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Overexpression of VpsS, a Hybrid Sensor Kinase, Enhances Biofilm Formation in *Vibrio cholerae*▽†

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Vibrio cholerae **causes the disease cholera and inhabits aquatic environments. One key factor in the environmental survival of *V. cholerae* is its ability to form matrix-enclosed, surface-associated microbial communities known as biofilms. Mature biofilms rely on *Vibrio* polysaccharide to connect cells to each other and to a surface. We previously described a core regulatory network, which consists of two positive transcriptional regulators, VpsR and VpsT, and a negative transcriptional regulator HapR, that controls biofilm formation by regulating the expression of *vps* genes. In this study, we report the identification of a sensor histidine kinase, VpsS, which can control biofilm formation and activates the expression of *vps* genes. VpsS required the response regulator VpsR to activate *vps* expression. VpsS is a hybrid sensor histidine kinase that is predicted to contain both histidine kinase and response regulator domains, but it lacks a histidine phosphotransferase (HPT) domain. We determined that VpsS acts through the HPT protein LuxU, which is involved in a quorum-sensing signal transduction network in *V. cholerae*. In vitro analysis of phosphotransfer relationships revealed that LuxU can specifically reverse phosphotransfer to CqsS, LuxQ, and VpsS. Furthermore, mutational and phenotypic analyses revealed that VpsS requires the response regulator LuxO to activate *vps* expression, and LuxO positively regulates the transcription of *vpsR* and *vpsT*. The induction of *vps* expression via VpsS was also shown to occur independent of HapR. Thus, VpsS utilizes components of the quorum-sensing pathway to modulate biofilm formation in *V. cholerae*.**

Vibrio cholerae, the causative agent of the diarrheal disease cholera, is a natural inhabitant of aquatic environments (14). The environmental survival and transmission of *V. cholerae* are facilitated by its ability to form biofilms (1, 15), which are surface-associated microbial communities composed of microorganisms and the extrapolymeric substances that they produce (10). The ability of *V. cholerae* to form biofilms depends on the production of the major extracellular matrix component *Vibrio* polysaccharide (VPS). VPS is produced by proteins encoded by the *vps* genes, which are organized into the *vps*-I (*vpsU* [VC0916], *vpsA* to *vpsK* [VC0917 to VC0927]) and *vps*-II (*vpsL* to *vpsQ* [VC0934 to VC0939]) coding regions (41).

The regulation of *vps* expression and biofilm formation in *V. cholerae* involves multiple two-component signal transduction proteins. A typical two-component system consists of a sensor histidine kinase (HK) and a response regulator (RR) (for a review, see reference 37). A stimulus received by the HK initiates a phosphorelay event. The HK autophosphorylates at a conserved histidine residue, and the phosphoryl group is then transferred to a conserved aspartate residue on the RR. Phosphorylation of the RR leads to activation and altered protein function or regulation of gene expression. More complex multicomponent systems can consist of hybrid HK proteins containing both HK and RR domains, proteins harboring histidine phosphotransferase (HPT) domains, and RRs. In these cases, the received stimulus results in autophosphorylation of a conserved histidine residue on the HK domain of the hybrid kinase, which in turn transfers the phosphoryl group intramolecularly to the conserved aspartate residue on the RR domain. The HPT then acts as an intermediate receiver and donor of the phosphoryl group for the RR domain of the hybrid kinase and another RR (24, 37). HPT domains can occur on the same protein with HK and RR domains or on separate proteins.

In *V. cholerae*, a quorum-sensing signal transduction system negatively regulates biofilm formation (17). In quorum sensing, bacteria produce signaling molecules termed autoinducers (AIs) that are secreted and accumulate in the medium in proportion to the population density. *V. cholerae* produces two AIs, known as cholerae autoinducer-1 (CAI-1) and autoinducer-2 (AI-2), that are detected via the hybrid HKs CqsS and LuxQ, respectively (17, 32). At a low cell density when the AI concentration is low, CqsS and LuxQ act as kinases and are autophosphorylated. Information from the sensors is then transduced through a phosphorelay, first to the HPT protein LuxU and then to the σ^{54}-dependent RR LuxO (32, 43). Phosphorylated LuxO then activates the transcription of four genes encoding the Qrr regulatory small RNAs (sRNAs), which destabilize the mRNA encoding the quorum-sensing master regulator, HapR (27). When the cell density is high, the AI concentration is high; LuxQ and CqsS bind their respective AIs and act as phosphatases. Under such conditions, LuxU and LuxO are unphosphorylated and HapR is produced at high levels, leading to a de-

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crease in *vps* expression. A mutation in HapR leads to increased production of VPS, indicating that one of the normal roles of HapR is to suppress biofilm formation (17, 40, 42).

VpsR, a positive regulator of *vps* expression, exhibits homology to the NtrC subclass of two-component response regulators (39). Disruption of *vpsR* prevents expression of the *vps* genes and production of VPS and abolishes formation of the typical three-dimensional biofilm structure, demonstrating that VpsR is required for biofilm formation. In a typical two-component system, VpsR is phosphorylated by an HK. Indeed, VpsR contains the conserved aspartate residue (D59) that is predicted to be necessary for phosphorylation. Conversion of this aspartate to alanine renders VpsR inactive, while conversion to glutamate generates a constitutively active VpsR, supporting the premise that phosphorylation controls the activity of VpsR (25). A second positive regulator of *vps* expression is VpsT, which is similar to proteins belonging to the UhpA (FixJ) family of transcriptional regulators (8). Disruption of *vpsT* reduces *vps* gene expression and reduces the capacity to form biofilms. While VpsR is essential for VPS production and biofilm formation, VpsT plays an accessory role, possibly by increasing the level or activity of VpsR (3, 8).

To better understand the regulatory network controlling biofilm formation, we attempted to identify the cognate HK for VpsR and searched for HKs regulating *vps* gene expression. In this study, we report the identification of a hybrid HK, VpsS, which positively regulates *vps* gene expression. VpsS requires the presence of VpsR and the quorum-sensing signal transduction proteins LuxU and LuxO to induce *vps* expression.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The bacterial strains and plasmids used in this study are listed in Table 1. All *V. cholerae* and *Escherichia coli* strains were grown aerobically at 30°C and 37°C, respectively, unless otherwise noted. The growth medium was Luria-Bertani (LB) medium (1% tryptone, 0.5% yeast extract, 1% NaCl; pH 7.5). LB agar contained 1.5% (wt/vol) granulated agar (Difco). The concentrations of antibiotics used, where appropriate, were as follows: ampicillin, 100 μg/ml; rifampin, 100 μg/ml; kanamycin, 50 μg/ml; and gentamicin, 30 μg/ml.

