The *Legionella pneumophila* Collagen-Like Protein Mediates Sedimentation, Autoaggregation, and Pathogen-Phagocyte Interactions

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Although only partially understood, multicellular behavior is relatively common in bacterial pathogens. Bacterial aggregates can resist various host defenses and colonize their environment more efficiently than planktonic cells. For the waterborne pathogen *Legionella pneumophila*, little is known about the roles of autoaggregation or the parameters which allow cell-cell interactions to occur. Here, we determined the endogenous and exogenous factors sufficient to allow autoaggregation to take place in *L. pneumophila*. We show that isolates from *Legionella* species which do not produce the *Legionella* collagen-like protein (Lcl) are deficient in autoaggregation. Targeted deletion of the Lcl-encoding gene (*lpg2644*) and the addition of Lcl ligands impair the autoaggregation of *L. pneumophila*. In addition, Lcl-induced autoaggregation requires divalent cations. *Escherichia coli* producing surface-exposed Lcl is able to autoaggregate and shows increased biofilm production. We also demonstrate that *L. pneumophila* infection of *Acanthamoeba castellanii* and *Hartmanella vermiformis* is potentiated under conditions which promote Lcl dependent autoaggregation. Overall, this study shows that *L. pneumophila* is capable of autoaggregating in a process that is mediated by Lcl in a divalent-cation-dependent manner. It also reveals that Lcl potentiates the ability of *L. pneumophila* to come in contact, attach, and infect amoebae.

The ability of bacterial cells to come into contact and form aggregates, or autoaggregation, has been implicated in environmental and host dissemination, and it is relatively common among Gram-negative pathogens (1–4). Autoaggregation plays a major role in the virulence of pathogens such as *Neisseria meningitidis* and *Listeria monocytogenes* (5–7). This phenomenon has properties similar to those of biofilms (8). Furthermore, the ability to autoaggregate is frequently correlated with the strength of biofilm production (9–11). Autoaggregation may involve surface-exposed proteins such as the self-associating autotransporter (SAAT) family of adhesins (12). SAATs are a versatile group of proteins which are involved in biofilm production and adherence to human cells in addition to autoaggregation (13–15). Despite the role that autoaggregation has in pathogenesis, the molecular determinants of interbacterial interactions remain only partially understood.

The Gram-negative pathogen *Legionella pneumophila* is a major cause of community-acquired pneumonia (16, 17) and has been shown to autoaggregate (18, 19). Legionellosis is acquired by inhaling contaminated aerosols (20), and to date there have been no reported cases of human-to-human transmission. In the environment, *L. pneumophila* is localized in the sediments of hot-water tanks and water distribution systems, which are believed to be sources of the pathogen during outbreaks (21, 22). In natural and in man-made water systems, *L. pneumophila* can be isolated from different protozoa (23–25). This intracellular stage plays a crucial role in *L. pneumophila*'s life cycle, since the bacteria utilize protozoan hosts as a means to replicate and disseminate in the environment (26). Despite the association that autoaggregation has with pathogenesis, there are only few studies to date exploring the molecular determinants, the nature, and the roles of this pro-

cess in *L. pneumophila*. The *Legionella* collagen-like protein (Lcl) is involved in both biofilm production and adherence to human cells (27–29), two processes which are characteristic of proteins implicated in autoaggregation (1, 12). Based on these initial findings, we hypothesized that the Lcl adhesin may play a role in autoaggregation. We show that Lcl is essential and sufficient for autoaggregation and that this process requires divalent cations. Using amoeba infection models, we reveal that Lcl-dependent autoaggregation and attachment may represent a key determinant of *L. pneumophila*'s life cycle and virulence by promoting contact and adhesion between the pathogen and its natural hosts.

MATERIALS AND METHODS

Chemicals, bacterial strains, and growth conditions. Unless otherwise indicated, all chemicals were purchased from Sigma. All *Legionella pneumophila* (Table 1) and other *Legionella* species (Table 2) isolates were cultured in buffered charcoal-yeast extract (BCYE) agar at 37°C and 5% CO_2 or with buffered yeast extract (BYE) broth at 37°C with shaking at 100 rpm (30). Cultures of Lp02 were supplemented with thymidine when required (31). *Escherichia coli* strains (Table 2) were cultured on Luria-

TABLE 1 *L. pneumophila* strains used in this study

Designation	Plasmid	Source or reference
Lp02		31
Lp02 Δ*lpg2644*		27
Lp02/pBH6119	pBH6119	27
Lp02 Δ*lpg2644*/pBH6119	pBH6119	27
Lp02/p*lpg2644*	pBH6119 *lpg2644*	27
Lp02 Δ*lpg2644*/p*lpg2644*	pBH6119 *lpg2644*	27
Lp02/pRFP	pKB288 (pBH6119 mCherry)	71
Lp02 Δ*lpg2644*/pRFP	pKB288 (pBH6119 mCherry)	71
Lp02/pGFP	pBH6119 GFP	28
Lp02 Δ*lpg2644*/pGFP	pBH6119 GFP	28
Lp02 Δ*lpg2644* c*lpg2644*/pGFP (chromosomal insertion of *lpg2644*)	pBH6119 GFP	28
sg1-9[a]		27
sg2		27
sg3		27
sg4		27
sg5		27
sg6		27
sg8		27
sg10		27
sg12		27

[a] Cross-reactive with serogroups 1 to 9.

TABLE 2 Other *Legionella* isolates and *E. coli* strains used in this study

Species	Designation	Plasmid	Source or reference
E. coli (TOP10)	*E. coli*/pTrc	pTrc	This study
	E. coli/p*lcl*	p*lcl*	This study
L. erythra	LR1359		27
L. feeleii	LR0568		27
		pBH6119	This study
		p*lpg2644*	This study
L. erythra	LR1317		27
L. rubrilucens	LR1406		27
L. maceachernii	LR0193		27
L. anisa	LR0398		27
L. bozemanii sg1	LR0651		27
L. bozemanii sg2	LR0405		27

Bertani (LB) agar at 37°C and 5% CO_2 or in LB broth at 37°C with shaking at 225 rpm.

General DNA techniques. Genomic DNA and plasmid DNA was purified using a QIAamp DNA Minikit and a QIA prep spin Miniprep kit (Qiagen), respectively. To quantify DNA, spectrophotometry was used. For PCR, 10 ng was used as a template, and PCRs were performed with *Taq* DNA polymerase as recommended by the manufacturer (Invitrogen). The PCR primers used are shown in Table S1 in the supplemental material. PCR amplifications for cloning were performed with Platinum *Taq* high-fidelity DNA polymerase as per the manufacturer's instructions (Invitrogen). All clones were verified by sequencing. Sequencing reactions were performed using a BigDye Terminator cycle sequencing kit, version 3.1, and products were purified with a BigDye X terminator purification kit and run on a 3130xl genetic analyzer (Applied Biosystems). To generate an *E. coli* strain expressing *lpg2644*, primers 3 and 4 were used to PCR amplify *lpg2644* from the PCR2.1-*lpg2644* vector. The resulting PCR product was cloned into the PCR2.1 vector and digested with Bsph1 and EcoRI. The digested fragment was then ligated into an NcoI- and EcoRI-digested pTrc plasmid under the regulation of the leaky IPTG (isopropyl-β-ᴅ-thiogalactopyranoside)-inducible promoter, *trc*.

Bacterial sedimentation assays. Sedimentation assays were performed as previously described with few modifications (18, 19). *L. pneumophila* strains were grown for 3 days, and colonies were suspended to an optical density at 600 nm (OD_{600}) of 1 in deionized water with 10% BYE or in deionized water with the addition of the indicated salts. For sedimentation assays with mixed populations, bacterial suspensions were adjusted to an OD_{600} of 1, and an equal volume of each suspension was added to test tubes and allowed to settle. To visualize sedimentation in *E. coli* strains, overnight plate cultures were suspended in deionized water with 10% LB or in deionized water with the addition of the indicated concentrations of $MgCl_2$ to an OD_{600} of 1. Images were taken immediately after the indicated time period, with all incubations being performed at room temperature. To measure sedimentation kinetics, sedimentation assays were performed as described above, and the OD_{600} was measured every hour with a spectrophotometer, where a decrease in OD_{600} indicates an increase in sedimentation.

SDS-PAGE and immunoblot analysis. SDS-PAGE was performed as previously described (32). Immunoblotting was performed according to the methods of Towbin et al. (33). To detect the presence of specific proteins from *Legionella* bacteria, cell lysates were prepared with broth cultures adjusted to an OD_{600} of 4.5, centrifuged at 5,000 rpm for 10 min, and washed twice with phosphate-buffered saline (PBS), and an equal volume of 2× Laemmli loading buffer with 10% 2-mercaptoethanol was added. *E. coli* cultures were induced with 5 mM IPTG during exponential phase, and after 3 h cells were centrifuged, washed twice with PBS, and mixed with an equal volume of 2× Laemmli loading buffer with 10% 2-mercaptoethanol. All samples were then boiled for 15 min before running on gels. Bound antibodies (1:20,000 for anti-Lcl and 1:50,000 for anti-IcdH) were detected with peroxidase-linked anti-rabbit IgG (1:20,000). To determine if Lcl is secreted during sedimentation, sedimentation assays were performed as described above, and after 5 h the suspension was pipetted vigorously to resuspend the cells. Afterwards, 1 ml of suspension was centrifuged for 5 min at 13,000 rpm, and the cell pellet was resuspended in 100 μl of PBS. The supernatant was then precipitated by adding 100% trichloroacetic acid (TCA) to a final concentration of 13% and incubated at −20°C overnight. Samples were then centrifuged for 15 min at 4°C and resuspended with an equal volume of PBS as the cell pellet.

