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The LonA Protease Regulates Biofilm Formation, Motility, Virulence, and the Type VI Secretion System in *Vibrio cholerae*

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ABSTRACT

The presence of the Lon protease in all three domains of life hints at its biological importance. The prokaryotic Lon protease is responsible not only for degrading abnormal proteins but also for carrying out the proteolytic regulation of specific protein targets. Posttranslational regulation by Lon is known to affect a variety of physiological traits in many bacteria, including biofilm formation, motility, and virulence. Here, we identify the regulatory roles of LonA in the human pathogen *Vibrio cholerae*. We determined that the absence of LonA adversely affects biofilm formation, increases swimming motility, and influences intracellular levels of cyclic diguanylate. Whole-genome expression analysis revealed that the message abundance of genes involved in biofilm formation was decreased but that the message abundances of those involved in virulence and the type VI secretion system were increased in a *lonA* mutant compared to the wild type. We further demonstrated that a *lonA* mutant displays an increase in type VI secretion system activity and is markedly defective in colonization of the infant mouse. These findings suggest that LonA plays a critical role in the environmental survival and virulence of *V. cholerae*.

IMPORTANCE

Bacteria utilize intracellular proteases to degrade damaged proteins and adapt to changing environments. The Lon protease has been shown to be important for environmental adaptation and plays a crucial role in regulating the motility, biofilm formation, and virulence of numerous plant and animal pathogens. We find that LonA of the human pathogen *V. cholerae* is in line with this trend, as the deletion of LonA leads to hypermotility and defects in both biofilm formation and colonization of the infant mouse. In addition, we show that LonA regulates levels of cyclic diguanylate and the type VI secretion system. Our observations add to the known regulatory repertoire of the Lon protease and the current understanding of *V. cholerae* physiology.

Vibrio cholerae is a facultative human pathogen that inhabits aquatic ecosystems around the world. This Gram-negative bacterium survives and persists in aquatic environments by living in aggregates enclosed by a self-produced matrix of exopolysaccharides, proteins, and nucleic acids, known as biofilms (1). It has been shown that growth in the biofilm state confers a range of benefits to *V. cholerae*, including resistances to protozoan predation, bacteriophage infection, and nutrient limitation and increases in the colonizing and virulence capabilities of the bacterium (2–4). The main components of *V. cholerae* biofilms are *Vibrio* polysaccharide (VPS) and the matrix proteins RbmA, RbmC, and Bap1 (5–9). The transcription of the genes that code for these matrix components and their biosynthetic enzymes is primarily controlled by the transcriptional regulatory proteins VpsR, VpsT, and HapR (10–12). VpsR and VpsT bind directly to the upstream regulatory regions of the *vps*-I and *vps*-II operons and directly activate the expression of *vps* genes (13). Quorum sensing (QS) acts to repress biofilm formation in *V. cholerae* at high cell densities by activating the production of HapR, which then directly affects the transcription of *vps* structural genes and *vpsT* (12, 13).

Regulatory circuits that control biofilm formation in *V. cholerae* are influenced by cellular levels of cyclic diguanylate (c-di-GMP) (14). An increase in intracellular c-di-GMP levels has been shown to positively regulate biofilm formation, while motility is decreased (15). The enzymes that synthesize c-di-GMP from two molecules of GTP are known as diguanylate cyclases (DGCs) and contain a GGDEF motif (16). The enzymes that degrade c-di-GMP into either pGpG or two molecules of GMP are known as phosphodiesterases (PDEs) and contain an EAL or HD-GYP motif, respectively (16). Collectively, the activities of these DGCs and PDEs control the amount of c-di-GMP in the cell in response to various environmental signals (16).

V. cholerae is the causative agent of the life-threatening infection known as cholera, which occurs when the bacterium colonizes the small intestine and produces the major virulence factors toxin-coregulated pili (TCP) and cholera toxin (CT), thereby inducing severe diarrhea (17). The regulation of CT and TCP is controlled by multiple regulatory circuits. The transcriptional regulator HapR, a protein whose production is determined by the various quorum-sensing pathways of *V. cholerae*, is one of the critical regulators of virulence (18). Under conditions of low cell

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density, HapR production is inhibited, and its repressive activity on the expression of the transcriptional regulator AphA is relieved. AphA, along with AphB, activates the production of TcpPH, and AphA and AphB then act in concert with ToxRS to activate the transcription of *toxT* (19). The production of CT and TCP is then directly activated by ToxT (20).

The type VI secretion system (T6SS) also contributes to virulence in *V. cholerae* and can provide protection and a competitive advantage in interactions with environmental competitors and predators (21, 22). The T6SS apparatus resembles a tube within a tube, in which the outer tube is a phage-like contractile sheath and the inner tube is made up of multiple units of hemolysin-coregulated protein (Hcp). The outer tube is believed to provide the mechanical force necessary to inject effector proteins into adjacent prokaryotic or eukaryotic cells in response to cell-to-cell contact in order to kill or inhibit the target cell (23, 24). The genes coding for the T6SS apparatus are located in one main cluster (VCA0105 to VCA0124) and two auxiliary clusters (VCA0017 to VCA0022 and VC1415 to VC1421) (21, 25). The genes of the T6SS are negatively regulated by the global regulator TsrA, the quorum-sensing (QS) regulator LuxO, and the osmolarity-responsive transcriptional regulator OscR, and they are positively regulated by HapR, cyclic AMP receptor protein (CRP), response regulator VxrB, and VasH (26–30).

While the transcriptional regulation and posttranscriptional regulation of motility, biofilm formation, and cellular levels of c-di-GMP have been studied intensely, we are just beginning to understand what effects posttranslational proteolytic regulation has on these processes (31–35). Recent studies documented that regulatory proteolysis in *V. cholerae* impacts virulence through modulation of the transcriptional regulators TcpP by YaeL (RseP) and Tsp proteases and ToxR by the RseP protease (36–38). Another protease that might play a significant role in *V. cholerae* physiology that has been linked to motility, biofilm formation, and virulence in a variety of other bacterial species is the Lon protease. This protease is composed of six identical subunits that form a hexameric ring structure characteristic of AAA^+ ATPase domains (39, 40). The LonA protein, encoded by *V. cholerae* VC1920, shares 82% identity with its *Escherichia coli* homolog. In *E. coli*, the Lon protease has been shown to be responsible for ~50% of the damaged protein turnover and the regulation of various processes, such as the SOS response and capsular polysaccharide synthesis (41). In a variety of bacterial species, including Gram-negative and Gram-positive organisms, Lon has been shown to be involved in the regulation of motility and virulence in relation to a range of host organisms and infection models (40).

In this work, we investigated the role that LonA plays in several behaviors central to the lifestyle of *V. cholerae*. We show that LonA contributes to the biofilm formation, motility, and virulence of *V. cholerae*. For the first time, we also show that levels of intracellular c-di-GMP and the expression of the T6SS are influenced by LonA.

MATERIALS AND METHODS

Ethics statement. All animal procedures used were in strict accordance with the *Guide for the Care and Use of Laboratory Animals* (42) and were approved by the University of California (UC), Santa Cruz, Institutional Animal Care and Use Committee, Santa Cruz, CA (approval number Yildf1206).

Strains and growth conditions. The strains used in this study are listed in Table S1 in the supplemental material. *V. cholerae* and *E. coli* strains were grown aerobically in Luria-Bertani (LB) broth (1% tryptone, 0.5% yeast extract, 1% NaCl [pH 7.5]) at 30°C and 37°C, respectively. LB agar contained granulated agar (Difco) at 1.5% (wt/vol). Medium additives were used when necessary at the following concentrations: rifampin, 100 μg/μl; ampicillin, 100 μg/μl; and chloramphenicol, 20 μg/μl for *E. coli* and 5 μg/μl or 2.5 μg/μl for *V. cholerae*.

Strain and plasmid construction. Plasmids were constructed using standard cloning methods or the Gibson Assembly recombinant DNA technique (New England BioLabs, Ipswich, MA). Gene deletions were carried out using allele exchange of the native open reading frame (ORF) with the truncated ORF, as previously described (43). The complementation of *lonA* was carried out using a Tn*7*-based system that inserted the ORF of *lonA* accompanied by 178 bp of upstream genomic sequence into the conserved Tn*7* site at the 3′ end of the *glmS* gene, as previously described (28). Transcriptional fusions were constructed by cloning the upstream regulatory regions of selected genes into the pBBR*lux* plasmid, as previously described (13). The exact lengths of the upstream regulatory region used for the construction of these fusions are listed in Table S1 in the supplemental material.