Generation of in-frame deletion mutants and *lacZ* reporter and *gfp*-tagged strains. Deletion mutants, reporter strains carrying promoter regions of either *vpsL*, *vpsT*, or *vpsR* fused to *lacZ* (*vpsLp-lacZ*, *vpsTp-lacZ*, and *vpsRp-lacZ*) inserted into the *lacZ* locus on the chromosome, and *gfp*-tagged strains were generated with the *V. cholerae* strains using the protocols described previously (16, 28, 35). *vpsR*(*D59A*) and *vpsR*(*D59E*) point mutations were introduced by amplifying coding regions of *vpsR* with primers *vpsR*_5′, *vpsR*_3′, *vpsR*_D59A_F, *vpsR*_D59A_R, *vpsR*_D59E_F, and *vpsR*_D59E_R and joining them together using splicing by overlap extension (26). To introduce the mutated version of *vpsR* into the chromosome, 5′ and 3′ regions of *vpsR* were amplified with *vpsR*_5′_F, *vpsR*_5′_R, *vpsR*_3′_F, and *vpsR*_3′_R, ligated to the splicing-by-overlap-extension product, and cloned into the suicide vector pGP704-*sacB*28. Primers used in this study are listed in Table S1 in the supplemental material. Deletion mutants, reporter strains, and *gfp*-tagged strains were verified by PCR.

Recombinant DNA techniques. DNA manipulations were carried out using standard molecular techniques (33). Restriction and DNA modification enzymes were purchased from New England Biolabs. PCRs were carried out using primers purchased from Operon Technologies (Alameda, CA) (see Table S1 in the supplemental material) and a high-fidelity PCR kit (Roche). Construction of *vpsR* site-directed mutagenesis plasmids harboring point mutations was carried out using a previously described protocol (5). Overexpression plasmids were generated using the pBAD/*myc*-His B vector (Invitrogen). Sequences of the plasmids that were constructed were verified by DNA sequencing.

Flow cell experiments and CLSM. Flow cell experiments were carried out using a procedure described previously (20, 28). Briefly, chambers were sterilized with 0.5% (vol/vol) hypochlorite overnight, and this was followed by addition of sterile MilliQ water and 2% LB medium (0.2 g/liter tryptone, 0.1 g/liter yeast extract, 1% NaCl) at a flow rate of 4.5 ml/h. Overnight cultures of *gfp*-tagged *V. cholerae* strains were diluted to obtain an optical density at 600 nm (OD_{600}) of 0.1, and 350-μl aliquots of the diluted cultures were inoculated by injection into the flow cell chambers. After inoculation, the chambers were allowed to stand inverted, with no flow, for 1 h. The flow was resumed at a rate of 4.5 ml/h with the chambers standing upright. Flow cell experiments were carried out at room temperature with 2% LB medium containing ampicillin (100 μg/ml) and arabinose (0.2%, wt/vol), when they were needed. Confocal laser scanning microscopy (CLSM) images of the biofilms were captured with an LSM 5 PASCAL system (Zeiss) using an excitation wavelength of 488 nm and an emission wavelength of 543 nm. Three-dimensional images of the biofilms were reconstructed using Imaris software (Bitplane) and quantified using COMSTAT (21). Flow cell experiments were carried out with at least two biological replicates.

β-Galactosidase assays. β-Galactosidase assays were performed using exponentially grown cultures by first growing *V. cholerae* overnight (18 to 20 h) aerobically in LB medium. The cells were then diluted 1:200 in fresh LB medium and grown aerobically to an OD_{600} of 0.3 to 0.4. Cells were then diluted again 1:200 in LB medium, allowed to grow to an OD_{600} of 0.3 to 0.4, and immediately harvested for assays. β-Galactosidase assays with cells overexpressing *vpsS* were carried out with overnight cultures grown in the absence of arabinose that were diluted 1:200 or 1:500 in fresh media with and without the inducer 0.2% arabinose. Assays were then performed 3 and 24 h after induction. The β-galactosidase assays were carried out in MultiScreen 96-well microtiter plates fitted onto a MultiScreen filtration system (Millipore) using a previously described procedure (16) which is similar to the procedure described by Miller (31). The assays were repeated with four biological replicates and eight technical replicates.

Biofilm formation assays. Biofilm formation assays in 96-well microtiter plates (polyvinyl chloride) were carried out with 100-μl portions of overnight cultures diluted to an OD_{600} of 0.2. The microtiter plates were incubated at 30°C for 8 h. Crystal violet staining and ethanol solubilization were carried out as previously described (16, 41). The assays were repeated with two different biological replicates and eight technical replicates.

Protease assays. *V. cholerae* cells harboring a *vpsS* overexpression plasmid were cultured by first growing cells in the absence of arabinose overnight and then diluting them 1:200 in fresh LB media with and without the inducer 0.2% arabinose. Protease assays were performed 24 h after induction. Protease activity was assayed using an EnzChek protease assay kit (Invitrogen) by following the manufacturer's protocol. Briefly, culture supernatants were collected by centrifugation and diluted 1:100 in LB media. Each diluted supernatant was then further diluted 1:10 in 1× digestion buffer, and 100-μl aliquots were mixed with 100 μl of a BODIPY FL casein solution. The reaction mixtures were incubated for 1 h at 30°C, and fluorescence was measured with a Victor3 microplate reader (PerkinElmer) and normalized to cell density. The assays were performed with three technical replicates per strain and repeated with three biological replicates.