Immunofluorescence assays. To detect Lcl on the surface of *E. coli* strains, bacteria from plate cultures were washed twice and resuspended in PBS. The inner-membrane-impermeative dye FM 4-64 (Molecular Probes) was used to label the cells, and afterwards bacteria were incubated with anti-Lcl antibodies (1:50) and anti-rabbit Alexa Fluor (Molecular Probes) antibodies (1:500), as previously described (28).

Biofilm quantification. All biofilm assays were performed using polystyrene 96-well plates (Costar). *E. coli* strains were grown for 16 h in LB broth and adjusted to a final OD of 0.2 in fresh LB with or without the addition of 5 mM IPTG. Ninety-six-well plates were incubated at 37°C and 5% CO_2 for 1 day. Biofilms were stained with 40 μl of 0.25% crystal violet per well for 15 min and washed three times with 200 μl of sterile deionized water. The crystal violet stain was then solubilized in 95% ethanol, and after 15 min absorbance was read at 600 nm. The results of three independent experiments were pooled, with 8 replicates each.

***Acanthamoeba castellanii* and *Hartmannella vermiformis* infection assays.** *Acanthamoeba castellanii* and *Hartmannella vermiformis* were grown in peptone-yeast extract-glucose (PYG) medium at room temperature and in *H. vermiformis* medium (modified PYNFH medium, ATCC medium 1034) at 35°C, respectively. One day before infection, 24-well tissue culture plates were seeded with 1 ml of amoeba suspension adjusted to a cell density of 5×10^5 cells/ml (34). *L. pneumophila* strains grown to stationary phase were added to *A. castellanii* at a multiplicity of infection (MOI) of 50 or to *H. vermiformis* at an MOI of 10 in deionized water and in deionized water with 500 μM $MgCl_2$ and allowed 2 h to infect. To determine if differences in infection between conditions were due to sedimentation or initial binding, infections were also performed with a cen-

trifugation at 880 × *g* for 10 min before the 2-h infection. Afterwards, *L. pneumophila* internalization was measured by flow cytometry as previously described (18, 19) with the addition of a 1-h 100-μg/ml gentamicin treatment to kill extracellular bacteria. All *L. pneumophila* strains used contained a plasmid expressing green fluorescent protein (GFP) under control of a constitutive promoter, and internalization was defined as the acquisition of fluorescence by *A. castellanii* compared to the uninfected control. To measure the infection of *A. castellanii* by microscopy, *A. castellanii* was seeded onto 12-mm glass coverslips inside 24-well tissue culture plates; the same procedure as described above was performed, and cells were fixed with 4% paraformaldehyde (PFA). One hundred *A. castellanii* cells were counted per replicate, and infection is presented as the percentage of total *A. castellanii* cells infected. Experiments were performed in triplicate. To determine the effect of aggregation on infection in the Lp02 Δ*lpg2644* strain, infection assays were performed as described above in deionized water with or without the addition of agglutinating anti-*L. pneumophila* serogroup 1 polyclonal antibodies (1:1,000) (35).

To measure intracellular growth of *L pneumophila* expressing GFP in *A. castellanii*, infection assays were performed as described above without gentamicin treatment using an MOI of 0.5 in *Acanthamoeba castellanii* buffer (AC buffer) (36) in the wells of 96-well plates. The plates were incubated at 37°C in a Tecan Infinite M200 Pro with a fluorescence module. Fluorescence was measured every 20 min for 86 h. Growth data shown are from one representative experiment with 3 or more inoculations per experiment. Intracellular replication was also measured by quantifying the number of *L. pneumophila* bacteria released into the supernatants of infected cultures of *A. castellanii* similarly to as previously described, with the following modifications (37, 38). Flow cytometry was used to enumerate the number of bacteria per ml by using Flow-Count fluorospheres (Beckman Coulter). Infection assays were performed as described above with an MOI of 10, and after 2 h the supernatant was aspirated and warm AC buffer was added. The number of bacteria in the supernatant was measured at 24, 48, and 72 h postinoculation. Experiments were performed in duplicate, with the results of one representative replicate being displayed.

RESULTS

Legionella pneumophila autoaggregates more than a selected panel of other *Legionella* species. Taking in account that autoaggregation is associated with increased virulence and biofilm formation in other bacterial species (14, 39), we first sought to determine whether *Legionella* species differ in their abilities to form autoaggregates. The conventional experimental strategy to evaluate bacterial autoaggregation is to perform sedimentation assays with bacterial suspensions as aggregates settle in suspension (5, 7, 13). To this end, we compared the sedimentation of *L. pneumophila* with that of *Legionella* isolates from other species using a sedimentation assay adapted from a previously described method (18, 19). *L. pneumophila* isolates of serogroups 1 (Lp02) and 1 to 9 (cross-reacting with serogroups 1 to 9) and eight *Legionella* isolates from other species were suspended in deionized water with 10% BYE and allowed to settle (Tables 1 and 2). After an overnight incubation, the *L. pneumophila* isolates sedimented, while *L. erythra*, *L. feeleii*, *L. rubrilucens*, *L. maceachernii*, *L. anisa*, and *L. bozemanii* strains showed poor sedimentation (Fig. 1A). Considering that these non-*pneumophila* species are poor biofilm producers and are rarely diagnosed in legionellosis patients, these data raise potential correlations between the ability to produce autoaggregates and the environmental colonization/persistence of *Legionella* bacteria (27–29). We next compared the sedimentations of *L. pneumophila* isolates from serogroups 1 (Lp02), 1 to 9, 2, 3, 4, 5, 6, 8, 10, and 12, which were previously shown to produce Lcl (27–29). All the tested isolates were capable of sedimentation,
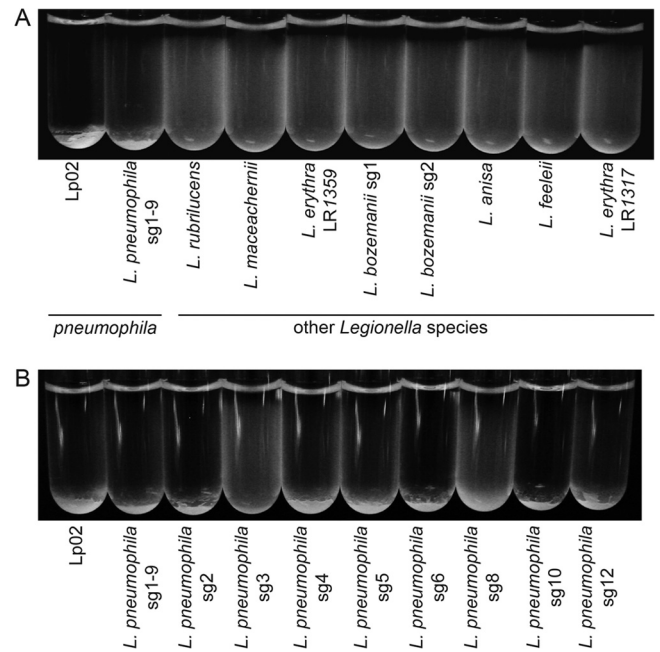

FIG 1 *L. pneumophila* sediments more efficiently than strains of other *Legionella* species. Results of sedimentation assays with Lp02, an *L. pneumophila* isolate that cross-reacts with serogroups 1 to 9 (*L. pneumophila* sg1-9), and eight isolates from other *Legionella* species (A) and with *L. pneumophila* isolates from serogroups 1(Lp02), 1 to 9, 2, 3, 4, 5, 6, 8, 10, and 12 (B) after overnight static incubation at room temperature in deionized water with 10% BYE are shown.

with the exception of *L. pneumophila* serogroup 3 and *L. pneumophila* serogroup 8, which showed only a moderate aggregation (Fig. 1B) and which have a low clinical prevalence (9, 40). Although most serogroups are able to autoaggregate, this suggests that differences in O-antigen decoration may have an impact on the aggregation of *L. pneumophila*, consistent with the effect of O-antigen differences on *E. coli* autoaggregation (9).