Biofilm assays. Biofilm formation assays in flow cell chambers were carried out as previously described, with slight modifications (44). To image the biofilms, the flow was stopped, tubing was disconnected, and each chamber was washed three times with 200 μl of phosphate-buffered saline (PBS). The biofilms were then stained in the chambers with a 1.3 μM solution of nucleic acid staining dye Syto9 (Thermo Fisher, Waltham, MA) in 1× PBS for 30 min. After being incubated with the stain, the chambers were washed three times with 200 μl of PBS and promptly imaged using confocal laser-scanning microscopy (LSM) (5-Pa LSM; Zeiss). The resulting image z-stacks were analyzed using Imaris (Bitplane, Concord, MA) and COMSTAT (45). Three independent biological replicates were each imaged three times for every time point. Statistical analysis of the COMSTAT results was carried out using an analysis of variance (ANOVA). Dunnett's multiple-comparison test identified samples that differed significantly from the biofilms formed by the wild-type strain.

Tube biofilms were grown in silicone tubing with an internal diameter of 0.125 in. under constant-flow conditions for the purpose of quantifying c-di-GMP and acquiring luminescence reporter readings from biofilm cells. To inoculate the tubing, overnight cultures of *V. cholerae* wild-type and Δ*lonA* mutant strains were diluted in LB broth to an optical density at 600 nm (OD_{600}) of 0.15, and 10-ml Luer-Lok syringes were used to deliver these dilutions to the entire length of silicone tubing. Cells were then allowed to attach to the surface of the tubing for 1 h before flow was started. A peristaltic pump was used to deliver constant flow at a rate of 4.4 ml per h. Full-strength LB broth was used to ensure sufficient growth. Biofilms were allowed to develop for 24 h at 25°C. To harvest the biofilms, the tubing was detached from flow, drained of excess medium and loosely adhering cells, and scraped from the tubing.

Motility assays. Soft agar motility plates were made using LB medium with 0.3% (wt/vol) agar. The plates were inoculated by briefly stabbing the agar from an overnight colony of the strains to be tested. The plates were then incubated at 30°C for 5 to 6 h until the diameters of the migration zones were measured. To compare the motility of different strains, two-tailed Student's *t* tests were used.

Determination of intracellular c-di-GMP levels. c-di-GMP extraction was performed as described previously (43). The *V. cholerae* wild-type, Δ*lonA* mutant, and Δ*lonA* Tn*7*::*lonA*- complemented strains were grown in LB broth to an OD_{600} of 0.4 before 40 ml was collected for c-di-GMP quantification from planktonic cells. In order to quantify c-di-GMP from the biofilm cells, biofilms were grown in 60-cm sections of tubing, as described above. The biofilms were resuspended in 44 ml of 1× PBS after 24 h of growth. c-di-GMP was quantified from 40 ml of this resuspension. The amount of c-di-GMP in samples was calculated with a standard curve generated from pure c-di-GMP suspended in 184 mM NaCl (BioLog Life Science Institute, Bremen, Germany). The concentrations used for standard curve generation were 50 nM, 100 nM, 500 nM, 2

μM, 3.5 μM, 5 μM, 7.5 μM, and 10 μM. The assay is linear from 50 nM to 10 μM, with an R^2 of 0.999. The c-di-GMP levels were normalized to total protein per milliliter of culture.

To determine protein concentration, 4 ml from each culture or biofilm resuspension was pelleted, the supernatant was removed, and cells were lysed in 1 ml of 2% sodium dodecyl sulfate (SDS). Total protein in the samples was estimated with a bicinchoninic acid (BCA) assay (Thermo Fisher, Waltham, MA) using bovine serum albumin (BSA) as the standard. Each c-di-GMP quantification experiment was performed with four biological replicates. Levels of c-di-GMP were compared between the samples using a two-tailed Student's *t* test.

Gene expression profiling. The microarrays used were composed of spotted 70-mer oligonucleotides representing the open reading frames of the *V. cholerae* strain N16961 genome and were printed at the University of California, Santa Cruz, Santa Cruz, CA (46). A common reference RNA sample was used for all whole-genome expression analyses. Reference RNA was sampled from *V. cholerae* grown overnight at 30°C in LB broth, diluted 1:200 in fresh medium, and harvested at mid-exponential phase at an OD_{600} of 0.3. Experimental RNA samples were prepared by diluting overnight-grown cultures of *V. cholerae* 1:200 in LB and incubated at 30°C until exponential phase (OD_{600}, 0.3). RNA was isolated as previously described (46). To remove contaminating DNA, total RNA was incubated with DNase I (Thermo Fisher, Waltham, MA), and the RNeasy minikit (Qiagen) was used to clean up the RNA after DNase digestion. Microarray hybridization and scanning were performed as described previously (46). Normalized signal ratios were obtained with locally weighted scatterplot smoothing (LOWESS) print-tip normalization, using the Bioconductor packages (47) in the R environment. Differentially regulated genes were determined using three biological replicates and two technical replicates for each treatment (six data points for each spot), using the Significance Analysis of Microarrays (SAM) program (48), with a 1.5-fold difference in gene expression and a 3% false-discovery rate (FDR) as cutoff values.

Luminescence assay. Wild-type *V. cholerae* and the Δ*lonA* mutant harboring transcriptional reporters were grown aerobically overnight in LB broth supplemented with chloramphenicol. For luminescence measurements from planktonic cells, overnight cultures were diluted 1:200 in LB broth plus chloramphenicol and grown at 30°C to an OD_{600} of 0.3 or 1.0. For luminescence measurements from the biofilm cells, biofilms were grown in 30-cm sections of tubing, as described above, using LB broth supplemented with chloramphenicol. Biofilms were grown for 24 h before being resuspended in 15 ml of 1× PBS, and measurements were taken. Luminescence was measured using a Victor3 multilabel counter (PerkinElmer, Waltham, MA) and is reported as counts per minute per milliliter divided by the OD_{600}. The assays were repeated, with at least three biological replicates for all strains tested. Four technical replicates were measured for all assays. Statistical analysis was performed using a two-tailed Student's *t* test.

Analysis of Hcp production and secretion. *V. cholerae* strains were grown to an OD_{600} of 2.0, and the culture (25 ml) was centrifuged at 20,000 × *g* for 15 min to obtain whole-cell pellets. The culture supernatant containing secreted proteins was filtered through 0.22-μm-pore-size membranes (Millipore, Billerica, MA), and secreted proteins in the culture supernatant were precipitated overnight at 4°C with 13% trichloroacetic acid (TCA), pelleted by centrifugation at 47,000 × *g* for 30 min at 4°C, washed with ice-cold acetone, and resuspended in 1× PBS containing Complete protease inhibitor (Roche, Basel, Switzerland). Bovine serum albumin (BSA) (100 μg/ml) was added to the culture supernatant prior to TCA precipitation as a control. Protein pellets from the whole-cell protein were suspended in 2% SDS, and protein concentrations were estimated using a Pierce BCA protein assay kit (Thermo Fisher, Waltham, MA). Equal amounts of total protein (20 μg) or equal volumes of TCA-precipitated supernatant samples were loaded into 12% SDS polyacrylamide gels for SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Western blotting analyses were performed, as described previously (9), using anti-Hcp polyclonal antiserum provided by the S. N. Wai lab (26), anti-RNA polymerase alpha (anti-RNAPα) (BioLegend, San Diego, CA), and anti-BSA (Santa Cruz Biotech, Santa Cruz, CA). For whole-cell Western blotting, Hcp and RNAPα were probed for on separate yet identical blots. For Western blots using supernatant samples, Hcp, RNAPα, and BSA were probed for in succession on the same blot. These experiments were conducted with at least three biological replicates.

Bacterial killing assay. Killing assays were performed as described previously (28). Briefly, bacterial strains were grown overnight on LB plates containing 340 mM NaCl, and single colonies were used to inoculate LB broth containing 340 mM NaCl. Overnight cultures of *V. cholerae* and *E. coli* MC4100 were mixed at a 10:1 ratio, and 25 μl was spotted onto 0.22-μm-pore-size-filter cellulose ester membranes (Merck Millipore, Billerica, MA) lying on LB agar plates containing 340 mM NaCl and incubated at 37°C for 4 h. *E. coli* diluted 1:10 in LB broth was plated and incubated under the same conditions as described above for comparison. All spots were harvested, serially diluted in PBS, and plated onto LB plates containing 50 μg/ml streptomycin to enumerate the surviving *E. coli* prey cells.