In vitro analysis of phosphotransfer relationships. Full-length coding regions for VpsS, VpsR, LuxU, and LuxO were cloned into the Invitrogen Gateway ENTRY vector pENTR/D-TOPO according to the manufacturer's instructions. We also cloned the coding regions of the HKs and receiver domains (RDs) of VpsS (VpsS-HK and VpsS-RD), LuxQ (LuxQ-HK and LuxQ-RD), and CqsS (CqsS-HK and CqsS-RD) separately, as well as the RDs of LuxO (LuxO-RD), VpsR (VpsR-RD), ChiS (ChiS-RD), VC0303 (VC0303-RD), VC1831 (VC1831-RD), VieS (VieS-RD), VC2369 (VC2369-RD), and VarS (VarS-RD). The sequences of all constructs were verified, and the constructs were then mobilized into expression vectors using the Gateway system. RD proteins were purified using an N-terminal thioredoxin-His_6 tag, while HK and HPT proteins were purified using an N-terminal His_6-maltose binding protein tag. Proteins were purified as described previously (36). Phosphotransfer reactions were carried out as described previously (6). Briefly, LuxQ-HK (2 μM), LuxQ-RD (2 μM), and LuxU (20 μM) were incubated together in HKEDG buffer supplemented with 5 mM $MgCl_2$ in the presence of 0.5 μCi [γ-^{32}P]ATP and 0.5 mM ATP for 30 min at 30°C in a 10-μl reaction mixture. This reaction mixture was then diluted 1:10 in HKEDG buffer supplemented with 5 mM $MgCl_2$. Aliquots (5 μl) of the reaction mixture were then added to 5 μl of each individual RD in HKEDG buffer supplemented with 5 mM $MgCl_2$ to obtain a final RD concentration of 10 μM in HKEDG buffer. The reaction mixtures were then incubated at room temperature for 1 or 5 min, and then the reactions were stopped and the mixtures were analyzed by using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and phosphorimaging.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant properties	Source or reference
E. coli strains		
TOP10	F$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80*lacZ*ΔM15 Δ*lacX74 recA1 araD139* Δ(*ara-leu*)*7697 galU galK rpsL* (Sm^r) *endA1 nupG*	Invitrogen
CC118(λ*pir*)	Δ(*ara-leu*) *araD* Δ*lacX74 galE galK phoA20 thi-1 rpsE rpoB argE*(Am) *recA1* λ*pir*	19
S17-1(λ*pir*)	Tp^r Sm^r, *recA thi pro* r_K^- m_K^+ RP4:2-Tc:MuKm Tn*7* λ*pir*	12
V. cholerae strains		
FY_Vc_1	*Vibrio cholerae* O1 El Tor A1552, smooth variant, Rif^r	39
FY_Vc_237	FY_Vc_1, mTn*7-gfp*, Gm^r	4
FY_Vc_616	*vpsLp-lacZ*, Rif^r	35
FY_Vc_2402	*vpsLp-lacZ* Δ*vpsS*, Rif^r	This study
FY_Vc_2715	*vpsLp-lacZ* Δ*vpsV*, Rif^r	This study
FY_Vc_2405	*vpsLp-lacZ* Δ*vpsR*, Rif^r	35
FY_Vc_3884	*vpsLp-lacZ vpsR*(*D59A*), Rif^r	This study
FY_Vc_3887	*vpsLp-lacZ vpsR*(*D59E*), Rif^r	This study
FY_Vc_3463	*vpsLp-lacZ* Δ*vpsT*, Rif^r	35
FY_Vc_3900	*vpsLp-lacZ* Δ*vpsR* Δ*vpsT*, Rif^r	This study
FY_Vc_2398	*vpsLp-lacZ* Δ*luxU*, Rif^r	This study
FY_Vc_2394	*vpsLp-lacZ* ΔVC1080, Rif^r	This study
FY_Vc_3445	*vpsLp-lacZ* ΔVC2038, Rif^r	This study
FY_Vc_3386	*vpsLp-lacZ* Δ*luxQ*, Rif^r	This study
FY_Vc_3389	*vpsLp-lacZ* Δ*cqsS*, Rif^r	This study
FY_Vc_3392	*vpsLp-lacZ* Δ*luxQ* Δ*cqsS*, Rif^r	This study
FY_Vc_3395	*vpsLp-lacZ* Δ*luxQ* Δ*vpsS*, Rif^r	This study
FY_Vc_3398	*vpsLp-lacZ* Δ*cqsS* Δ*vpsS*, Rif^r	This study
FY_Vc_3401	*vpsLp-lacZ* Δ*luxQ* Δ*cqsS* Δ*vpsS*, Rif^r	This study
FY_Vc_2469	*vpsLp-lacZ* Δ*luxO*, Rif^r	This study
FY_Vc_3841	*vpsLp-lacZ* Δ*hapR*, Rif^r	This study
FY_Vc_3466	*vpsLp-lacZ* Δ*hapR* Δ*luxO*, Rif^r	This study
FY_Vc_4169	*vpsTp-lacZ*, Rif^r	This study
FY_Vc_4178	*vpsTp-lacZ* Δ*luxO*, Rif^r	This study
FY_Vc_4166	*vpsRp-lacZ*, Rif^r	This study
FY_Vc_4175	*vpsRp-lacZ* Δ*luxO*, Rif^r	This study
FY_Vc_4286	*vpsLp-lacZ* mTn*7-luxO*, Rif^r	This study
FY_Vc_4288	*vpsLp-lacZ* Δ*luxO* mTn*7-luxO*, Rif^r	This study
FY_Vc_4290	*vpsLp-lacZ* Δ*hapR* mTn*7-luxO*, Rif^r	This study
FY_Vc_4292	*vpsLp-lacZ* Δ*hapR* Δ*luxO* mTn*7-luxO*, Rif^r	This study
FY_Vc_4310	*vpsLp-lacZ* ΔVCA0939, Rif^r	This study
FY_Vc_4313	*vpsLp-lacZ* ΔVCA0939 Δ*luxO*, Rif^r	This study
Plasmids		
pBAD/*myc*-His-B	Arabinose-inducible expression vector with C-terminal *myc* epitope and six-His tags	Invitrogen
pFY-672	pBAD/*myc*-His-B::VC0303, Ap^r	This study
pFY-673	pBAD/*myc*-His-B::VC1831, Ap^r	This study
pFY-873	pBAD/*myc*-His-B::VCA0709, Ap^r	This study
pFY-774	pBAD/*myc*-His-B::VCA0719, Ap^r	This study
pFY-674	pBAD/*myc*-His-B::*chiS*, Ap^r	This study
pFY-670	pBAD/*myc*-His-B::*cqsS*, Ap^r	This study
pFY-671	pBAD/*myc*-His-B::*luxQ*, Ap^r	This study
pFY-875	pBAD/*myc*-His-B::*varS*, Ap^r	This study
pFY-564	pBAD/*myc*-His-B::*vpsS*, Ap^r	This study
pFY-565	pBAD/*myc*-His-B::*vpsV*, Ap^r	This study
pGP704-*sacB*28	pGP704 derivative, *mob*/*oriT sacB*, Ap^r	8
pFY-217	pGP704-*sac*28::*vpsLp-lacZ* transcriptional fusion, Ap^r	35
pFY-717	pGP704-*sac*28::*vpsTp-lacZ* transcriptional fusion, Ap^r	This study
pFY-716	pGP704-*sac*28::*vpsRp-lacZ* transcriptional fusion, Ap^r	This study
pFY-475	pGP704-*sac*28::Δ*vpsS*, Ap^r	This study
pFY-563	pGP704-*sac*28::Δ*vpsV*, Ap^r	This study
pFY-277	pGP704-*sac*28::Δ*vpsS*Δ*vpsV*, Ap^r	This study
pFY-16	pGP704-*sac*28::Δ*vpsR*, Ap^r	39
pFY-214	pGP704-*sac*28::*vpsR*(*D49A*), Ap^r	This study
pFY-215	pGP704-*sac*28::*vpsR*(*D49E*), Ap^r	This study
pFY-15	pGP704-*sac*28::Δ*vpsT*, Ap^r	8
pFY-9	pGP704-*sac*28::Δ*hapR*, Ap^r	40
pFY-477	pGP704-*sac*28::Δ*luxU*, Ap^r	This study
pFY-538	pGP704-*sac*28::ΔVC1080, Ap^r	This study
pFY-732	pGP704-*sac*28::ΔVC2038, Ap^r	This study
pFY-153	pGP704-*sac*28::ΔVCA0939, Ap^r	This study