Lcl mediates *Legionella pneumophila* autoaggregation. BLAST searches of genomic data from *Legionella* species available in NCBI databases revealed that genes encoding homologues of Lcl are present in all sequenced *L. pneumophila* serogroup 1 strains (strain 130b, strain LPE 509, strain Lorraine, strain HL06041035, ATCC strain 43290, strain Corby, strain 2300/99 Alcoy, strain Lens, and strain Paris), in *L. pneumophila* serogroup 6 strain Thunder Bay, and in *L. pneumophila* serogroup 12 (strain 570-CO-H) (amino acid sequence identity of 65% to 85%), while it is absent from *L. longbeachae* (strains NSW150 and D-4968) (27) and *L. drancourtii* (data not shown) genomes. We also previously reported that homologues of the *L. pneumophila* biofilm mediator Lcl cannot be detected in species which are poor biofilm producers, such as *L. erythra*, *L. feeleii*, *L. rubrilucens*, *L. maceachernii*, *L. anisa*, and *L. bozemanii* (27–29). On the basis of studies correlating biofilm formation and autoaggregation, we next sought to test the hypothesis that the autoaggregation phenotype observed with *L. pneumophila* is mediated by Lcl (encoded by *lpg2644*). While Lp02 was able to form autoaggregates as observed by this strain's ability to sediment, this was not the case for Lp02 Δ*lpg2644* (Fig. 2A). Complementation of Lp02 Δ*lpg2644* by transformation with a plasmid expressing *lpg2644* (p*lpg2644*) resulted in recovery of its
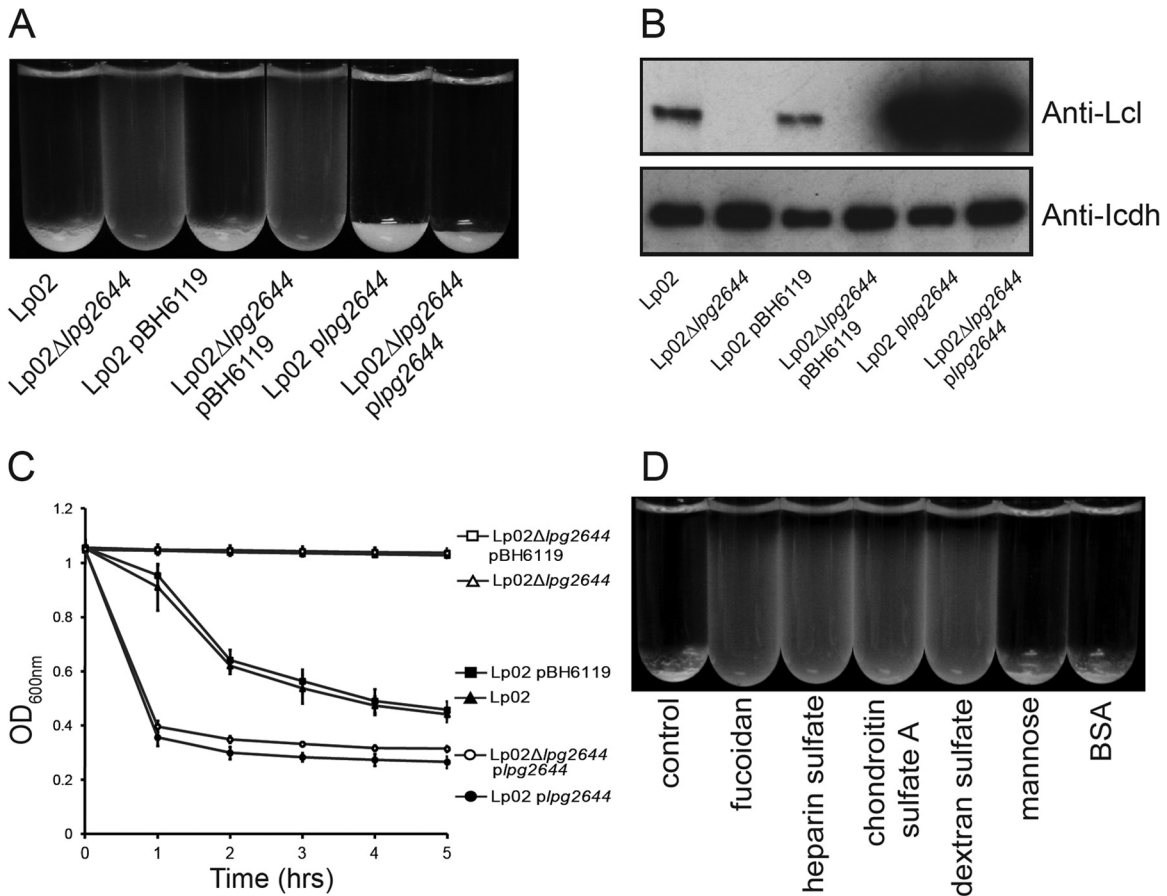
FIG 2 Lcl is essential for *L. pneumophila* autoaggregation. (A and C) Sedimentation with the wild type (Lp02), an *lpg2644* knockout (Lp02 Δ*lpg2644*), Lp02 and Lp02 Δ*lpg2644* transformed with an empty vector (Lp02/pBH6119 and Lp02 Δ*lpg2644*/pBH6119, respectively), Lp02 overexpressing Lcl (Lp02/p*lpg2644*), and the complemented knockout (Lp02 Δ*lpg2644*/p*lpg2644*), measured macroscopically in test tubes (A) and with sedimentation kinetics (C). The OD$_{600}$ values in panel C have been normalized to Lp02. (B) Anti-Lcl immunoblot demonstrating different expression levels of Lcl in Lp02, Lp02 Δ*lpg2644*, Lp02/pBH6119, Lp02 Δ*lpg2644*/pBH6119, Lp02/p*lpg2644*, and Lp02 Δ*lpg2644*/p*lpg2644*. IcdH was used as a loading control. (D) Sedimentation assays with Lp02 in the presence of 0.25 mg/ml GAGs. Mannose (0.25 mg/ml) and BSA (0.25 mg/ml) were used as negative controls.

autoaggregation properties. Together, these results suggest that Lcl is essential for *L. pneumophila* autoaggregation. According to CFU assays, the sedimentation phenotype was not due to cell death (data not shown). Importantly, the empty-vector controls (Lp02/pBH6119 and Lp02 Δ*lpg2644*/pBH6119) did not have differences in sedimentation compared to their respective untransformed strains. The complemented mutant Lp02 Δ*lpg2644*/p*lpg2644* and the wild-type strain transformed with p*lpg2644* (Lp02/p*lpg2644*) produced more Lcl than Lp02 and Lp02/pBH6119 as estimated by anti-Lcl immunoblotting (Fig. 2B). Taking these data in account, we next compared the sedimentation kinetics of these *L. pneumophila* strains. After 1 h, Lp02 Δ*lpg2644*/p*lpg2644* and Lp02/p*lpg2644*, which express higher levels of Lcl, had sedimented more rapidly than Lp02 (Fig. 2C). There were no significant differences in sedimentation rate or degree between Lp02/p*lpg2644* and Lp02 Δ*lpg2644*/p*lpg2644*, which is consistent with similar levels of Lcl production. This suggests that the rate and degree of autoaggregation are influenced by the amount of Lcl produced.

Previously, we reported that Lcl is capable of binding to several glycosaminoglycans (GAGs), including fucoidan, chondroitin sulfate A, dextran sulfate, and heparin sulfate, whose presence was shown to inhibit the biofilm formation by *L. pneumophila* (27). To determine whether the GAG binding properties of Lcl are required for the autoaggregation of *L. pneumophila*, sedimentation assays were performed with Lp02 in the presence of 0.25 mg/ml of these GAGs. Fucoidan, chondroitin sulfate A, dextran sulfate, and heparin sulfate all inhibited Lp02 sedimentation. In contrast, the negative controls, mannose (0.25 mg/ml) and bovine serum albumin (BSA) (0.25 mg/ml), did not alter Lp02 autoaggregation (Fig. 2D). Thus, the addition of Lcl ligands is able to prevent the autoaggregation of Lp02.

Divalent cations are required for Lcl-induced autoaggregation. Divalent cations were previously implicated in cell-cell interactions of both prokaryotes and eukaryotes (41–43). Therefore, we hypothesized that divalent cations may also play a role in Lcl-dependent *L. pneumophila* autoaggregation. To test this hypothesis, we assessed the sedimentation of Lp02, Lp02 Δ*lpg2644*, and Lp02 Δ*lpg2644*/p*lpg2644* in deionized water or in 500 μM solutions of CaCl$_2$, MgCl$_2$, ZnCl$_2$, or KCl in deionized water. While autoaggregation did not occur in deionized water alone, Lp02 sedimented in assay mixtures supplemented with 500 μM CaCl$_2$, MgCl$_2$, or ZnCl$_2$ (Fig. 3A). In contrast, Lp02 did not autoaggregate with the addition of 500 μM KCl, indicating that potassium and chloride ions have no effect on the autoaggregation of *L. pneumo-*
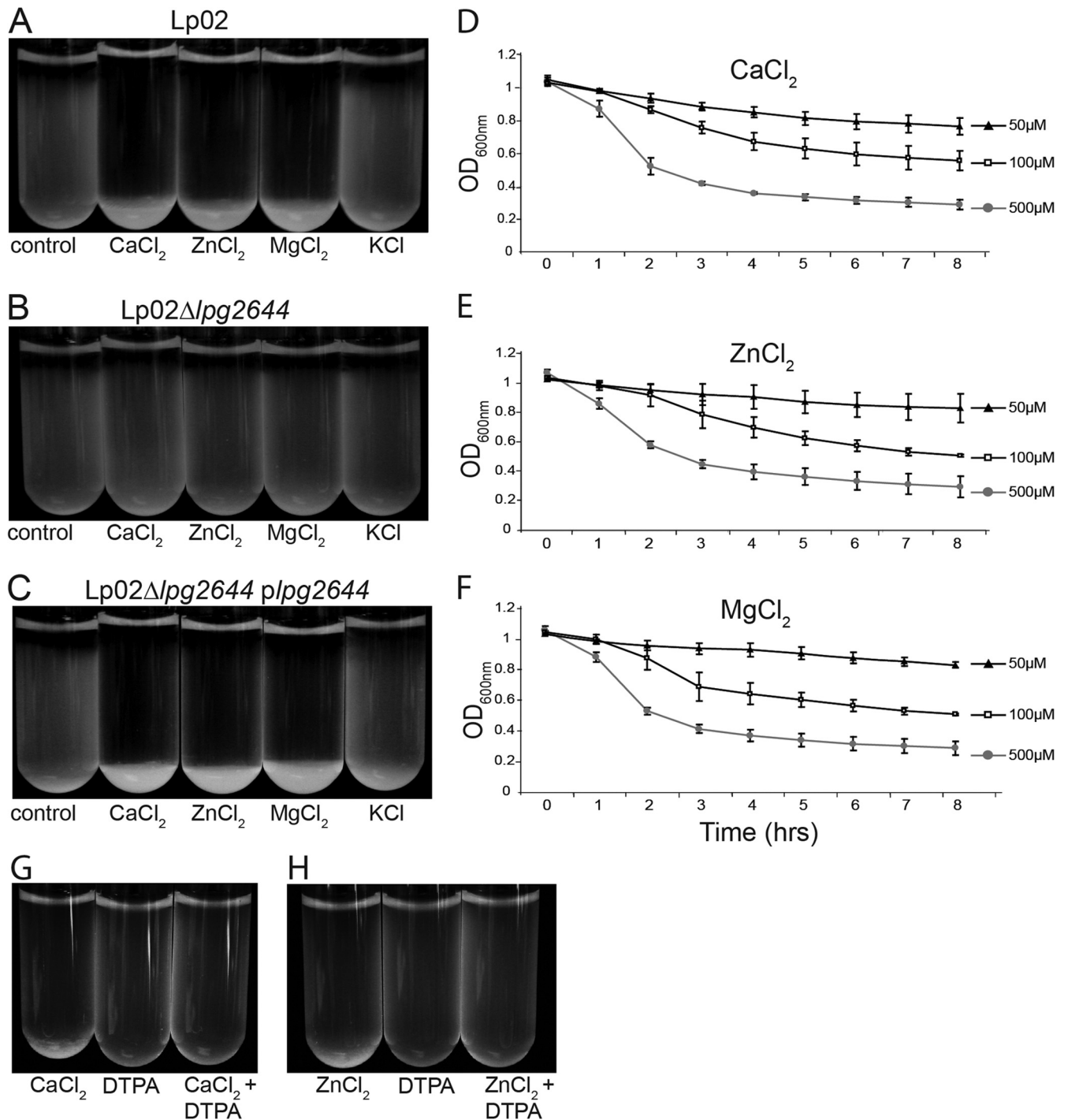
FIG 3 Divalent cations are required for Lcl-dependent autoaggregation. (A to C) Sedimentation assays with Lp02 (A), Lp02 Δ*lpg2644* (B), and Lp02 Δ*lpg2644*/p*lpg2644* (C) in deionized water (control) and deionized water supplemented with 500 μM CaCl₂, ZnCl₂,MgCl₂, or KCl. (D to F) Sedimentation kinetics of Lp02 with 500 μM, 100 μM, and 50 μM CaCl₂ (D), ZnCl₂ (E), or MgCl₂ (F). (G and H) Sedimentation assays with and without 50 μM DTPA in the presence of 100 μM CaCl₂ (G) and 100 μM ZnCl₂ (H) in deionized water.