Intestinal colonization assay. An *in vivo* competition assay for intestinal colonization was performed as described previously (49). A *V. cholerae lonA* mutant strain (*lacZ*$^+$) and the fully virulent otherwise-wild-type strain (*lacZ* mutant) were grown to stationary phase at 30°C with aeration in LB broth. The Δ*lonA* mutant and wild-type strain were mixed to approach a 1:1 CFU ratio in PBS. The inoculum was plated on LB agar plates containing 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-Gal) to differentiate wild-type and mutant colonies and to determine the input ratio. Approximately 10^5 CFU was intragastrically administered to groups of seven anesthetized 5-day-old CD-1 mice (Charles River Laboratories, Hollister, CA). After 20 h of incubation, the small intestine was removed, weighed, homogenized, and plated on appropriate selective and differential media to enumerate mutant and wild-type cells recovered and to obtain the output ratios. *In vivo* competitive indices were calculated by dividing the small intestine output ratio of the mutant to wild-type strains by the inoculum input ratio of the mutant to wild-type strains. Statistical analyses were performed using the Prism 5 software (GraphPad Software, Inc., San Diego, CA) using a Wilcoxon signed-rank test. *P* values of <0.05 were determined to be statistically significant.

Microarray data accession number. The microarray data have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus (GEO) database (accession no. GSE73023).

RESULTS

LonA contributes to biofilm formation in *V. cholerae*. To assess what effects LonA might have on *V. cholerae* physiology, a strain lacking *lonA* in the wild-type genetic background and a complementation strain harboring a single copy of *lonA* at the target site of the Tn*7* transposon in the Δ*lonA* mutant genetic background were created.

Biofilm formation was analyzed for the Δ*lonA* mutant, *lonA*-complemented, and wild-type strains by growing them in flow cells. Biofilms were grown under flow conditions, stained with Syto9, and imaged using confocal laser-scanning microscopy at 6, 24, and 48 h postinoculation. At 6 h postinoculation, the wild-type and *lonA*-complemented strains had begun to form compact clusters of cells, known as microcolonies, while the Δ*lonA* mutant biofilm was composed of single cells that showed no obvious signs of cell-to-cell adhesion (Fig. 1A). Quantitative analysis of the biofilm images was made possible by the program COMSTAT, which was designed specifically for the quantification of three-dimensional biofilm images (45). COMSTAT allowed us to analyze the biomass, average and maximum thicknesses, substratum coverage, and roughness of each biofilm image stack. Quantitative analysis by COMSTAT revealed that the 6-h Δ*lonA* mutant biofilm

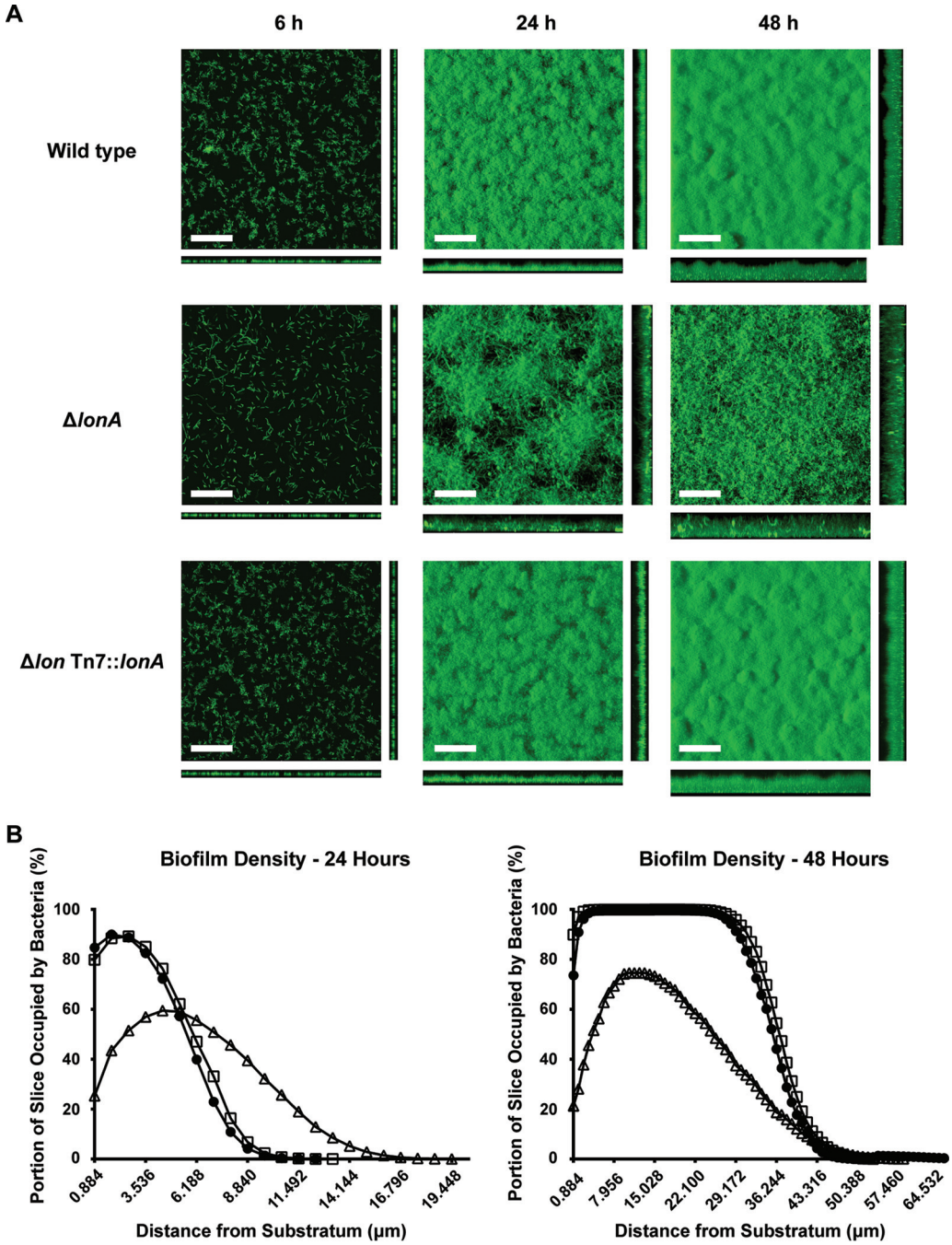

FIG 1 Biofilm formation of the Δ*lonA* and complemented strains. (A) Top-down and orthogonal views of biofilms formed by wild-type, Δ*lonA*, and *lonA*-complemented strains after 6, 24, and 48 h. Scale bars = 40 μm. Biofilms were grown in flow cells and stained with Syto9 prior to being imaged. (B) Biofilm density was determined using COMSTAT analysis of 24- and 48-h biofilms. The results shown are from one representative biofilm of three biological replicates. Circles, wild type; triangles, Δ*lonA* mutant; squares, *lonA* Tn*7*::*lonA* strain.

had significantly less biomass and less average thickness than those of the 6-h wild-type and complemented-strain biofilms (Table 1). Differences in the biofilms formed by the Δ*lonA* mutant became apparent at 24 h. While the wild-type and complemented strains had formed compact mats of cells with uniform pillars and crevices characteristic of a mature *V. cholerae* biofilm, the Δ*lonA* mutant had grown into a porous web-like formation of filamentous cells with large gaps in the biomass, in which the substratum was still visible (Fig. 1A). These Δ*lonA* mutant biofilms did not have a significant decrease in overall biomass compared to that of the wild-type and complemented strains, while the thickness of the biofilms was visibly greater for the Δ*lonA* mutant (Fig. 1A). The COMSTAT analysis corroborated this observation by revealing that the maximum thickness of the biofilm is nearly doubled for the Δ*lonA* mutant compared to that of the wild type, with mean ± standard deviation heights of 20.04 ± 1.40 μm and 10.71 ±

TABLE 1 COMSTAT analysis of biofilms formed after 6, 24, and 48 h by the wild-type, Δ*lon*, and Δ*lon* Tn*7*::*lon* strains[a]