Continued on following page

TABLE 1—*Continued*

Strain or plasmid	Relevant properties	Source or reference
pFY-734	pGP704-*sac*28::Δ*luxQ*, Ap^r	This study
pFY-733	pGP704-*sac*28::Δ*cqsS*, Ap^r	This study
p28-*luxO*	pGP704-*sac*28::Δ*luxO*, Ap^r	30
pUX-BF13	oriR6K helper plasmid, *mob*/*oriT*, provides the Tn*7* transposition function in *trans*, Ap^r	2
pMCM11	pGP704::mTn*7*-*gfp*, Gm^r Ap^r	G. Schoolnik
pFY-720	pGP704::mTn*7*, Gm^r Ap^r	This study
pFY-724	pGP704-Tn*7*-*luxO*, Gm^r Ap^r, pGP704-Tn*7* containing *luxO* promoter and coding regions	This study
pENTR/D-TOPO	ENTRY vector for Gateway cloning system, Kan^r	Invitrogen
pFY-607	pENTR-CqsS-HK, HK domain of *cqsS* in pENTR/D-TOPO	This study
pFY-608	pENTR-CqsS-RD, RR RD of *cqsS* in pENTR/D-TOPO	This study
pFY-611	pENTR-LuxQ-HK, HK of *luxQ* in pENTR/D-TOPO	This study
pFY-612	pENTR-LuxQ-RD, RD of *luxQ* in pENTR/D-TOPO	This study
pFY-609	pENTR-LuxO-RD, RD of *luxO* in pENTR/D-TOPO	This study
pFY-610	pENTR-LuxO-Full, full length of *luxO* in pENTR/D-TOPO	This study
pFY-613	pENTR-LuxU, full length of *luxU* in pENTR/D-TOPO	This study
pFY-614	pENTR-VpsR-RD, RD of *vpsR* in pENTR/D-TOPO	This study
pFY-615	pENTR-VpsR-Full, full length of *luxO* in pENTR/D-TOPO	This study
pFY-616	pENTR-VpsS-HK, HK of *vpsS* in pENTR/D-TOPO	This study
pFY-617	pENTR-VpsS-RD, RD of *vpsS* in pENTR/D-TOPO	This study
pFY-618	pENTR-VpsS-Full, full length of *vpsS* in pENTR/D-TOPO	This study
pFY-619	pENTR-VC0303-RD, RD of VC0303 in pENTR/D-TOPO	This study
pFY-620	pENTR-VC1831-RD, RD of VC1831 in pENTR/D-TOPO	This study
pFY-727	pENTR-ChiS-RD, RD of *chiS* in pENTR/D-TOPO	This study
pFY-728	pENTR-VieS-RD, RD of *vieS* in pENTR/D-TOPO	This study
pFY-730	pENTR-VC2369-RD, RD of VC2369 in pENTR/D-TOPO	This study
pFY-731	pENTR-VarS-RD, RD of *varS* in pENTR/D-TOPO	This study

RESULTS

Identification of a sensor HK positively regulating *vps* gene expression. In many two-component signal transduction systems, genes encoding cognate HKs and RRs are arranged in operons. However, *vpsR* (VC0665), encoding the most downstream RR for *vps* gene regulation, does not occur in an operon with an HK gene, and no cognate HK has been identified. We therefore hypothesized that an orphan HK (an HK protein whose gene is not found in an operon with or next to a gene encoding an RR domain-containing protein) would be a likely phosphodonor for VpsR. We searched Pfam and InterPro databases and found 42 genes encoding proteins with an HK domain in the *V. cholerae* genome. Nine of the proteins are predicted to be orphan HKs. We therefore screened each orphan HK for the ability to induce *vps* expression upon overexpression. To this end, each HK gene was cloned into an inducible pBAD expression vector, and the plasmids were introduced individually into a reporter strain harboring a *vpsLp-lacZ* transcriptional fusion at the *lacZ* locus on the *V. cholerae* chromosome. Each HK was then assessed to determine its ability to upregulate *vpsL* expression by determining the β-galactosidase activity in cells that were grown for 24 h in the presence and absence of the inducer arabinose. This initial screen revealed that overexpression of *luxQ* (VCA0736) and VC1445 resulted in increased *vpsL* expression (Fig. 1).

We then focused on VC1445 as it has not been studied previously. To further confirm these results, we performed β-galactosidase assays with the *vpsLp-lacZ* strain harboring the VC1445 (designated *vpsS* for *v*ibrio *p*oly*s*accharide biosynthesis *s*ensor) overexpression plasmid or the vector in the presence or absence of the inducer arabinose. Overexpression of *vpsS* resulted in an increase in *vpsL* expression after 3 and 24 h of induction (Fig. 2A). As expected, strains carrying the vector did not exhibit elevated *vpsL* expression in the presence of arabinose. Strains carrying either the vector or p*vpsS* grown in the absence of arabinose did not show elevated *vpsL* expression (data not shown). Because *vpsL* expression was greater after 24 h of induction and the production of Myc-tagged VpsS was also found to be greater after 24 h of induction when it was analyzed by Western analysis (data not shown), we chose this time point for subsequent overexpression studies.

To better evaluate the involvement of VpsS in the regulation

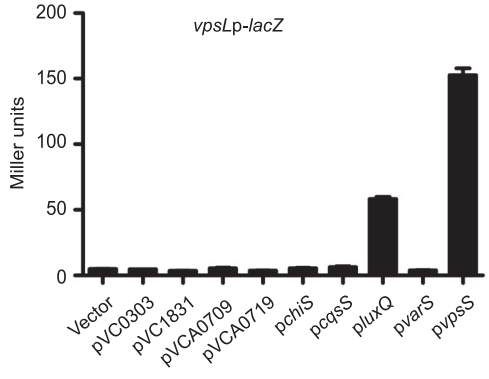

FIG. 1. Overexpression of *vpsS* and *luxQ* activates *vps* expression. Expression of *vpsL* in a *vpsLp-lacZ* reporter strain harboring either the vector pBAD or pVC0303, pVC1831, pVCA0709, pVCA0719, p*chiS*, p*cqsS*, p*luxQ*, p*varS*, or p*vpsS* was examined. Overnight cultures were diluted 1:500 and grown for 24 h in LB medium supplemented with ampicillin and 0.2% arabinose to induce HK overexpression. The error bars indicate standard deviations of eight technical replicates. The results of one representative experiment of four biological replicates are shown.