phila (Fig. 3A). Importantly, Lp02 Δ*lpg2644* did not autoaggregate under any conditions (Fig. 3B). The deficiency in sedimentation of Lp02 Δ*lpg2644* was restored upon complementation with p*lpg2644* (Fig. 3C). These data suggest that divalent cations induce *L. pneumophila* autoaggregation in an Lcl-dependent manner. Consistent with results obtained with Lp02, autoaggregation of

Lp02 Δ*lpg2644*/p*lpg2644* did not occur with the addition of 500 μM KCl (Fig. 3C). In addition, Lp02 and Lp02 Δ*lpg2644*/p*lpg2644* also sedimented in the presence of 500 μM MgSO₄, confirming that divalent cations and not chloride anions are required for the autoaggregation of *L. pneumophila* (data not shown).

We next determined whether the concentrations of specific

cations have an impact on the sedimentation kinetics of *L. pneu-mophila*. Lp02 colonies were suspended in deionized water with 500 μM, 100 μM, and 50 μM CaCl$_2$, ZnCl$_2$, or MgCl$_2$, and the OD$_{600}$ of the bacterial suspensions was monitored for 8 h. Lp02 sedimentation appeared to be dose dependent with the addition of CaCl$_2$, ZnCl$_2$, and MgCl$_2$ (Fig. 3D to F). The addition of up to 500 μM KCl, however, did not rescue Lp02 autoaggregation, suggest-ing that the lack of autoaggregation in the previous assays was not due to a lower chloride or potassium ion concentration (data not shown). In addition, no significant difference was observed in the degree and rate of sedimentation between the different cations (Fig. 3D to F). Furthermore, sedimentation of Lp02 supplemented with CaCl$_2$ and ZnCl$_2$ was inhibited by the addition of a sublethal concentration of the metal chelator diethylenetriaminepenta-acetic acid (DTPA) (Fig. 3G and H), confirming that the aggrega-tion of *L. pneumophila* is dependent on divalent cations. Autoag-gregation in the presence of MgCl$_2$, however, could not be inhibited by the addition of DTPA (data not shown). This is con-sistent with the reported low affinity of DTPA for magnesium compared to Ca^{2+} and Zn^{2+} (44).

Lcl secreted in the extracellular milieu is not sufficient to in-duce the autoaggregation of Lp02 Δ*lpg2644*. Based on evidence that Lcl is both surface exposed and secreted into the extracellular milieu using the type II secretion system (28, 29, 45), we next tested whether Lcl is secreted into the extracellular milieu during sedimen-tation assays. Anti-Lcl immunoblot assays detected Lcl in the super-natant fractions after sedimentation assays with Lp02 and with a mixed population of Lp02 and Lp02 Δ*lpg2644*, while Lcl could not be detected in assays with Lp02 Δ*lpg2644* alone (Fig. 4A). We next in-vestigated if Lcl secreted into the extracellular milieu by wild-type bacteria could be sufficient to autoaggregate Lp02 Δ*lpg2644* in a mixed population of Lp02 Δ*lpg2644* expressing red fluorescent pro-tein (RFP) (Lp02 Δ*lpg2644*/pRFP) and Lp02 expressing GFP (Lp02/pGFP). When sedimented cells were visualized by fluorescence mi-croscopy, the vast majority of the bacteria that were observed were Lp02 expressing GFP, with few being Lp02 Δ*lpg2644*/pRFP, indicat-ing that Lp02 primarily had sedimented (Fig. 4B). Plasmids were next swapped to ensure that the observed phenotype was not indirectly linked to the heterologous expression of the respective fluorescent proteins. Sedimentation assays were performed with RFP-expressing Lp02 (Lp02/pRFP) and GFP-expressing Lp02 Δ*lpg2644* (Lp02 Δ*lpg2644*/pGFP). Most of the bacteria found at the bottom of the test tube were Lp02/pRFP (Fig. 4C), suggesting that the lack of cell-cell interactions observed between Lp02 and Lp02 Δ*lpg2644* is not an indirect consequence of the heterologous expression of RFP and GFP. These results were further confirmed by measuring the propor-tion of each labeled bacterial cell after sedimentation using fluores-cence microscopy. Among 5 representative microscopy fields, Lp02 accounted for 91 to 93% of the visualized cells (Fig. 4D and E). In control assays, the expression of RFP or GFP did not alter the indi-vidual sedimentation rates of any of the strains used, indicating that the expression of neither GFP nor RFP affects autoaggregation (data not shown). Taken together, these results show that Lp02 Δ*lpg2644* bacteria are not able to aggregate with wild-type bacteria and suggest that the Lcl secreted in the extracellular milieu is not sufficient for the incorporation of *L. pneumophila* into autoaggregates.

Lcl is sufficient to induce autoaggregation and to increase biofilm production in *E. coli* and autoaggregation in *L. feeleii*. To determine whether Lcl is sufficient to induce autoaggregation, we next measured the impact of heterologous expression of Lcl on

the sedimentation of *E. coli*. The Lcl gene (*lpg2644*) was cloned into the plasmid pTrc under the control of the leaky *trc* IPTG-inducible promoter. This plasmid was then transformed into *E. coli* TOP 10 to create *E. coli*/p*lcl* (Table 2). As a negative control, the *E. coli* TOP10 strain was transformed with the empty pTrc vector to obtain *E. coli*/pTrc (Table 2). In immunoblot assays, anti-Lcl antibodies reacted with a 50-kDa protein in *E. coli*/p*lcl* lysates, and an increased expression level was observed in the pres-ence of IPTG (Fig. 5A). The cellular localization of Lcl was exam-ined by live imaging using spinning-disk confocal microscopy and live cell anti-Lcl immunofluorescence assays with *E. coli*/p*lcl*. Con-focal analysis revealed that Lcl is expressed at the surface of *E. coli*/p*lcl* cells (antibodies are not outer membrane permeative), albeit not uniformly distributed (Fig. 5B). This heterogeneous cluster distribution of recombinant Lcl in *E. coli* is consistent with its previously reported distribution in *L. pneumophila* (28). No fluorescence signal was detected with *E. coli*/pTrc and anti-Lcl antiserum (Fig. 5B). The cellular distribution of Lcl was further analyzed using deconvolved confocal *xy* planes (Fig. 5B). Inner membranes were labeled with the dye FM4-64 (46). The scanning of Lcl fluorescence intensities along cross sections of the bacteria and the comparison with the fluorescence intensities for FM 4-64 confirmed that heterologous Lcl is expressed at the cell surface of *E. coli* (Fig. 5B). To determine if surface-exposed Lcl is sufficient to mediate bacterial cell-cell interactions, the autoaggregations of *E. coli*/p*lcl* and *E. coli*/pTrc colonies were compared in sedimentation assays. After 5 h of incubation, control strain *E. coli*/pTrc re-mained in suspension, whereas *E. coli*/p*lcl* showed a marked abil-ity to autoaggregate (Fig. 5C). By differential interference contrast (DIC) microscopy, cells from settled *E. coli*/pTrc appeared to be distributed evenly and homogenously (Fig. 5D). In contrast, set-tled *E. coli*/p*lcl* cells were found to cluster into large aggregates, suggesting that heterologous expression of Lcl is sufficient to in-duce autoaggregation and results in sedimentation (Fig. 5D). This finding was further confirmed by sedimentation kinetic assays, where *E. coli*/p*lcl* started to settle after an hour of incubation while *E. coli*/pTrc remained in suspension for the 5-h time course (Fig. 5E). Importantly, *E. coli*/p*lcl* also sedimented in deionized water in the presence of MgCl$_2$ (see Fig. S1A in the supplemental material); this demonstrates that in *E. coli*, Lcl-dependent autoaggregation is also divalent cation dependent. Additionally the sedimentation of *E. coli*/p*lcl* was inhibited in the presence of 0.25-mg/ml fucoidan, suggesting that heterologously expressed Lcl may be properly folded as it retains its glycosaminoglycan binding ability (see Fig. S1B in the supplemental material). Differences in sedimentation between these strains were not due to differences in cell viability as demonstrated by comparing CFU of cell suspensions collected before and after sedimentation assays (data not shown).