Time postinoculation and strain	Biomass ($\mu m^3/\mu m^2$)	Mean thickness (SD) (μm)		Substrate coverage[b]	Roughness coefficient
		Avg	Maximum		
6 h					
Wild type	0.34 (0.06)	0.21 (0.07)	4.71 (1.10)	0.15 (0.11)	1.71 (0.05)
Δ*lon* mutant	0.16 (0.03)***	0.11 (0.02)**	4.32 (0.70)^{NS}	0.06 (0.01)***	1.87 (0.02)***
Δ*lon* Tn*7*::*lon* strain	0.35 (0.04)^{NS}	0.22 (0.04)^{NS}	5.30 (0.44)^{NS}	0.14 (0.02)^{NS}	1.71 (0.03)^{NS}
24 h					
Wild type	4.08 (1.03)	3.69 (0.88)	10.71 (1.28)	0.69 (0.15)	0.47 (0.14)
Δ*lon* mutant	3.81 (1.55)^{NS}	5.09 (1.43)*	20.04 (1.40)***	0.19 (0.11)***	0.69 (0.20)*
Δ*lon* Tn*7*::*lon* strain	5.25 (0.56)^{NS}	4.67 (0.57)^{NS}	13.47 (1.06)**	0.75 (0.07)^{NS}	0.36 (0.05)^{NS}
48 h					
Wild type	31.81 (3.17)	32.40 (2.43)	52.16 (5.27)	0.70 (0.11)	0.12 (0.02)
Δ*lon* mutant	20.02 (2.54)***	31.94 (2.15)^{NS}	56.77 (2.72)*	0.21 (0.06)***	0.22 (0.03)***
Δ*lon* Tn*7*::*lon* strain	35.15 (1.62)*	34.88 (1.50)*	50.49 (2.98)^{NS}	0.89 (0.09)**	0.10 (0.01)^{NS}

[a] The total biomass, average and maximum thicknesses, substrate coverage, and roughness coefficient were calculated using COMSTAT. The values presented are the means and standard deviations of data from at least nine z-series image stacks. Significance was determined by an ANOVA. Dunnett's multiple-comparison test identified samples whose values differ significantly from those of biofilms formed by the wild-type strain. NS, differences were not significant, *, $P \leq 0.05$; **, $P \leq 0.001$; ***, $P \leq 0.0001$.
[b] A value of 0 indicates no coverage (equivalent to 0%), while a value of 1 indicates full coverage (equivalent to 100%).

1.28 μm, respectively (Table 1). The trends in biomass and maximum thickness revealed by COMSTAT in the 24-h biofilms did not reappear at 48 h. The biomass of the Δ*lonA* mutant biofilms had significantly decreased compared to that of both the wild-type and complemented strains, while the average and maximum heights of all three strains had become roughly equal (Fig. 1 and Table 1). Substratum coverage at all three time points was visually and quantitatively decreased in the Δ*lonA* mutant biofilms compared to that of the wild-type or complemented biofilms (Table 1).

Due to the change in the Δ*lonA* mutant's biofilm thickness and substratum coverage, with no change in biomass at 24 h, we wondered if we could better analyze the compactness of the biofilms. By using COMSTAT to determine how much of each slice of each image z-stack was occupied by bacteria from the substratum to the top of the biofilm, we constructed a graph representing the cellular density of the biofilms (Fig. 1B). This was done for both 24- and 48-h biofilms in order to assess what structural changes might be taking place between the wild-type and Δ*lonA* mutant biofilms between these time points (Fig. 1B). The 24-h-biofilm density graphs made it clear that the Δ*lonA* mutant formed biofilms that had maximum cell density further away from the substratum than the wild-type strain while also growing to an increased height, effectively creating a more porous biofilm with decreased density in respect to biomass (Fig. 1B). The 48-h-biofilm density graph revealed that while the Δ*lonA* mutant biofilms had increased in height and biomass compared to the 24-h biofilms, they were never able to achieve >80% slice coverage at any distance from the substratum. Wild-type biofilms achieved close to 100% slice coverage from 0 to 30 μm from the substratum, revealing a significant difference in biofilm density between the Δ*lonA* mutant and wild type. Overall, these results suggest that the ability of a *V. cholerae* Δ*lonA* mutant to produce a biofilm is significantly altered.

LonA affects motility in *V. cholerae*. After seeing major differences in biofilm formation in the absence of LonA, and considering that this process is inversely regulated with motility in *V. cholerae*, we wanted to know if motility was also affected by this intracellular protease. We measured swimming motility in the Δ*lonA* mutant by performing a motility assay on soft agar plates. When grown for 5.5 h at 30°C, the average migration zone diameter of the Δ*lonA* mutant was 5.2 cm, while both the wild-type and complemented strains had diameters of 3.1 cm (Fig. 2). Therefore, *V. cholerae* motility nearly doubles in the absence of LonA and returns to wild-type levels with the introduction of the *lonA* gene.

Levels of intracellular c-di-GMP are altered in planktonic and biofilm cells of a Δ*lonA* mutant. The hypermotile and abnormal biofilm formation phenotypes of a Δ*lonA* mutant prompted us to evaluate c-di-GMP levels. To determine if levels of c-di-GMP were being affected in the Δ*lonA* strain, we extracted and quantified c-di-GMP from planktonic cells of the wild-type, Δ*lonA* mutant, and *lonA*-complemented strains. The quantification of c-di-GMP revealed that exponentially growing planktonic Δ*lonA* mutant cells had a small but significant decrease in intracellular c-di-GMP levels compared to those of the wild type (Fig. 3A). We also quantified intracellular c-di-GMP in the biofilms of wild-type and Δ*lonA* strains to assess whether LonA affects the characteristic increase in intracellular c-di-GMP found in biofilm cells. Biofilms were allowed to grow in silicone tubing for 24 h under constant flow before being collected for c-di-GMP quantification. As expected, wild-type biofilm cells had more c-di-GMP than their planktonic counterparts (Fig. 3). In contrast to planktonic cells, biofilms of the Δ*lonA* strain had a significant increase in c-di-GMP levels compared to those of the wild type (Fig. 3B). These results suggests that LonA plays a role in regulating levels of c-di-GMP in both planktonic and biofilm cells of *V. cholerae*.

LonA affects the transcriptome of *V. cholerae*. LonA is known to regulate various transcriptional regulators in other bacterial organisms (40). We hypothesized that a similar regulation may exist in *V. cholerae*, in which a negative regulator of biofilm formation, positive regulator of motility, or phosphodiesterase involved in c-di-GMP degradation may be a target protein of LonA in *V. cholerae*. To understand whether LonA affects transcription at the whole-genome level and whether these transcriptional changes may provide clues regarding the targets of LonA protease, we performed whole-genome-level transcriptional profiling experiments and compared the transcriptomes of the planktonic Δ*lonA* mutant and wild-type strains.

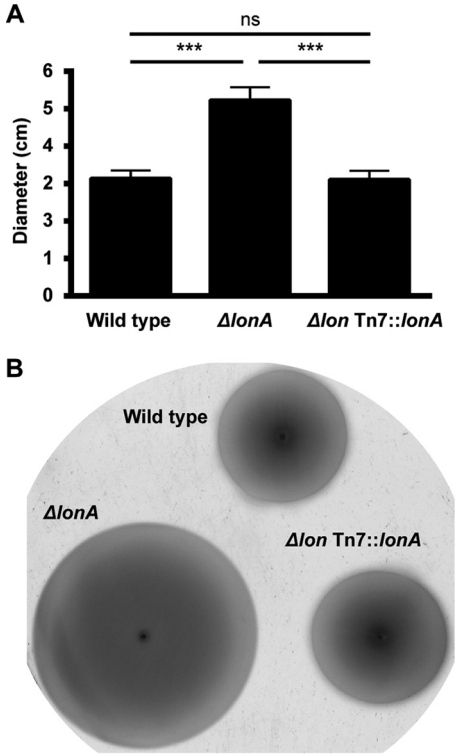

FIG 2 Swimming motility of the Δ*lonA* and complemented strains. (A) The wild-type, Δ*lonA*, and *lonA*-complemented strains were grown on LB agar plates containing 0.3% agar at 30°C for 5.5 h before migration zones were measured. The error bars indicate the standard deviations of the results from 9 biological replicates. ***, $P \leq 0.0001$ by Student's *t* test; ns, differences were not significant. (B) Migration zones of wild-type, Δ*lonA*, and *lonA*-complemented strains after 5.5 h of growth on 0.3% agar plates.