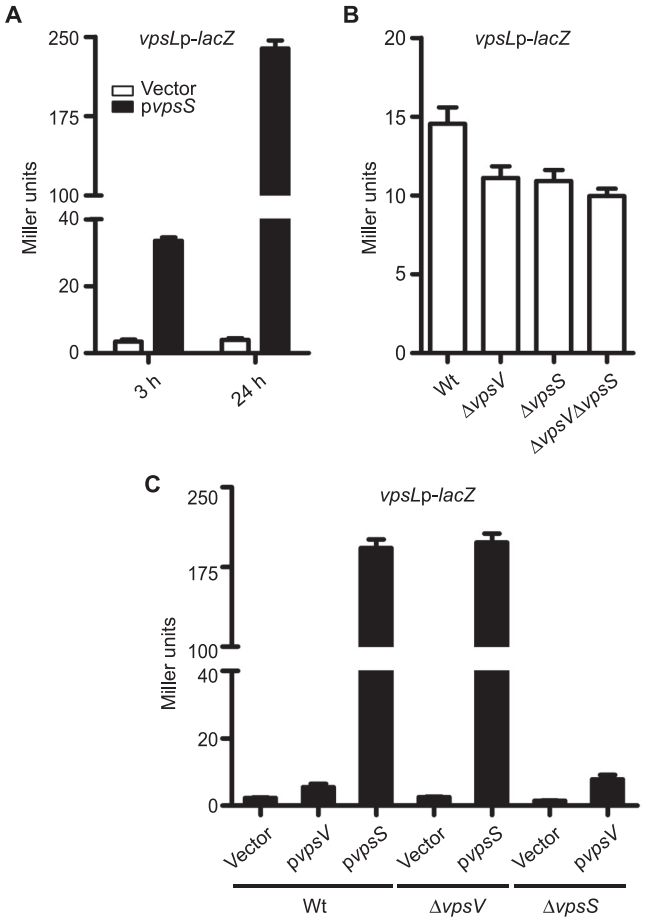

FIG. 2. VpsS activates *vps* expression. (A) Expression of *vpsL* in a *vpsLp-lacZ* reporter strain harboring either the vector pBAD (open bars) or p*vpsS* (filled bars). Overnight cultures were diluted 1:500 and grown for 3 or 24 h in LB medium supplemented with ampicillin and 0.2% arabinose. (B) Expression of *vpsL* in wild-type (Wt), Δ*vpsV*, Δ*vpsS*, and Δ*vpsV* Δ*vpsS* strains harboring a single copy of the *vpsLp-lacZ* reporter grown to exponential phase in LB medium. (C) Expression of *vpsL* in wild-type, Δ*vpsV*, and Δ*vpsS* strains carrying a *vpsLp-lacZ* reporter fusion harboring either the vector pBAD, p*vpsV*, or p*vpsS*. The error bars indicate standard deviations of eight technical replicates. The results of one representative experiment of four biological replicates are shown.

of *vps* gene expression, we generated an in-frame *vpsS* deletion mutant in the *vpsLp-lacZ* reporter strain and analyzed *vps* gene expression in cells grown to exponential phase using β-galactosidase assays. We determined that *vpsL* expression was slightly decreased in the Δ*vpsS* mutant compared to the wild type (Fig. 2B). Expression of *vps* genes is negatively regulated by the quorum-sensing signal transduction system in stationary phase, and as expected, we did not see a significant difference in *vps* gene expression between the wild type and the *vpsS* mutant when expression was analyzed in cells grown to stationary phase (data not shown). Taken together, these results indicate that VpsS positively regulates *vps* expression.

vpsS is the second gene in a predicted two-gene operon with VC1444. Transcriptional linkage analysis by reverse transcription-PCR revealed that VC1444 (designated *vpsV*) and *vpsS* are in an operon (data not shown). We therefore hypothesized that *vpsV* might be necessary for VpsS function. To test this possibility, we generated *vpsV* and *vpsV vpsS* in-frame deletion mutants in the *vpsLp-lacZ* reporter strain and analyzed *vpsL* gene expression. In exponential phase, the level of expression of *vpsL* was lower in the Δ*vpsV* and Δ*vpsV* Δ*vpsS* strains than in the wild type, similar to the data for the Δ*vpsS* strain (Fig. 2B). This finding suggests that *vpsV* and *vpsS* affect *vpsL* expression similarly. To determine whether *vpsS* required *vpsV* to upregulate *vpsL*, we overexpressed *vpsS* in the Δ*vpsV* background. The expression of *vpsL* in the Δ*vpsV* strain was similar to that in the wild-type strain when *vpsS* was overexpressed (Fig. 2C). Furthermore, overexpression of *vpsV* did not induce *vpsL* expression in the wild type and in the Δ*vpsS* strain. As expected, strains carrying the vector or the overexpression plasmids did not activate *vpsL* expression in the absence of arabinose (data not shown). This finding suggests that, when overexpressed, VpsS can function without VpsV.

VpsS modulates biofilm formation. Since both deletion and overexpression of *vpsS* altered *vpsL* transcription, we reasoned that VpsS might also modulate biofilm formation in *V. cholerae*. To examine this possibility, we initially compared the biofilm-forming ability of the Δ*vpsS* mutant to that of the wild type using a crystal violet staining assay. We determined that the biofilm phenotypes of the Δ*vpsS* and wild-type strains were similar (data not shown). However, using the same assay, we determined that overexpression of *vpsS* in the wild-type strain carrying p*vpsS* markedly increased the biofilm formation compared to that of the same strain containing the vector (Fig. 3A). In the absence of arabinose, strains carrying the vector or p*vpsS* exhibited similar biofilm-forming capacities.

We further evaluated the contribution of VpsS to biofilm formation by analyzing biofilm phenotypes. The biofilm formation by the Δ*vpsS* strain was similar to that by the wild-type strain over a period of 48 h (Fig. 3B). However, overexpression of *vpsS* in the wild-type strain carrying p*vpsS* markedly increased biofilm formation compared to the biofilm formation by the same strain containing the vector. Quantitative analysis of biofilm images with COMSTAT revealed that the total biomass and average and maximum thicknesses of the biofilm formed by the wild-type strain overexpressing *vpsS* differed from the total biomass and average and maximum thicknesses of the biofilm formed by the wild-type strain harboring the control vector after 48 h (Table 2). No significant differences in the total biomass and average and maximum thicknesses were observed between the wild-type and Δ*vpsS* strains (Table 2). It should be noted that the growth rates of these strains are identical (data not shown). These results indicate that overexpression, but not deletion, of *vpsS* modulates biofilm formation. The absence of a clear biofilm phenotype in strains lacking *vpsS* may indicate that there is functional redundancy in the VpsS regulatory network, that the level of *vpsS* expression may not be high enough under the experimental conditions used in this study, or that the decreased *vps* expression in a Δ*vpsS* mutant is not significant enough to alter biofilm formation.