We know from our previous studies that surface-exposed Lcl is essential for the biofilm production by *L. pneumophila* (27, 28). Based on the ability of Lcl to induce the autoaggregation of *E. coli*, we next sought to determine whether heterologous expression of *lpg2644* could also be sufficient to influence *E. coli* biofilm produc-tion. In comparison with *E. coli*/pTrc, *E. coli*/p*lcl* produced signif-icantly more biofilm (Fig. 5F). Moreover, in the presence of IPTG, increased synthesis of Lcl was correlated with a significantly larger amount of biofilm production by the *E. coli*/p*lcl* strain (Fig. 5F). The addition of IPTG to *E. coli*/pTrc did not alter the biofilm production of *E. coli*/pTrc, while it slightly decreased the growth of both *E. coli*/pTrc and *E. coli*/p*lcl* as measured by the absorbance of
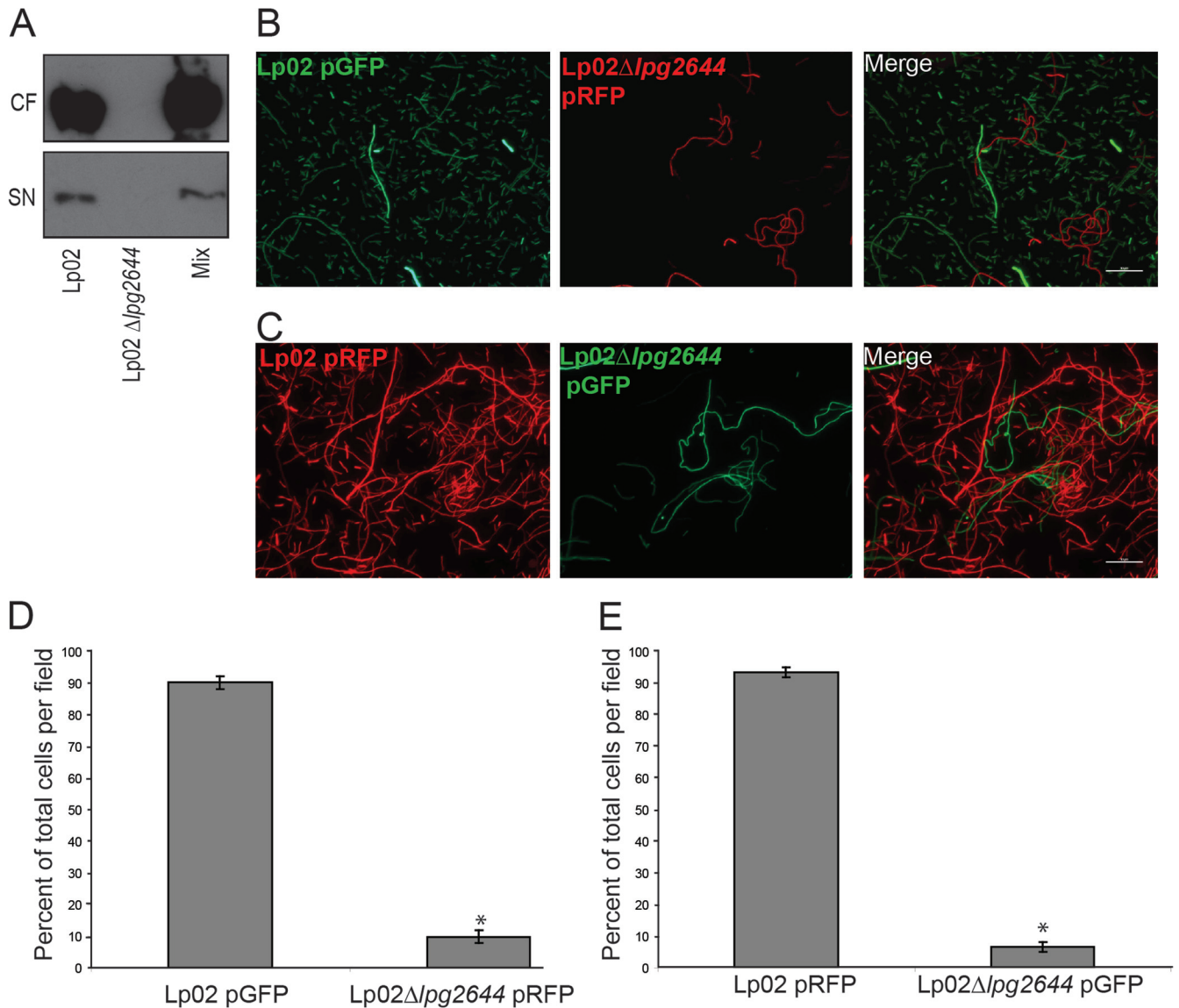
FIG 4 Lcl secreted in the extracellular milieu is not sufficient to induce the aggregation of Lp02 Δ*lpg2644*. (A) Anti-Lcl immunoblots of the supernatant (SN) and cell fraction (CF) from sedimentation assays with Lp02, Lp02 Δ*lpg2644*, and mixed suspensions. (B and C) Fluorescence microscopy analysis of labeled autoaggregates after 5-h static incubations with Lp02/pGFP and Lp02 Δ*lpg2644*/pRFP (B) and Lp02/pRFP and Lp02 Δ*lpg2644*/pGFP (C) mixed suspensions in deionized water with 500 μM MgCl$_2$. (D and E) Relative quantifications of Lp02 and Lp02 Δ*lpg2644* in mixed sedimentation assays from panels B and C, respectively. Scale bars, 10 μm. *, statistically significant differences ($P \leq 0.001$ by the two-tailed Student t test).

bacterial suspensions at 600 nm (data not shown). This decrease in growth, however, did not appear to impact the adherent biomass as detected by crystal violet staining. We then evaluated whether heterologous expression of *lpg2644* could also be sufficient to influence autoaggregation in other *Legionella* species. *Legionella feeleii* was transformed with p*lpg2644* to obtain *L. feeleii*/p*lpg2644* (Table 2). The synthesis of recombinant Lcl in *L. feeleii*/p*lpg2644* was confirmed by anti-Lcl immunoblot analysis (Fig. 5G). Similarly to *E. coli*/p*lcl*, *L. feeleii*/p*lpg2644* showed a marked ability to autoaggregate in sedimentation assays, while *L. feeleii* transformed with the empty plasmid (*L. feeleii*/pBH6119) remained in suspension (Fig. 5G).

Lcl mediates the attachment of *L. pneumophila* to *Acanthamoeba castellanii* and *Hartmanella vermiformis*. Once an