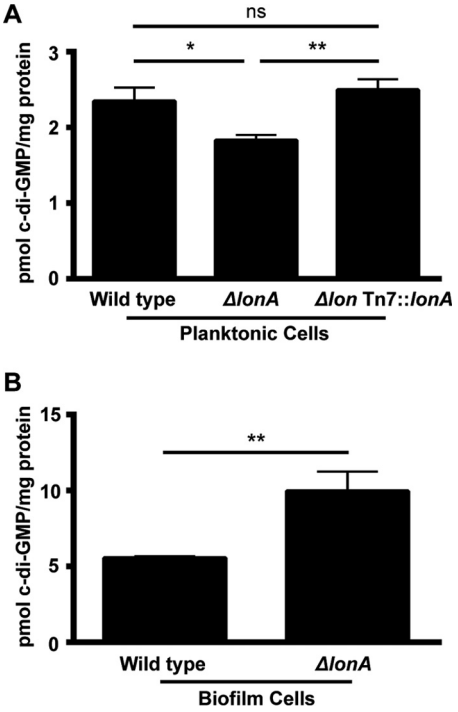

FIG 3 Quantification of intracellular c-di-GMP concentrations. (A) The wild-type, Δ*lonA*, and *lonA*-complemented strains were grown aerobically to an OD_{600} of ~0.4 before c-di-GMP was extracted from whole-cell protein and quantified by high-performance liquid chromatography–tandem mass spectrometry (HPLC-MS/MS). (B) Wild-type and Δ*lonA* biofilms were grown in silicone tubing under constant flow for 24 h before being collected for c-di-GMP extraction and quantification by HPLC-MS/MS. The error bars indicate the standard deviations of the results from 3 biological replicates. *, $P \leq 0.05$; **, $P \leq 0.005$ by Student's *t* test; ns, differences were not significant.

Analysis of the whole-genome expression data revealed that the expression of 45 genes was significantly upregulated, and the expression of 17 genes was significantly downregulated, in the Δ*lonA* mutant compared to the wild-type strain by at least 1.5-fold (Table 2). This finding suggests that the stability of transcriptional regulators may be altered in the Δ*lonA* mutant.

Expression of biofilm matrix genes is altered in planktonic and biofilm cells of a Δ*lonA* mutant. We found that the message abundance of 10 genes that are involved in VPS and biofilm matrix production was lower in the Δ*lonA* mutant than in the wild type. Similarly, levels of VC1185, a gene encoding a GGDEF family protein that may act as a DGC, was decreased in the Δ*lonA* mutant. These findings suggest that the decrease in the expression of *vps* and biofilm matrix genes and VC1185 may be due to the decreased c-di-GMP levels observed in planktonic cells of the Δ*lonA* mutant.

In light of the whole-genome expression results, which suggest that the expression of *vps* and matrix protein genes is downregulated in planktonic cells of the Δ*lonA* mutant, and the abnormal biofilm structure phenotype of the mutant, we hypothesized that the transcriptional regulation of the master biofilm regulators might be impacted by the absence of LonA. To test this hypothesis, we evaluated the expression of *vpsL*, *vpsR*, *vpsT*, and *hapR* using transcriptional fusions to the *lux* operon (*luxCDABE*) carried on a plasmid. Luminescence assays were first conducted in the wild-type and Δ*lonA* mutant strains from exponentially growing planktonic cells. The expression of *vpsL* in the Δ*lonA* mutant was decreased compared to that in the wild type, in agreement with the downregulation of *vps* genes seen in the whole-genome expression results (Fig. 4A and Table 2). Both *vpsR* and *hapR* expression did not change significantly between the two strains, while *vpsT* expression had a small but significant decrease in the Δ*lonA* mutant (Fig. 4A).

Due to the increase in intracellular c-di-GMP levels in the biofilms of the Δ*lonA* strain, we also analyzed the expression of *vpsL*, *vpsR*, *vpsT*, and *hapR* in biofilms of the wild type and the Δ*lonA* mutant. We found that *vpsL* expression was significantly increased in Δ*lonA* biofilms, while *vpsR* and *hapR* expression was significantly decreased compared to that in the wild-type strain (Fig. 4B). The expression of *vpsT* showed no difference between the wild-type and Δ*lonA* strains (Fig. 4B).

These data confirm that *vps* genes are being downregulated in planktonic cells of the Δ*lonA* mutant and reveal that the wild-type patterns of expression for these genes are not fully conserved in a Δ*lonA* mutant between planktonic and biofilm growth states.

Expression of T6SS genes is increased in the Δ*lonA* mutant. The genes whose expression was upregulated in the Δ*lonA* mutant included genes involved in biogenesis and function of the type VI secretion system (Table 2), with the *hcp1* and *hcp2* genes that code for the inner tube proteins of the T6SS being some of the most upregulated genes in the Δ*lonA* mutant compared to the wild type (Table 2). It was of major interest to us that such a large number of

TABLE 2 Genes that are differentially expressed in the Δ*lonA* mutant compared to the wild-type strain[a]

Gene ID by cellular role category	Specific role(s), gene	Fold change (Δ*lon* mutant/WT)
Pathogenesis		
VC0828	Toxin-coregulated pilin, *tcpA*	2.46
VC0830	Toxin-coregulated pilus biosynthesis protein Q, *tcpQ*	1.81
VC0831	Toxin-coregulated pilus biosynthesis outer membrane protein C, *tcpC*	1.68
VC0832	Toxin-coregulated pilus biosynthesis protein R, *tcpR*	1.99
VC0833	Toxin-coregulated pilus biosynthesis protein D, *tcpD*	1.87
VC0834	Toxin-coregulated pilus biosynthesis protein S, *tcpS*	1.84
VC0837	Toxin-coregulated pilus biosynthesis protein F, *tcpF*	1.66
VC0838	TCP pilus virulence regulatory protein, *tcpN* or *toxT*	1.72
VC1456	Cholera enterotoxin B subunit, *ctxB*	2.68
VC1457	Cholera enterotoxin A subunit, *ctxA*	2.25
VCA0219	Hemolysin, *hlyA*	2.38
Secretion		
VC1415	Type VI secretion system, Hcp protein, *hcp-1*	4.80
VCA0017	Type VI secretion system, Hcp protein, *hcp-2*	5.29
VCA0021	Type VI secretion system, T6SS immunity, *tsiV2*	1.52
VCA0108	Type VI secretion system, conserved hypothetical protein, *vipB*	1.53
VCA0109	Type VI secretion system, hypothetical protein	1.84
VCA0112	Type VI secretion system, hypothetical protein, *fha*	1.70
VCA0114	Type VI secretion system, hypothetical protein, *vasE*	1.52
VCA0124	Type VI secretion system, T6SS immunity, *tsiV3*	2.32
VPS and biofilm matrix biosynthesis		
VC0916	Phosphotyrosine protein phosphatase, *vpsU*	0.62
VC0918	UDP-*N*-acetyl-D-mannosaminuronic acid dehydrogenase, *vpsB*	0.50
VC0919	Serine acetyltransferase-related protein, *vpsC*	0.53
VC0926	Exopolysaccharide, hypothetical protein, *vpsJ*	0.54
VC0928	Matrix proteins, hypothetical protein, *rbmA*	0.52
VC0930	Matrix proteins, hemolysin-related protein, *rbmC*	0.52
VC0932	Matrix proteins, hypothetical protein, *rbmE*	0.57
VC0933	Matrix proteins, hypothetical protein, *rbmF*	0.51
VC0935	Exopolysaccharide, hypothetical protein, *vpsM*	0.37
VC1888	Matrix proteins, hemolysin-related protein, *bap1*	0.50
Metabolism		
VC1516	Iron-sulfur cluster-binding protein	0.60
VCA0013	Maltodextrin phosphorylase, *malP*	1.94
VCA0014	4-Alpha-glucanotransferase, *malQ*	4.26
VCA0016	1,4-Alpha-glucan-branching enzyme, *glgB*	2.72
VCA0523	Aminotransferase class II	1.73
Protein fate		
VC0018	16-kDa heat shock protein A, *ibpA*	1.70
VC1674	Periplasmic linker protein, putative	0.52
VC2675	Protease HslVU, subunit HslV, *hslV*	1.52
Transport and binding proteins		
VC1927	C_4-dicarboxylate transport protein, *dctM*	1.91
VC2081	Zinc ABC transporter, periplasmic zinc-binding protein, *znuA*	0.61
VCA0943	Maltose ABC transporter, permease protein, *malG*	2.15
VCA0945	Maltose ABC transporter, periplasmic maltose-binding protein, *malE*	4.23
VCA1028	Maltoporin, *ompS*	4.04
Hypothetical proteins		
VC0102	Hypothetical protein	1.72
VC0191	Conserved hypothetical protein	2.24
VC0353	Conserved hypothetical protein	2.01
VC0469	Conserved hypothetical protein	1.55
VC1151	Conserved hypothetical protein	0.60

(Continued on following page)

TABLE 2 (Continued)

Gene ID by cellular role category	Specific role(s), gene	Fold change (Δ*lon* mutant/WT)
VC1191	Hypothetical protein	1.50
VC1262	Hypothetical protein	3.65
VC1510	Hypothetical protein	0.57
VC2473	Conserved hypothetical protein	1.84
VCA0883	Hypothetical protein	1.81
VCA1065	Conserved hypothetical protein	1.87
Other functions		
VC0175	Deoxycytidylate deaminase-related protein	1.67
VC1185	GGDEF family protein	0.60
VC1413	Methyl-accepting chemotaxis protein	0.56
VC1935	CDP-diacylglycerol–glycerol-3-phosphate 3-phosphatidyltransferase-related protein	1.81
VC2389	Carbamoyl-phosphate synthase, large subunit, *carB*	2.20
VC2390	Carbamoyl -phosphate synthase, small subunit, *carA*	1.91
VC2749	Nitrogen regulation protein NR(I), *ntrC*	1.50
VCA1027	Maltose operon periplasmic protein, putative	1.84
VCA1069	Methyl-accepting chemotaxis protein	2.19

[a] Differentially expressed genes were determined using SAM software with a ≥1.5-fold change in gene expression and an FDR of ≤0.03 as the criteria. ID, identifier; WT, wild type.

