 50% of the cell monolayer (CT_{50}) was determined by nonlinear regression analysis (GraphPad Prism).

TEM translocation assays. Measurement of Icm/Dot-dependent substrate translocation was performed as previously described, using the pXDC61-LepA and pXDC61-SdeA TEM fusion-carrying plasmids (69).

Tissue culture and cell lines. *Acanthamoeba castellanii* (ATCC 30234) bacteria were cultured in proteose peptone-yeast extract-glucose (PYG) medium at 30°C. THP-1 cells were grown in RPMI GlutaMAX medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS) at 37°C in a 5% CO_2 incubator. Differentiation of THP-1 cells was induced with phorbol myristate acetate (PMA; Sigma) at a final concentration of 10^{-8} M for 48 h.

Light and fluorescence microscopy. Images were acquired with a Nikon TE-200 inverted microscope with an attached Hamamatsu ORCA camera using OpenLab 5 software from Perkin-Elmer. Images were analyzed, quantified, and edited using the open-source ImageJ software program and the appropriate plug-ins (http://rsbweb.nih.gov/ij/download.html).

Immunofluorescence and confocal scanning laser microscopy. THP-1 cells were maintained in RPMI GlutaMax medium containing 10% FBS. Cells were seeded at 5×10^5 cells/well (with PMA) on poly-lysine-covered 12-mm coverslips in a 24-well microplate and differentiated for 48 h at 37°C in a 0.5% CO_2 incubator. Differentiated THP-1 cells were infected with 5×10^6 (MOI, 10) *L. pneumophila* strains for 1 h. Cells were washed twice with phosphate-buffered saline (PBS; pH 7.4), fixed with 3.7% PBS-buffered formalin for 20 min, washed with PBS, and blocked with 2% bovine serum albumin (BSA) in PBS. Anti-MOMP polyclonal antibodies were added (1:200) to the wells. Primary antibodies were washed with PBS, and an Alexa Fluor 350-conjugated anti-rabbit antibody (Invitrogen) was added (1:500). This step allowed the labeling of the extracellular bacteria. Cells were washed and blocked/permeabilized with 2% BSA in PBS containing 0.1% saponin. Cells were incubated with blocking buffer containing UH1, a monoclonal antibody against hamster Lgp-A/LAMP-1 (1:200) (DSHB; University of Iowa) for 1 h, washed, and incubated with goat anti-mouse Alexa Fluor 597 (1:500) (Invitrogen) for 30 min. After the wash, the coverslips were fixed to glass slides using Vectashield HardSet mounting medium (Vector Laboratories) for confocal microscopy. Confocal images were acquired with a Zeiss LSM510 Meta laser-scanning microscope. Images were processed and merged using ImageJ (http://rsb.info.nih.gov/ij/index.html).

Evaluation of intracellular multiplication. Intracellular multiplication was tested in the protozoan *Acanthamoeba castellanii* and the human monocyte cell line THP-1. The principles of this method were described in reference 13. Monolayers of *A. castellanii* or THP-1 human monocyte cells were formed in 96-well plates at a cell density of 1×10^5 cell per well and infected with *L. pneumophila* strains harboring GFP-expressing plasmids at different MOIs. Infection of *A. castellanii* was carried out in AC buffer as described previously (18). Infection of THP-1 cells was carried out in RPMI or in RPMI CO_2-independent medium. Prior to infection, growth medium was removed and replaced with fresh medium containing *L. pneumophila* strains at the desired MOI. Plates were centrifuged for 10 min at 1,500 rpm. Intracellular multiplication was monitored automatically by measuring GFP fluorescence at an excitation wavelength of 480 nm and an emission wavelength of 520 nm with a Tecan Infinite M200 plate reader every 90 min for the duration of the experiment. Fluorescence data were collected using Magellan software.

HPLC quantification of cyclic di-GMP. Cyclic di-GMP extraction was performed as previously described (33). Briefly, 2.0 ml of *L. pneumophila* cell cultures (OD_{600}, ~0.5) was harvested by centrifugation for 30 s at 14,000 rpm, and the supernatant was discarded. The cell pellet was dis-

solved in 200 μl 1 M formic acid, and nucleotides were extracted for 10 min at 4°C. Insoluble cellular components were then pelleted, and the supernatant was filtered through a 0.22-μm filter. Flowthrough was directly analyzed by chromatography.

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SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00316-10/-/DCSupplemental.

Table S1, PDF file, 0.132 MB.
Table S2, PDF file, 0.196 MB.
Figure S1, PDF file, 0.464 MB.
Figure S2, PDF file, 0.629 MB.
Figure S3, PDF file, 0.393 MB.
Figure S4, PDF file, 1.567 MB.

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