intracellular pathogen is in contact with a host cell, it next adheres intimately to host surface using adhesins. Lcl is an adhesin that mediates the attachment of *L. pneumophila* to human lung epithelial cells (27). We thus hypothesized that it may also mediate the attachment of Lp02 to *A. castellanii*. To test this hypothesis independently of the aggregation/sedimentation phenotype, we performed *A. castellanii* infection experiments where cell contacts were promoted by mild centrifugation. After incubation, the infections of *A. castellanii* with Lp02/pGFP, Lp02 Δ*lpg2644*/pGFP, and a chromosomally complemented *lpg2644* deletion mutant labeled with GFP (Lp02 Δ*lpg2644* c*lpg2644*/pGFP) were measured by flow cytometry as previously described (18, 19). Under these experimental conditions, Lp02/pGFP was found to infect 76 to 83% of *A. castellanii* cells regardless of the presence of MgCl$_2$ (Fig. 6A).
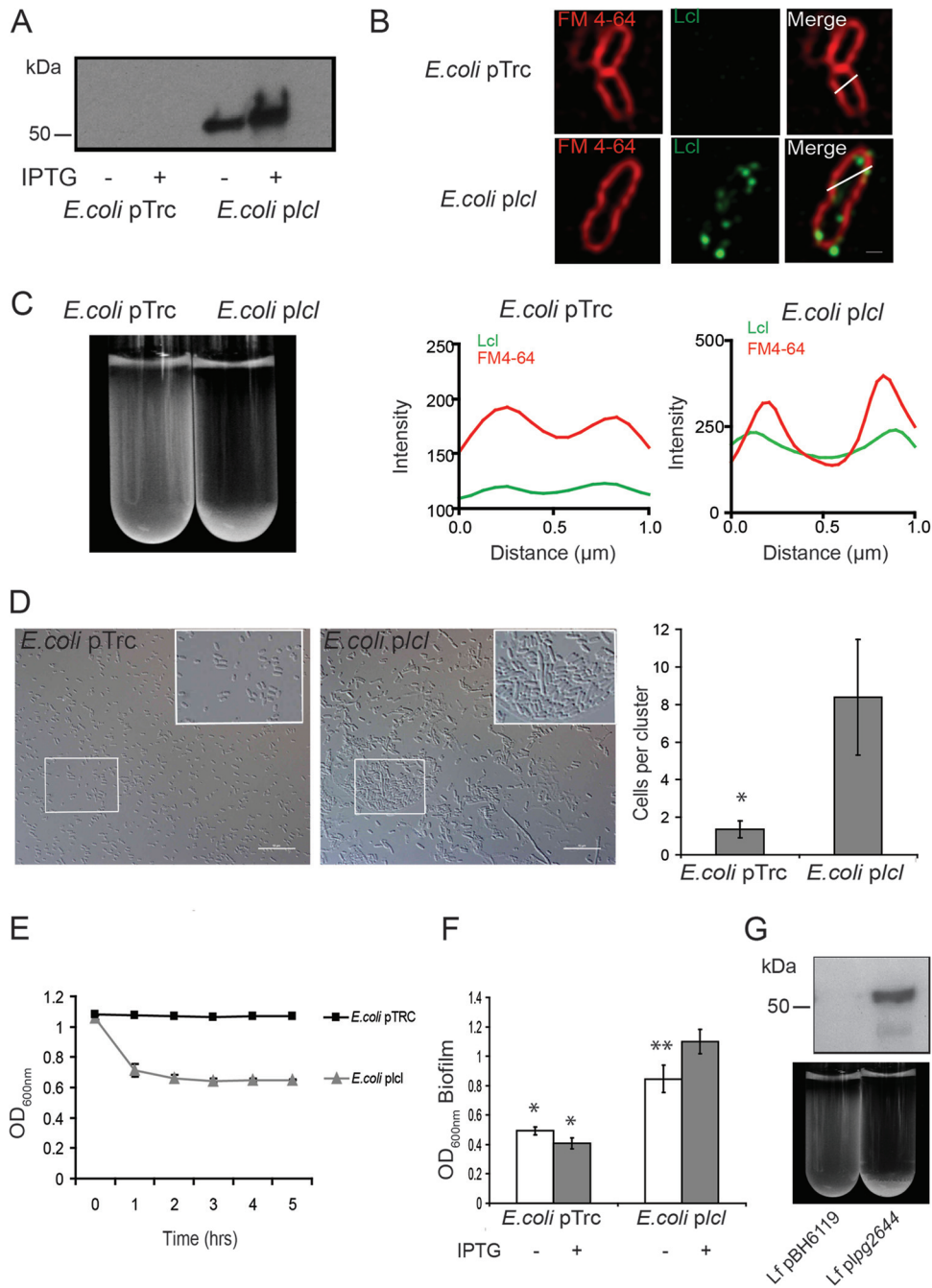
FIG 5 Lcl alone is sufficient to induce autoaggregation and biofilm production in *E. coli* and autoaggregation in *L. feeleii*. (A) Anti-Lcl immunoblot of *E. coli* containing the empty plasmid (*E. coli*/pTrc) and *E. coli* transformed with a plasmid containing *lpg2644* under the control of a leaky IPTG-inducible promoter (*E. coli*/p*lcl*). (B) Anti-Lcl immunofluorescence and FM 4-64 staining of live *E. coli*/pTrc and *E. coli*/p*lcl* (top). The localization of Lcl (green lines) was determined by comparing its fluorescence intensity along the white lines with the inner membrane marker FM 4-64 (red lines). (C to E) Sedimentation of *E. coli*/pTrc and *E. coli*/p*lcl* measured by test tube sedimentation assays (C), DIC microscopy of sediment (D), and sedimentation kinetics (E). (F) Biofilm production of *E. coli*/pTrc and *E. coli*/p*lcl* as measured by crystal violet staining. ∗, statistically significant differences between the indicated condition and *E. coli*/p*lcl* without IPTG; ∗∗, statistically significant differences with *E. coli*/p*lcl* with IPTG ($P \leq 0.05$ by the two-tailed Student t test). (G) Anti-Lcl immunoblot and sedimentation assays with *L. feeleii* transformed with the empty vector (*Lf*/pBH6119) and with the p*lpg2644* plasmid (*Lf*/p*lpg2644*). IPTG was added to a final concentration of 1 mM when indicated for sedimentation experiments and immunoblotting. For biofilm assays, IPTG was added to a final concentration of 5 mM. Scale bars, 0.6 μm and 10 μm for panels B and D, respectively.

This suggests that magnesium does not influence the infection of amoebae with *L. pneumophila* once they are in contact. In contrast, only 5 to 13% of the *A. castellanii* cells were infected in assays with Lp02 Δ*lpg2644*/pGFP. Considering the short-term incubation of our assays (2 h), these results presumably reflect a reduced adhesion of Lp02 Δ*lpg2644* and are consistent with the reported decreased *L. pneumophila* infection of *A. castellanii* in the presence of blocking anti-Lcl antibodies (29). The infection of *A. castellanii*
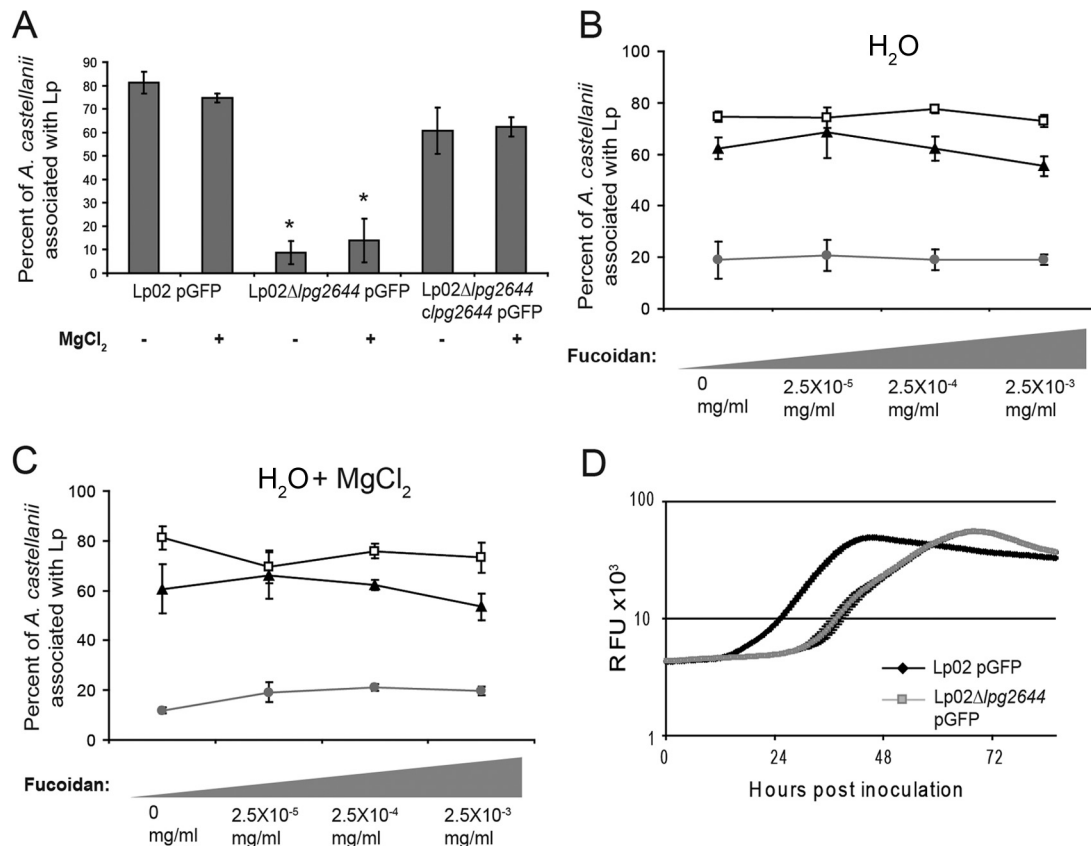
FIG 6 Lcl mediates the attachment of *L. pneumophila* to *Acanthamoeba castellanii*. (A) Infection of *A. castellanii* by Lp02/pGFP, Lp02 Δ*lpg2644*/pGFP, and Lp02 Δ*lpg2644* c*lpg2644* (chromosomal insertion of gene *lpg2644*)/pGFP after centrifugation in deionized water in the absence or presence of 500 μM MgCl$_2$ as measured by flow cytometry. *, statistically significant differences with Lp02 Δ*lpg2644* c*lpg2644* without MgCl$_2$ by the two-tailed Student *t* test ($P \leq 0.01$). (B and C) Infection of *A. castellanii* measured by flow cytometry in the presence of 2.5×10^{-3} to 2.5×10^{-5} mg/ml fucoidan after centrifugation in deionized water (B) and in deionized water with 500 μM MgCl$_2$ (C) with Lp02/pGFP (white squares), Lp02 Δ*lpg2644*/pGFP (gray circles), and Lp02 Δ*lpg2644* c*lpg2644*/pGFP (black triangles). (D) Intracellular growth of Lp02/pGFP (black diamonds) and Lp02 Δ*lpg2644*/pGFP (gray squares) in *A. castellanii*.

with the complemented mutant was significantly higher than that with Lp02 Δ*lpg2644*/pGFP ($P < 0.01$) and followed the same trend as the wild-type strain under all experimental conditions (Fig. 6A). To confirm this result, the number of *A. castellanii* associated with *L. pneumophila* was next monitored by confocal laser scanning microscopy (CLSM) using the same experimental strategy. This approach revealed the same trend as our flow cytometry assays, suggesting that the Lcl adhesin mediates the attachment of *L. pneumophila* to *A. castellanii* (see Fig. S2A in the supplemental material). To evaluate if Lcl plays a role in attachment to other *L. pneumophila* natural hosts, we next compared the short-term infections of *H. vermiformis* with Lp02/pGFP, Lp02 Δ*lpg26444*/pGFP, and Lp02 Δ*lpg2644* c*lpg2644*/pGFP. In contrast to Lp02/pGFP (71 to 79%) and Lp02 Δ*lpg2644* c*lpg2644*/pGFP (57 to 68%), Lp02 Δ*lpg26444*/pGFP could infect only 16 to 24% of the *H. vermiformis* cells, suggesting that Lcl is also involved in the attachment to this amoeba species (see Fig. S2B in the supplemental material).

When infection assays with centrifugation were performed in the presence of fucoidan concentrations that inhibit the autoaggregation of Lp02 (see Fig. S3 in the supplemental material), the infection of *A. castellanii* with Lp02/pGFP, Lp02 Δ*lpg26444*/pGFP, and Lp02 Δ*lpg2644* c*lpg2644*/pGFP remained unchanged (Fig. 6B and C). These results suggest that fucoidan does not inhibit the

attachment to *A. castellanii* while it can specifically inhibit *L. pneumophila* aggregation.