T6SS genes was so highly upregulated in the Δ*lonA* mutant, as LonA has never been shown to regulate this system in other organisms (Table 2). To further confirm that T6SS genes were upregulated in the Δ*lonA* mutant, we analyzed *hcp* expression by using a transcriptional fusion of the *hcp2* gene to the *lux* operon. The luminescence output from P_{hcp2}-*lux* was found to be significantly higher in the Δ*lonA* mutant than in the wild type during the exponential and stationary phases of growth (Fig. 5A). This result confirmed that the transcription of genes involved in the T6SS was induced to a higher level in the Δ*lonA* mutant, but whether or not the system was more active was not addressed.

To determine if more Hcp was actually being produced by the Δ*lonA* mutant, we analyzed Hcp production by Western blotting using whole-cell and supernatant protein samples. We found that a greater amount of Hcp was present in both the whole-cell and supernatant samples of the Δ*lonA* mutant than in the wild type, while complementation of the Δ*lonA* mutant restored Hcp to wild-type levels (Fig. 5B). A Δ*hcp1* Δ*hcp2* mutant was included as a negative control, as it is unable to produce Hcp (28). As expected, no Hcp was detectable in the samples collected from this mutant.

In order to determine if the activity of the T6SS system was affected in the Δ*lonA* mutant, we carried out an interbacterial killing assay in which *E. coli* was used as prey for T6SS-mediated killing by *V. cholerae*. We found that the level of surviving *E. coli* strain MC4100 was modestly but significantly lower in the Δ*lonA* mutant than that in the wild type (Fig. 5C). This finding suggests that LonA affects the transcription, production, and activity of the T6SS.

Expression of virulence genes is increased in the Δ*lonA* mutant, and LonA plays a role in colonization of the infant mouse. The message abundance of virulence regulators *toxT* and *ctxAB* and of most of the genes responsible for TCP production was also significantly higher in the Δ*lonA* mutant (Table 2). Due to a multitude of virulence-related genes being upregulated in the Δ*lonA* mutant and LonA having been shown to play a role in the virulence of many bacterial species, we wanted to determine if LonA plays a role in the virulence of *V. cholerae* (Table 2). We first chose *ctxA* and *tcpA*, genes essential for the production of CT and TCP, respectively, as representatives of the virulence genes that were upregulated in the expression profiling study and analyzed their expression using *lux* transcriptional fusions. Both *ctxA* and *tcpA*, in agreement with the expression profiling data, were significantly upregulated in the Δ*lonA* mutant compared to the wild type during both exponential- and stationary-phase growth (Fig. 6A).

The overexpression of virulence genes in the Δ*lonA* mutant suggested that it may have the ability to outcompete the wild type in an infection setting. To test this hypothesis, we assessed the ability of the Δ*lonA* mutant to colonize the infant mouse intestine by competing it with the wild-type strain. The Δ*lonA* mutant was found to be severely defective in colonizing the infant mouse, with a competitive index of 0.08 (Fig. 6B). To ensure that any defect in colonization observed by analysis of the Δ*lonA* mutant was dependent on the environment of the host's digestive tract, we competed the strains *in vitro* with the same strain mixture used for the infection studies. The output ratios of the *in vitro* competitions were found to be the same as the input ratios (data not shown). This result suggests that LonA controls the processes involved in the virulence of *V. cholerae*.

DISCUSSION

The body of research addressing the roles of the enigmatic Lon protease has continued to develop since it was first described as a regulator of capsular polysaccharide production in *E. coli* in 1963 (50). Our understanding of LonA's regulatory repertoire has since grown to include a wide array of cellular processes in bacteria, including cell division, the SOS response, quorum sensing, motility, biofilm formation, and virulence (51–61). The observations reported here serve not only to characterize the roles of LonA in *V. cholerae* but also to expand the available knowledge of the regulatory roles of LonA in bacteria. We have determined that a Δ*lonA* mutant of *V. cholerae* displays alterations in cell morphology, biofilm, motility, and virulence phenotypes similar to and different from those of Δ*lonA* mutants in other bacterial species. Additionally, we show for the first time that this protease is involved in the regulation of global c-di-GMP pools and the T6SS.

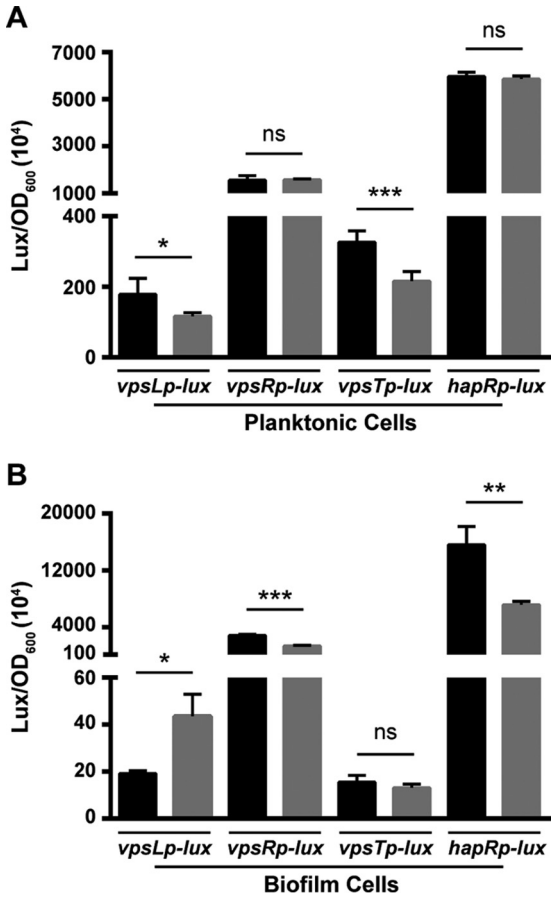

FIG 4 Analysis of biofilm gene expression in the wild-type and Δ*lonA* strains. Transcriptional reporters harboring regulatory regions of *vpsL*, *vpsR*, *vpsT*, and *hapR* upstream of a promoterless *lux* reporter were used to analyze the expression of key biofilm genes. (A) Cultures of the wild-type and Δ*lonA* strains containing P_{vpsL}-*lux*, P_{vpsR}-*lux*, P_{vpsT}-*lux*, or P_{hapR}-*lux* were grown to exponential phase (OD_{600}, ~0.3), and luminescence was measured. (B) Biofilms of the wild-type and Δ*lonA* strains containing P_{vpsL}-*lux*, P_{vpsR}-*lux*, P_{vpsT}-*lux*, or P_{hapR}-*lux* were grown for 24 h before luminescence was measured. The black bars represent the wild type, and the gray bars represent the Δ*lonA* mutant. The graphs present the average numbers of relative light units (RLU) and standard deviations obtained from four technical replicates of two independent biological samples. *, $P \leq 0.01$; **, $P \leq 0.001$; ***, $P \leq 0.0001$ by Student's *t* test; ns, differences were not significant.