VpsS requires VpsR, but not VpsT, to activate *vps* expression. VpsR is an RR and requires phosphorylation to activate *vps* gene expression and biofilm formation (25; J. Meir and F. H. Yildiz, unpublished data). We reasoned that VpsS could positively regulate *vps* expression through phosphorylation of VpsR. We therefore evaluated whether VpsS induces *vps* ex-

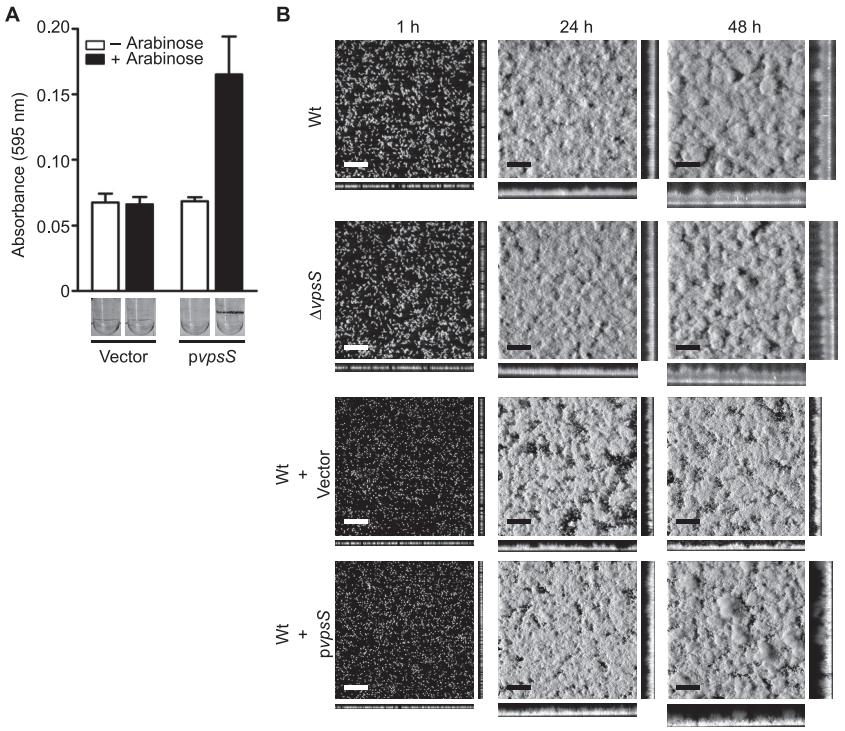

FIG. 3. Overexpression of *vpsS* activates biofilm formation. (A) Quantitative comparison of biofilm formation by wild-type strains harboring the vector or p*vpsS*. Strains were grown for 8 h in LB medium supplemented with ampicillin in the absence (open bars) or presence (filled bars) of arabinose at 30°C under static conditions. Crystal violet-stained biofilms formed in the wells of polyvinyl chloride microtiter plates are also shown. The error bars indicate standard deviations of eight technical replicates. The results of one representative experiment of two biological replicates are shown. (B) CSLM images of horizontal (*xy*) and vertical (*xz*) projections of biofilm structures formed by the wild-type strain (Wt), the Δ*vpsS* mutant, and wild-type strains carrying the vector or p*vpsS*. Strains were grown for the times indicated in 2% LB medium in the presence or absence of ampicillin and 0.2% arabinose at room temperature. Bars = 40 μm.

pression in a VpsR-dependent manner. To this end, we overexpressed *vpsS* in the *vpsLp-lacZ* reporter strain harboring an in-frame deletion of *vpsR* or in mutants in which the chromosomal copy of *vpsR* was mutated such that aspartate 59 was replaced with alanine [*vpsR*(*D59A*), constitutively inactive] or with glutamate [*vpsR*(*D59E*), constitutively active] and evaluated *vpsL* expression using β-galactosidase assays. No significant increase in *vpsL* expression was observed in the Δ*vpsR* strain overexpressing *vpsS* or harboring the vector alone (Fig. 4A), indicating that VpsR is required for VpsS to upregulate *vpsL* expression. Overexpression of *vpsS* in a strain harboring a "constitutively inactive" version of VpsR [*vpsR*(*D59A*)] resulted in increased *vpsLp-lacZ* activity compared to the same strain harboring the control vector. The strain harboring a "constitutively active" version of VpsR [*vpsR*(*D59E*)] and carrying the control vector exhibited a high level of *vpsL* expression compared to the wild-type strain carrying the control vector, supporting the idea that VpsR modification modulates its activity. Moreover, a marked increase in *vpsL* expression was observed in the *vpsR*(*D59E*) strain when *vpsS* was overexpressed compared to the same strain harboring the control vector. Taken together, these results suggest that VpsS has functions other than phosphorylating the canonical aspartate (D59) of VpsR. It is also likely that VpsS can indirectly activate VpsR through another two-component system or control its expression.

Since VpsS does not appear to directly phosphorylate VpsR, we hypothesized that overexpression of VpsS might instead modulate the expression of *vpsR*. When *vpsS* was overexpressed in a strain carrying a chromosomal *vpsRp-lacZ* reporter fusion, the expression was similar to that of a strain

TABLE 2. COMSTAT analysis of biofilms of wild-type and Δ*vpsS* strains and wild-type strains harboring a vector or *vpsS* overexpression plasmid[a]

Strain	Time (h)	Total biomass ($\mu m^3/\mu m^2$)	Thickness (μm)	
			Avg	Maximum
Wild type	1	11.8 (1.37)	10.9 (1.37)	11.0 (1.45)
	24	23.4 (2.43)	22.5 (2.43)	22.5 (2.46)
	48	37.4 (4.08)	36.5 (4.08)	36.7 (3.93)
Δ*vpsS*	1	11.2 (1.46)	10.3 (1.46)	10.4 (1.61)
	24	23.5 (3.93)	22.6 (3.93)	22.7 (3.91)
	48	39.8 (4.39)	38.9 (4.39)	39.1 (4.25)
Wild type/vector[b]	1	2.0 (0.44)	3.2 (0.88)	10.1 (1.87)
	24	21.1 (2.23)	23.6 (2.76)	25.2 (3.12)
	48	25.8 (5.10)	29.4 (5.19)	30.9 (5.19)
Wild type/p*vpsS*[b]	1	3.0 (0.37)	5.2 (0.83)	12.8 (2.07)
	24	23.6 (2.45)	24.6 (3.40)	25.3 (3.60)
	48	32.0 (2.17)	37.4 (4.72)	42.0 (8.06)

[a] The values are the means (standard deviations) of data from at least six *z*-series image stacks.
[b] Cultures were grown in the presence of ampicillin and 0.2% arabinose.