To evaluate the role of Lcl in *L. pneumophila* intracellular growth, *A. castellanii* was challenged with Lp02 and Lp02 Δ*lpg26444* producing GFP. Fluorescence was monitored over 72 h of incubation to determine the growth dynamics of each strain. Although the growth of Lp02 Δ*lpg26444* was significantly delayed, presumably due to a lower initial infection rate compared to that of the wild-type strain, Lp02 and Lp02 Δ*lpg2644* showed growth curves with similar slopes in *A. castellanii* (Fig. 6D). This result is consistent with previous studies suggesting that Lcl does not play a role in intracellular growth using an *in vitro Drosophila* cell infection model (47).

Lcl-dependent autoaggregation potentiates the infection of *A. castellanii* and *H. vermiformis* by *L. pneumophila*. Amoeba species, such as the *L. pneumophila* host *A. castellanii*, are preferentially found attached to surfaces in aquatic environments, where they feed more effectively on their prey (48, 49). Given that Lcl is a mediator of autoaggregation and bacterial aggregates tend to settle, we hypothesized that this process may also assist the bacteria in more efficiently encountering and thus invading amoebae. To test this hypothesis, Lp02/pGFP, Lp02 Δ*lpg2644*/pGFP and Lp02 Δ*lpg2644* c*lpg2644*/pGFP were left to settle for 2 h on *A. castellanii* monolayers. To evaluate the role of Lcl-dependent au-
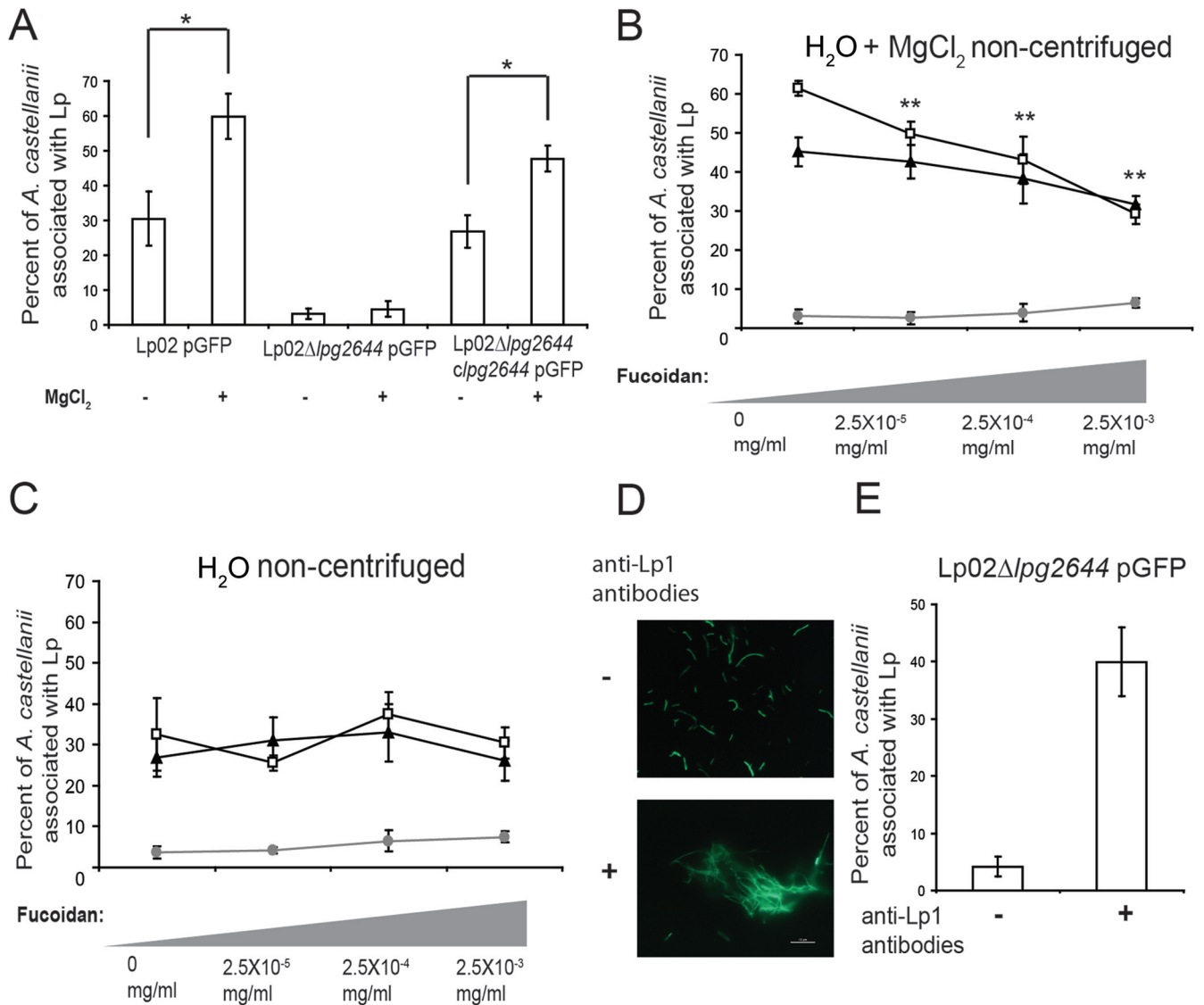
FIG 7 Lcl-dependent autoaggregation potentiates the internalization of *L. pneumophila* in *A. castellanii*. (A) Infection of *A. castellanii* by Lp02/pGFP, Lp02 Δ*lpg2644*/pGFP, and Lp02 Δ*lpg2644* c*lpg2644*/pGFP in the absence or presence of 500 μM MgCl₂ as measured by flow cytometry. (B and C) Infection of *A. castellanii* as measured by flow cytometry in the presence of 2.5×10^{-3} to 2.5×10^{-5} mg/ml fucoidan in deionized water with the addition of 500 μM MgCl₂ (B) and in deionized water alone (C) with Lp02/pGFP (white squares), Lp02 Δ*lpg2644*/pGFP (gray circles), and Lp02 Δ*lpg2644* c*lpg2644*/pGFP (black triangles). (D) Fluorescence microscopy of Lp02 Δ*lpg2644*/pGFP in the absence or presence of anti-*Legionella pneumophila* serogroup1 (anti-Lp1) agglutinating antibodies (1:1,000). (E) Infection of *A. castellanii* with isolated planktonic Lp02 Δ*lpg2644*/pGFP and artificially aggregated Lp02 Δ*lpg2644*/pGFP (without and with anti-Lp1 antibodies, respectively). Scale bar, 10 μm. * and **, statistically significant differences between the indicated strains (A) and compared to assays with 0 mg/ml fucoidan (B), respectively, by the two-tailed Student *t* test ($P \leq 0.01$).

toaggregation in *A. castellanii* infection, assays were performed in deionized water and in deionized water with 500 μM MgCl₂, which are nonpermissive and permissive, respectively, for Lcl-dependent autoaggregation. Lp02/pGFP infected 108% ($P < 0.01$) more amoebae in the presence of 500 μM MgCl₂ than in deionized water alone (Fig. 7A). This result suggests that Lcl-dependent autoaggregation of wild-type Lp02 increases the ability of *L. pneumophila* to come in contact with *A. castellanii*. Lp02 Δ*lpg2644*/pGFP showed a marked and significant decrease in internalization in both the absence and presence of magnesium in comparison to Lp02/pGFP. Infection assays of *A. castellanii* with the complemented mutant Lp02 Δ*lpg2644* c*lpg2644*/pGFP followed the same trend as with wild-type Lp02 (Fig. 7A). This find-

ing was further confirmed by CLSM analyses suggesting that the Lcl-dependent autoaggregation potentiates the infection of *A. castellanii* by *L. pneumophila* (see Fig. S2C in the supplemental material). These results were further confirmed in the *H. vermiformis* infection model, suggesting that the role of Lcl-dependent auto-aggregation in initial contact with amoebae may be extrapolated to other host species (see Fig. S2D in the supplemental material).

Taking into account that Lcl also mediates the attachment of Lp02 to *A. castellanii*, the reduced infectivity of Lp02 Δ*lpg2644* in these experiments may reflect a sum of deficiencies in aggregation-dependent contact as well as attachment. To experimentally separate these two processes, we took advantage of the dose-dependent inhibitory effect of soluble GAGs on Lcl-mediated auto-

aggregation, which does not affect the attachment of Lp02 (Fig. 2D and 6B and C; see Fig. S3 in the supplemental material). When Lp02/pGFP and Lp02 Δ*lpg2644* c*lpg2644*/pGFP were left to auto-aggregate and settle for 2 h in 500 μM MgCl$_2$, a dose-dependent inhibition of *A. castellanii* infection was observed in the presence of fucoidan concentrations that inhibit the autoaggregation of Lp02 (Fig. 7B). Consistent with the results observed in the infection assays with centrifugation described above (Fig. 6B and C), fucoidan had no effect on the infection of *A. castellanii* under conditions which do not promote autoaggregation (Fig. 7C). As expected, the infection of *A. castellanii* with the Lp02 Δ*lpg26444*/pGFP mutant remained unchanged despite the presence of fucoidan (Fig. 7B and C). Bearing in mind that fucoidan does not inhibit the attachment of wild-type Lp02 to *A. castellanii* but inhibits Lcl-dependent autoaggregation, these data suggest that Lcl-dependent autoaggregation potentiates the infection of these host cells. The intracellular replication was next assessed by determining the number of *L. pneumophila* bacteria released from lysed *A. castellanii* into the supernatant of infected cultures (37, 38) using flow cytometry. This revealed that although the concentrations of Lp02/pGFP and Lp02 Δ*lpg2644* c*lpg2644*/pGFP were higher than that of Lp02 Δ*lpg2644*/pGFP in the supernatant at 72 h postinoculation, the rate of intracellular replication was similar for all strains and was unchanged in the presence of 500 μM MgCl$_2$ (see Fig. S2E and F in the supplemental material).