While using a transposon mutant library of *Pseudomonas aeruginosa* to find mutants defective for biofilm formation with and without the presence of a sub-MIC of gentamicin, Marr et al. found that insertions in the *lonA* gene drastically reduced the ability of the organism to form a biofilm (56). Our assessment of the biofilm-forming capacities of a *V. cholerae* Δ*lonA* mutant under constant-flow conditions revealed that the mutant formed biofilms with abnormal characteristics compared to those of the wild-type strain, accompanied by a significant defect in accumulated biomass after 48 h of growth. The decrease in biofilm density exhibited by the Δ*lonA* mutant suggests that adhesion between cells in the biofilm has been compromised. The elongated cell morphology apparent within the Δ*lonA* mutant biofilm may be a contributing factor to the porous biofilm phenotype, although the specific characteristics of the biofilm that would be affected by this filamentous cell type are unclear. We also observed that levels of c-di-GMP and *vpsL* expression were decreased in planktonic cells and increased in biofilm cells of the Δ*lonA* mutant. Higher *vpsL* expression would suggest that the Δ*lonA* mutant should be able to produce more VPS and thus exhibit an enhanced ability to form biofilms. However, the biofilms produced by the Δ*lonA* mutant were structurally quite different than those of the wild type, leading us to believe that both the structure of biofilms and the c-di-GMP-mediated regulation of biofilm formation are compromised in the absence of LonA.

Due to the presence of significant alterations in biofilm phenotype, cellular c-di-GMP levels, and message abundance of biofilm matrix genes in the Δ*lonA* mutant, we reasoned that one or more of the three master regulators of biofilm formation, VpsR, VpsT, and HapR, may be impacted. We found that in planktonic cells, the expression of biofilm genes and *vpsT* was significantly decreased in the Δ*lonA* mutant, while the expression of *vpsR* and *hapR* was not altered. It is possible that the modest decrease in global c-di-GMP levels in planktonic Δ*lonA* mutant cells is the sole factor in the downregulation of these biofilm genes, as the level of intracellular c-di-GMP is directly linked to the induction of these genes through direct binding to VpsT, whose activity is controlled by cellular levels of c-di-GMP (62). It remains to be determined if VpsT or a PDE is a target of LonA. We determined that in biofilms of the Δ*lonA* mutant, the expression of biofilm matrix genes was increased, *vpsR* and *hapR* expression was decreased, and *vpsT* expression was not altered compared to that in the wild type. We previously showed that wild-type *V. cholerae* responds to an increase in c-di-GMP levels with an increase in *vpsL*, *vpsT*, and *vpsR* expression (46). While the Δ*lonA* mutant is still able to increase *vpsL* expression when there is an increase in c-di-GMP, the lack of an increase in *vpsR* and *vpsT* expression further supports that c-di-GMP-mediated regulation of biofilm formation is altered significantly in the absence of LonA. It is unclear how *vpsL* expression is being increased in the Δ*lonA* mutant, but it is possible that the decrease in *hapR* expression, a negative regulator of *vpsL*, allows the observed increase in *vpsL* expression (12). Taken together, these expression data reveal that LonA is required for the full complement of transcriptional regulation that is characteristic of the switch from planktonic to biofilm growth states in *V. cholerae*. Future studies are planned to determine if VpsR, VpsT, or other regulators of *V. cholerae* biofilm formation are direct targets of LonA.

To date, the effects of the Lon protease on biofilm formation in bacteria have been studied only in *P. aeruginosa* and *V. cholerae* (this work and reference 56). In these two organisms, the contribution of LonA differs, in that *P. aeruginosa* Δ*lonA* mutants exhibit a severe defect in biofilm formation, while *V. cholerae* Δ*lonA* mutants still produce biofilms but with compromised structure (56). The exact mechanism by which *lonA* regulates these processes remains to be determined.

The regulation of motility by the Lon protease has been well studied. In *Proteus mirabilis*, *Vibrio parahaemolyticus*, and *P. aeruginosa*, the Lon protease has been found to affect swarming motility (54–56). Additionally, in *P. aeruginosa*, LonA has been shown to affect swimming and twitching motility. While the mechanisms for these effects are not clear, it is interesting to note that in the absence of the Lon protease, all motility behaviors are inhibited (56, 59). In contrast, swarming motility behaviors of *Bacillus subtilis* and *P. mirabilis* are upregulated in the absence of LonA. A recent study by Mukherjee and colleagues (63) described

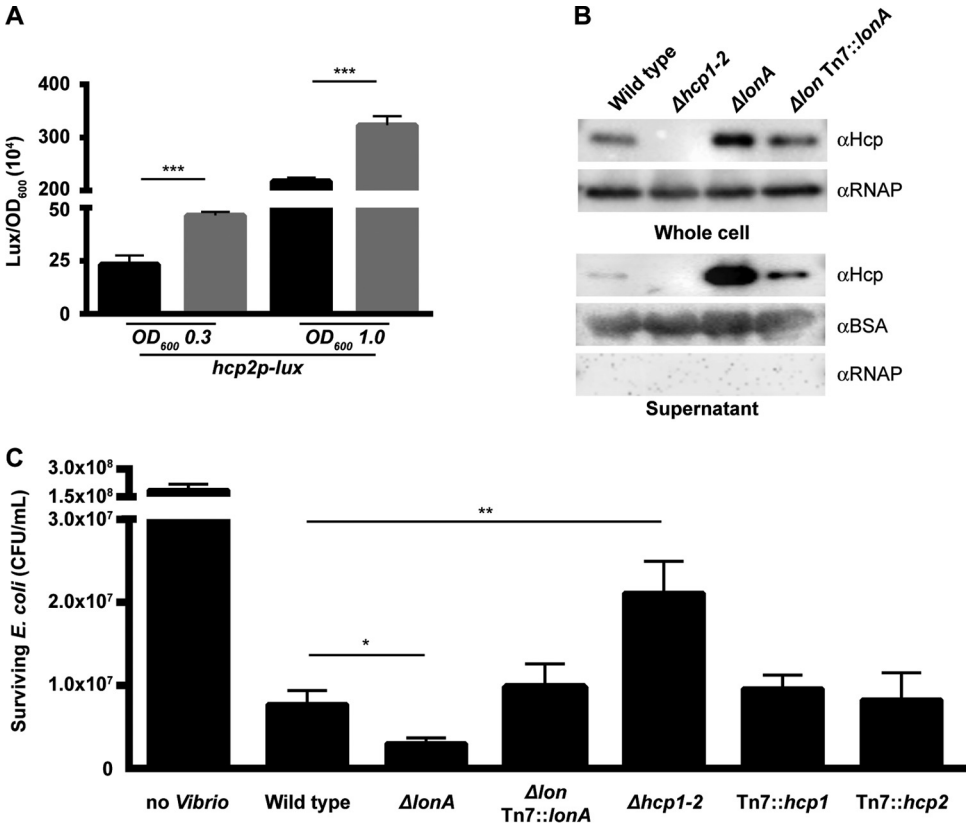

FIG 5 Analysis of T6SS expression and activity. (A) The transcriptional reporter harboring the regulatory region of *hcp2* upstream of a promoterless *lux* reporter was used to represent expression of the T6SS. Cultures of the wild-type and Δ*lonA* strains containing P_{hcp2}-*lux* were grown aerobically, and luminescence was measured at exponential phase (OD_{600}, ~0.3) and early stationary phase (OD_{600}, ~1.0). The graph presents the average numbers of RLU and standard deviations obtained from four technical replicates of two independent biological samples. *, $P \leq 0.01$; ***, $P \leq 0.0001$ by Student's *t* test. (B) Hcp production and secretion were analyzed in whole-cell and culture supernatants of the wild-type, Δ*lonA*, and *lonA*-complemented strains by immunoblotting. Equal amounts of whole-cell protein (determined by BCA assay) were loaded onto a 12% SDS-polyacrylamide gel to analyze production. Prior to TCA precipitation and total protein quantification, 100 μg/ml BSA was added to the supernatant. Equal volumes of TCA-precipitated protein were loaded onto a 12% SDS-polyacrylamide gel to analyze secretion. The blots were probed for Hcp, while duplicate blots were probed for RNAPα and BSA. The data shown are representative of the results from three independent experiments. (C) Interbacterial killing was analyzed by mixing *V. cholerae* strains and prey *E. coli* strain MC4100 in a 10:1 ratio, incubating them on LB agar plates containing 340 mM NaCl for 4 h at 30°C, and quantifying the surviving *E. coli* MC4100 cells. The data represent the averages and standard deviations of the results from three biological replicates. *, $P \leq 0.05$; **, $P \leq 0.005$ by Student's *t* test.

a system present in *B. subtilis* in which LonA inhibits swarming motility by degrading SwrA, the master activator of swarming motility in that species, an event requiring the novel substrate-specific adaptor protein SmiA. In the case of *P. mirabilis*, it was found that cells deficient in LonA had a hyperswarming phenotype due to increased stabilization of the swarming activators FlhDC (55). It is not entirely clear what LonA might be targeting in *V. cholerae* to alter motility. The absence of LonA may lead to the stabilization of an activator of the flagellar regulatory cascade in a fashion similar to that of FlhDC stabilization, but we found no evidence of differential regulation of the flagellar regulatory cascade between the wild-type and Δ*lonA* mutant strain. It may be that LonA affects motility in *V. cholerae* in an entirely transcriptionally independent manner. As motility is negatively regulated by c-di-GMP, the decrease in c-di-GMP levels in planktonic Δ*lonA* cells may be responsible for the increased motility phenotype. If this is the case, LonA may be targeting the enzymes or regulators of the enzymes that synthesize and degrade c-di-GMP. A number of these enzymes, known as diguanylate cyclases (DGCs) and phosphodiesterases (PDEs), have been shown to affect the motility of *V. cholerae* in a c-di-GMP-dependent manner (43).