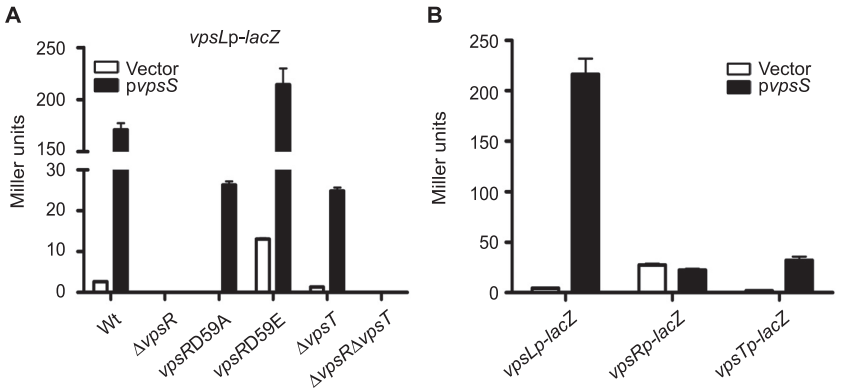

FIG. 4. VpsS requires VpsR to activate *vps* expression. (A) Expression of *vpsL* in wild-type (Wt), Δ*vpsR*, *vpsR*(*D59A*), *vpsR*(*D59E*), Δ*vpsT*, and Δ*vpsR* Δ*vpsT* strains harboring a *vpsLp-lacZ* reporter carrying the vector (open bars) or p*vpsS* (filled bars). (B) Expression of *vpsL*, *vpsR*, and *vpsT* from a single-copy chromosomal *vpsLp-lacZ*, *vpsRp-lacZ*, or *vpsTp-lacZ* reporter in the wild-type strain harboring the vector (open bars) or p*vpsS* (filled bars). *vpsS* was overexpressed in LB medium supplemented with ampicillin and 0.2% arabinose. The error bars indicate standard deviations of eight technical replicates. The results of one representative experiment of four biological replicates are shown.

carrying the vector alone (Fig. 4B). These results suggest that VpsS does not regulate the expression of *vpsR* but nonetheless requires VpsR to regulate *vpsL* expression.

VpsT is another known positive regulator of *vps* genes. We therefore asked whether the effect of VpsS, in addition to the effect of VpsR, was mediated through VpsT. To examine this possibility, we overexpressed *vpsS* in the *vpsLp-lacZ* reporter strain harboring Δ*vpsT* or Δ*vpsR* Δ*vpsT* mutations. As expected, the Δ*vpsT* strain carrying the control vector had reduced *vpsL* expression compared to the wild type carrying the control vector (Fig. 4A). However, when *vpsS* was overexpressed in the Δ*vpsT* strain, *vpsL* expression was induced. Furthermore, the Δ*vpsR* Δ*vpsT* double mutant showed no change in *vpsL* expression when *vpsS* was overexpressed compared to the same strain carrying the control vector. We then hypothesized that VpsS might modulate *vpsL* expression through the expression of *vpsT*. Indeed, when VpsS was overexpressed in a chromosomal *vpsTp-lacZ* strain, the *vpsT* expression increased compared that in a strain harboring the vector alone (Fig. 4B). These results suggest that VpsS regulates *vpsT* expression and that VpsT contributes to, but is not essential for, the VpsS-dependent activation of *vps* genes.

VpsS requires LuxU to activate *vps* genes. VpsS is a hybrid kinase that has both HK and RR domains, but it lacks the HPT domain that normally mediates movement of phosphoryl groups from a hybrid HK to a downstream RR. We thus speculated that VpsS does not phosphorylate its cognate RR directly but instead acts via a separate HPT. We therefore searched the genome to identify genes encoding potential cytoplasmic HPT proteins by looking for proteins with characteristics of other HPTs based on the following criteria: (i) smaller than 250 amino acids, (ii) >70% predicted alpha helical secondary structure, and (iii) the presence of a conserved HXXKG motif within the predicted alpha helices (6). This analysis identified LuxU and VC1080 as possible candidates. We also searched the *V. cholerae* genome for genes predicted to encode HPT domains (PF01627) and identified another HPT, VC2038, that is predicted to localize to the cytoplasmic membrane.

To determine whether these HPT proteins were involved in the phosphorelay inducing *vps* expression, we generated in-frame deletions of genes encoding each HPT in the *vpsLp-lacZ* reporter strain. A previous study reported that LuxU positively regulates biofilm formation (17). Indeed, a *luxU* mutant showed decreased *vpsL* expression compared to the wild type when the organisms were grown to exponential phase (Fig. 5A). Mutants with in-frame deletions of VC1080 and VC2038, however, did not exhibit differences in *vpsL* expression compared to the wild type, suggesting that VC1080 and VC2038 are not involved in the regulation of *vpsL* expression. We then overexpressed *vpsS* and analyzed *vpsL* expression in each HPT mutant strain. As expected, strains lacking *luxU* showed no VpsS-dependent activation of *vpsLp-lacZ* when *vpsS* was overexpressed, while the *vpsL* expression was similar to that of the wild type in VC1080 and VC2038 deletion mutants (Fig. 5B). Strains carrying only the vector did not exhibit activation of *vpsL* in the presence of arabinose. These results indicate that

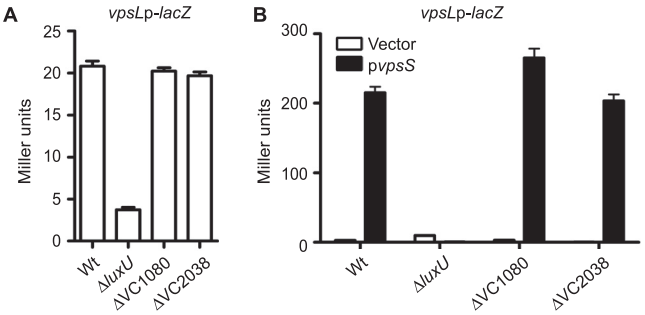

FIG. 5. VpsS requires the HPT LuxU to activate *vps* expression. (A) Expression of *vpsL* from a *vpsLp-lacZ* reporter in wild-type (Wt), Δ*luxU*, ΔVC1080, and ΔVC2038 strains grown to exponential phase in LB medium. (B) Expression of *vpsL* from a *vpsLp-lacZ* reporter in wild-type, Δ*luxU*, ΔVC1080, and ΔVC2038 strains harboring the vector (open bars) or p*vpsS* (filled bars). *vpsS* was overexpressed in LB medium supplemented with ampicillin and 0.2% arabinose. The error bars indicate standard deviations of eight technical replicates. The results of one representative experiment of four biological replicates are shown.