We next asked if aggregation alone could potentiate the host internalization of the adhesion-deficient mutant Lp02 Δ*lpg2644*. *A. castellanii* was challenged with aggregates of Lp02 Δ*lpg2644*/pGFP formed in the presence of agglutinating anti-*L. pneumophila* polyclonal antibodies (Fig. 7D). Compared to a suspension of planktonic bacteria, the infection of *A. castellanii* with Lp02 Δ*lpg26444*/pGFP aggregates was 8 times greater (Fig. 7E). Taken together, these data suggest that aggregation of *L. pneumophila* can potentiate the infection of *A. castellanii* independently of attachment.

Lcl-dependent autoaggregation increases the number of *L. pneumophila* bacteria per infected *A. castellanii* cell. In initial CLSM analyses, *A. castellanii* infected with Lp02/pGFP and Lp02 Δ*lpg2644* c*lpg2644*/pGFP appeared to contain higher numbers of intracellular *L. pneumophila* bacteria under conditions permissive for autoaggregation (Fig. 8; see Fig. S2G in the supplemental material). Meanwhile, only individual bacteria were internalized in *A. castellanii* infected with Lp02 Δ*lpg2644* (Fig. 8). These initial results led us to speculate that the Lcl-dependent autoaggregation may also have an impact on the number of internalized *L. pneumophila* bacteria per infected *A. castellanii* cell by potentiating the contact of host cells with multicellular aggregates rather than isolated planktonic bacteria. To investigate this, the numbers of internalized *L. pneumophila* bacteria per infected *A. castellanii* cell were counted with CLSM. Infection assays performed with Lp02/pGFP and complemented mutant Lp02 Δ*lpg2644* c*lpg2644* showed a greater number of internalized *L. pneumophila* bacteria per *A. castellanii* cell under conditions allowing Lcl-dependent autoaggregation (deionized water with MgCl$_2$) than in the absence of Lcl-dependent autoaggregation (deionized water alone) (Fig. 8). In contrast, in infection assays performed with Lp02 Δ*lpg2644*/pGFP, most infected *A. castellanii* amoebae contained only individual bacteria regardless of the absence or presence of magnesium. This is consistent with the inability of this strain to autoaggregate.
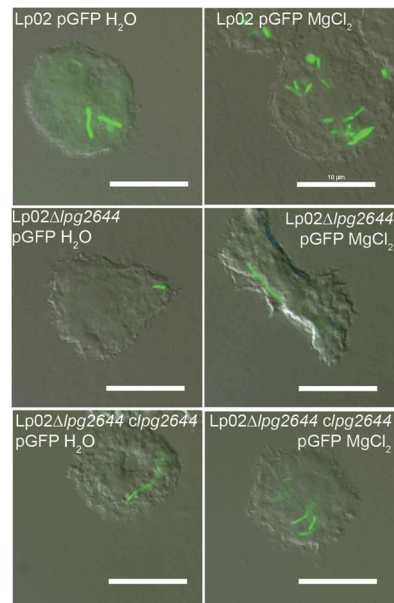

FIG 8 Lcl-dependent autoaggregation increases the number of *L. pneumophila* bacteria per infected *A. castellanii* cell. Visualization of *A. castellanii* infection by Lp02/pGFP, Lp02 Δ*lpg2644*/pGFP, and Lp02 Δ*lpg2644* c*lpg2644*/pGFP in the absence or presence of 500 μM MgCl$_2$ in deionized water by fluorescence microscopy is shown. Scale bars, 10 μm.

DISCUSSION

Bacterial biofilms are major determinants in host colonization for several pathogens (7, 50). Although bacterial autoaggregation is commonly linked to biofilm production (2, 9, 11, 51), a recent study suggests that these two processes may have phenotypic differences (8). Here, we demonstrate that among eight *Legionella* species, *L. pneumophila* is exclusively capable of autoaggregation. These seven other *Legionella* species were previously shown to neither produce a homologue of Lcl nor contain a detectable *lpg2644* gene (27). Thus, the rare occurrence of these *Legionella* species in legionellosis patients may be explained by their deficiency in Lcl-dependent aggregation, which may lead to a reduced capacity for environmental dissemination in anthropogenic water systems and therefore reduced contact with humans. To test the role of Lcl in *L. pneumophila* autoaggregation, we evaluated the autoaggregation of an *L. pneumophila* site-directed mutant deficient in Lcl. These assays revealed that Lcl is essential for the autoaggregation of *L. pneumophila*. Keeping in mind that Lcl is also a mediator of biofilm formation (27), this finding is consistent with the reported role of autoaggregation in the formation of *L. pneumophila* biofilms (52). Furthermore, it has been previously reported that the *lqs* quorum-sensing gene cluster is involved in the regulation of sedimentation and may be linked to the sedimentation phenotype we described (18, 19).

Amoebae, the natural hosts of *L. pneumophila*, often reside on surfaces in stagnant environmental water, where they are also frequently recovered from biofilms with *L. pneumophila* (48, 49). Upon phagocytosis, *L. pneumophila* is able to replicate within the protozoan hosts, a process which promotes the environmental colonization of water systems with this pathogen (24, 53). The early time points of phagocytosis by amoebae include the following sequence of events: initial contact of the pathogen with the phagocyte, intimate adherence, and ingestion (54). Among the

virulence factors promoting these early phases of phagocytosis, *L. pneumophila*'s flagellin was shown to promote initial contact with host cells such as *A. castellanii*, although it does not act as adhesin (54, 55). Here, control immunoblot analyses suggested that the loss of autoaggregation in Lp02 Δ*lpg2644* is not linked to a change in flagellin synthesis compared to that of wild-type Lp02 (see Fig. S4 in the supplemental material). Upon initial contact, the intimate adherence of *L. pneumophila* to the surface of its natural hosts may involve binding to surface receptors by adhesin proteins. Thus, Lcl may serve as an adhesin for the attachment of *L. pneumophila* to its environmental hosts. Consistent with this hypothesis, we show that the deletion of *lpg2644* in *L. pneumophila* greatly reduces the ability of Lp02 to infect *A. castellanii* and *H. vermiformis*. In addition to its role in the initial binding to *A. castellanii* and *H. vermiformis*, Lcl also promotes the autoaggregation of *L. pneumophila*, which appeared to potentiate the infection of amoebae by facilitating contact between the pathogen and its host. Similarly, the aggregation of *Chlamydia trachomatis* and *Chlamydia pneumoniae* cells mediated by host collagenous lectins was previously shown to enhance bacterial uptake per human phagocyte (56). Interestingly, many cases of legionellosis are linked to the presence of *L. pneumophila* in stagnant water (57, 58). Thus, the Lcl adhesin may play a dual role in the life cycle of *L. pneumophila* by promoting conditions where the bacteria can both settle and also infect amoebae, increasing the ability of *L. pneumophila* to replicate and disseminate in the environment.

In this study, we showed that heterologous expression of *lpg2644* in *E. coli* leads to autoaggregation and increased biofilm production. This strongly suggests that heterologous expression of *lpg2644* is sufficient to promote cell-cell interactions and that surface expression of Lcl is required for *L. pneumophila* autoaggregation, as suggested by sedimentation assays with a mixed suspension of Lp02 and Lp02 Δ*lpg2644*. Taken together, these data suggest that cell-cell interactions may result from homophilic interactions between surface-exposed Lcl proteins of neighboring cells. Alternatively, it is possible that Lcl requires unidentified bridging factors that are conserved among *E. coli* and *L. pneumophila* species, as in the instance of *Aerobacter aerogenes* autoaggregation (59). The phenomenon of heterologous expression of proteins inducing autoaggregation is similar to what was observed with the *Staphylococcus aureus* adhesin protein A, where production of protein A in *Lactococcus lactis* was sufficient to induce both autoaggregation and biofilm production (60).

We observed that autoaggregation mediated by Lcl requires the presence of divalent cations. This finding is correlated with the reported ability of calcium and magnesium to increase the attachment of *L. pneumophila* to abiotic surfaces (61). It is thus possible that the role of divalent cations in initial attachment may be directly related to the ability of *L. pneumophila* to form autoaggregates. Divalent cations have previously been shown to affect the autoaggregation and the biofilm production of several bacterial species by acting as a structural element (43, 62–64) and also by acting as a signaling molecule (65, 66). Interestingly in all the aforementioned examples, there is specificity in the divalent cation which elicits the response. This is not the case here, since calcium, zinc, and magnesium were equally efficient in inducing Lcl-dependent *L. pneumophila* autoaggregation. The halophile *Halobacterium salinarum* has also been shown to aggregate in the presence of several different divalent cations (67, 68). Interestingly, both *L. pneumophila* and *H. salinarum* are aquatic organ-isms, and it is possible that the selective pressure for the reliance on several different cations could arise from differences in the environments where their respective biofilms are being produced. Organisms which produce biofilms in aquatic environments may have acquired the ability to use several different cations because of the scarcity of these ions in their natural environment. During mammalian infections, however, microorganisms that produce biofilms are in environments where there is an abundance of different divalent cations. For *L. pneumophila*, although the production of biofilms or aggregates in the human host remains unexplored, the presence of zinc is associated with contamination of water sources with *L. pneumophila* (69). Moreover, zinc potentiates *L. pneumophila* attachment to human alveolar epithelial cells (70). Altogether, the requirement of divalent cations for Lcl-dependent autoaggregation, the reported roles of Lcl in attachment to epithelial cells and GAGs, and the role of cations in *L. pneumophila* adhesion suggest that these correlated Lcl-dependent mechanisms may contribute to both the environmental dissemination of and the host colonization by *L. pneumophila*.

ACKNOWLEDGMENTS

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