To our knowledge, all Δ*lonA* mutants assessed for virulence capabilities in standard *in vivo* models have exhibited a decreased ability to cause infection (57, 58, 60, 61). The upregulation of key virulence genes in a *V. cholerae* Δ*lonA* strain that we observed suggested that this organism may exhibit enhanced virulence compared to that of the wild type; yet, when we tested the ability of the Δ*lonA* strain to compete with the wild type in the infant mouse infection model, we found that it had a severe defect in colonization. It may be that the altered virulence gene expression of the Δ*lonA* mutant is detrimental when it attempts to survive and colonize the infant mouse small intestine, as it has been shown that genes in the small intestine, such as *tcpA* and *ctxAB*, are temporally and spatially regulated during infection (64). Alternatively, it may be that the *in vivo* fitness of the Δ*lonA* mutant is compromised and that it lacks the ability to survive host defenses encountered in the infant mouse digestive tract, such as antimicrobial peptides or reactive nitrogen species (65). The inability of the Δ*lonA* mutant to form a normal biofilm may also affect its ability to survive in the

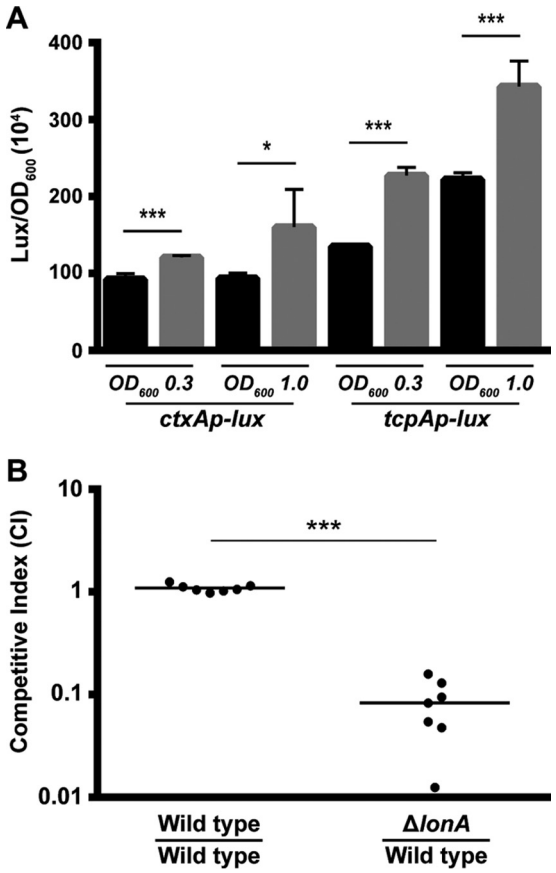

FIG 6 Analysis of virulence gene expression and intestinal colonization of the Δ*lonA* mutant. (A) Transcriptional reporters harboring regulatory regions of *ctxA* and *tcpA* upstream of a promoterless *lux* reporter were used to represent the expression of key virulence genes. Cultures of the wild-type and Δ*lonA* strains containing P_{ctxA}-*lux* or P_{tcpA}-*lux* were grown aerobically to exponential phase (OD_{600}, ~0.3) and early stationary phase (OD_{600}, ~1.0), and luminescence was measured. The graph presents the average numbers of RLU and standard deviations obtained from four technical replicates of two independent biological samples. *, $P \leq 0.01$; ***, $P \leq 0.0001$ by Student's *t* test. (B) The wild-type strain was coinoculated with a Δ*lonA* mutant at a ratio of ~1:1 into infant mice. The number of bacteria per intestine was determined 20 to 22 h postinoculation. The competitive index (CI) was determined as the output ratio of mutant to wild-type cells divided by the input ratio of mutant to wild-type cells. Each point represents results from an individual mouse. Statistical analysis was carried out using the Wilcoxon signed-rank test, comparing the CI of each strain to the CI of the wild-type *lacZ*$^+$ strain divided by the CI of the wild-type *lacZ*-negative strain.

infant mouse small intestine, leading to an inability to compete with the wild type. Tsou et al. (66) showed that the regulation of motility behaviors plays a significant role in the regulation of virulence gene expression during a *V. cholerae* infection. Therefore, the hypermotile phenotype of the Δ*lonA* strain may impede this coordinated regulation, leading to a decrease in colonization ability.

There is a precedent for the degradation of virulence regulators by LonA in other species, including the virulence regulators RovA of *Yersinia* and HilC and HilD of *Salmonella* (67, 68). Possible candidates for targets of LonA that might upregulate virulence gene expression in *V. cholerae* when stabilized include AphA, ToxRS, TcpPH, and ToxT. We did not find there to be a statistically significant difference in the amount of HapR produced by the Δ*lonA* mutant compared to that produced by the wild type (data not shown), so it is unlikely that LonA regulates the virulence genes by targeting a quorum-sensing pathway. Exactly how LonA regulates the CT and TCP pathways in *V. cholerae* remains to be determined.

Until now, the Lon protease has never been shown to regulate the T6SS. LonA of *V. cholerae* appears to negatively regulate the transcription of all three gene clusters of the system, the production and secretion of Hcp, and the activity of the system against *E. coli* prey. We speculate that LonA regulates one of the transcriptional regulators of the T6SS, such as TsrA, VasH, VxrB, or CRP. Hcp was named hemolysin-coregulated protein before the characterization of the T6SS, because it was found to be coregulated with the virulence factor HlyA in an HlyU-dependent manner (69). Because *hlyA* and both *hcp1* and *hcp2* were upregulated in the Δ*lonA* mutant, the winged helix-turn-helix (wHTH) transcriptional regulator HlyU is another intriguing candidate for degradation by LonA (70). While we did not investigate any links between the aberrant biofilm phenotype and the upregulation of T6SS expression and activity in this study, it is interesting that LonA appears to play a regulatory role in both of these systems. Schwarz et al. (71) showed that the T6SS is required for *Burkholderia thailandensis* to proliferate in a biofilm state when in the presence of *Pseudomonas putida*, and one study done with *V. cholerae* showed that T6SS expression and activity are upregulated when *V. cholerae* is allowed to form a biofilm on chitinous surfaces (71, 72). While these studies do not imply that there is a direct regulatory connection between the T6SS and biofilm formation, they certainly suggest that the two processes are contextually related. Taken together with the LonA-dependent phenotypes we report here, we speculate that there may be some degree of coregulation of biofilm formation and the T6SS in *V. cholerae*.

Although there is precedence for the direct degradation of transcriptional regulators by the Lon protease, it has yet to be determined whether all of the phenotypes of a Δ*lonA* mutant of *V. cholerae* are due to directed proteolysis by LonA. The Lon protease is known to be important for maintaining proper protein homeostasis in *E. coli*, in which it degrades ~50% of all damaged proteins (41). Therefore, it is possible that LonA of *V. cholerae* plays a similar role, and in the absence of this protein quality control, cells become sick due to proteotoxic stress. If Δ*lonA* mutant cells are highly stressed by the absence of LonA, this might lead to some of the phenotypes we observed, such as compromised biofilm structure and an inability to colonize infant mice. In order to resolve the uncertainty associated with the origins of the phenotypes we have described in a Δ*lonA* mutant, we plan to carry out further studies, with the hope of identifying regulatory proteins that are degraded directly by LonA.

It is clear that LonA plays an important role in the life cycle of *V. cholerae*. Although we were not able to reveal any of the specific mechanisms by which LonA regulates biofilm formation, motility, virulence, or the T6SS in *V. cholerae*, we believe that this work is the first step in understanding the seemingly important role that this intracellular ATP-dependent protease plays in the physiology of this human pathogen.

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