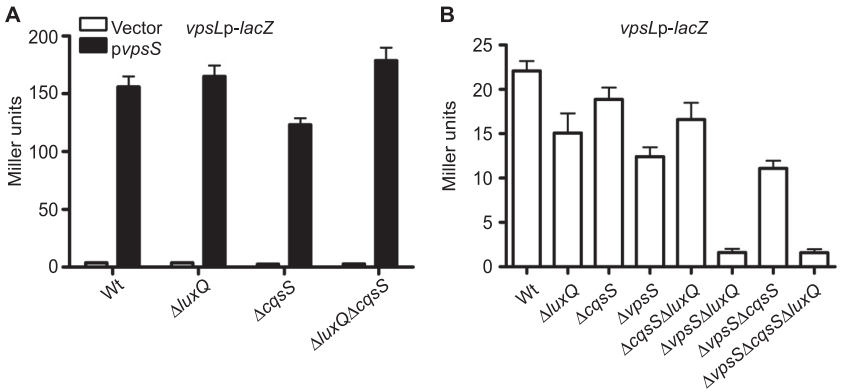

FIG. 6. LuxQ, CqsS, and VpsS regulate *vps* expression in parallel. (A) Expression of *vpsL* from a *vpsLp-lacZ* reporter in wild-type (Wt), Δ*luxQ*, Δ*cqsS*, and Δ*luxQ* Δ*cqsS* strains harboring the vector (open bars) or p*vpsS* (filled bars). *vpsS* was overexpressed in LB medium supplemented with ampicillin and 0.2% arabinose. The error bars indicate standard deviations of eight technical replicates. The results of one representative experiment with four biological replicates are shown. (B) Expression of *vpsL* from a *vpsLp-lacZ* reporter in wild-type, Δ*luxQ*, Δ*cqsS*, Δ*vpsS*, Δ*cqsS* Δ*luxQ*, Δ*vpsS* Δ*luxQ*, Δ*vpsS* Δ*cqsS*, and Δ*vpsS* Δ*cqsS* Δ*luxQ* strains grown to exponential phase in LB medium. The error bars indicate standard deviations of eight technical replicates. The results of one representative experiment of four biological replicates are shown.

the VpsS phosphorelay system functions through the HPT LuxU.

VpsS contributes to the quorum-sensing phosphotransfer cascade. The quorum-sensing hybrid HKs CqsS and LuxQ were previously shown to require LuxU to control quorum-sensing-regulated gene expression (32). We therefore asked whether LuxQ or CqsS contributes to *vps* upregulation through VpsS. To answer this question, we generated Δ*cqsS*, Δ*luxQ*, and Δ*cqsS* Δ*luxQ* deletion mutants of the *vpsLp-lacZ* reporter strain, overexpressed *vpsS* in these strains, and quantified *vpsL* expression. Overexpression of *vpsS* in any of these deletion strains resulted in upregulation of *vpsL* similar to that in the wild-type strain (Fig. 6A), suggesting that CqsS and LuxQ are not required for VpsS to activate *vpsL* expression.

We further evaluated the contribution of *cqsS*, *luxQ*, and *vpsS* to *vps* gene expression using epistasis analysis. To this end, we constructed individual Δ*luxQ*, Δ*cqsS*, and Δ*vpsS* deletion mutants and combination Δ*cqsS* Δ*luxQ*, Δ*vpsS* Δ*luxQ*, Δ*vpsS* Δ*cqsS*, and Δ*vpsS* Δ*cqsS* Δ*luxQ* deletion mutants of the *vpsLp-lacZ* reporter strain and measured *vpsL* expression in cells grown to exponential phase. Deletion of *luxQ*, *cqsS*, or *vpsS* individually moderately decreased *vpsL* expression (Fig. 6B). The Δ*cqsS* Δ*luxQ* and Δ*vpsS* Δ*cqsS* double mutations additively decreased *vpsL* expression, and the Δ*vpsS* Δ*luxQ* double-deletion mutant exhibited the most drastic decrease in *vpsL* expression, similar to the Δ*vpsS* Δ*cqsS* Δ*luxQ* triple-deletion mutant. These results indicate that CqsS, LuxQ, and VpsS act in parallel through LuxU to regulate *vpsL* expression.

LuxU participates in phosphotransfer with VpsS. To further analyze the relationships between VpsS and LuxU, we evaluated the in vitro phosphotransfer relationship between LuxU, VpsS, CqsS, and LuxQ. To this end, we separately purified LuxU and the HK domains and RDs from VpsS, CqsS, and LuxQ. Only the kinase domain of LuxQ (LuxQ-HK) exhibited significant autophosphorylation in vitro. Incubation of LuxQ-HK, LuxQ-RD, and LuxU led to accumulation of phosphorylated LuxU, as expected if LuxQ can transfer phosphoryl groups to LuxU. As we could not test whether VpsS phosphotransfers to LuxU, we then tested whether LuxU~P could serve as a phosphodonor to the RDs of VpsS, CqsS, and LuxQ. To assess the specificity of an in vitro LuxU~P phosphotransfer, we also performed the assay with the RDs of six other hybrid histidine kinases, VC0303, ChiS, VC1831, VieS, VC2369, and VarS. Incubation of LuxU~P with the RDs of VpsS, CqsS, and LuxQ led to depletion of radiolabel from LuxU, indicating that there was phosphotransfer (Fig. 7). A band corresponding to the phosphorylated RD was seen in each case, although this band was faint for LuxQ-RD and CqsS-RD. For these two RDs, the residual LuxQ-HK in the reactions (used to produce LuxU~P) may drive their dephosphorylation, or they may be intrinsically less stable. For the other six RDs, there was no evidence of phosphotransfer, except for VC1831. It has not been determined whether VC1831 is part of the quorum-sensing pathway in *V. cholerae* or whether LuxU exhibits some promiscuity in vitro. We also tested for phosphotransfer from LuxU~P to the RDs of LuxO and VpsR but did not observe any significant transfer. LuxO, a predicted substrate for LuxU~P in vivo, may require additional factors for phosphotransfer in vitro. Whether VpsR is a substrate for LuxU~P is unclear. Our data do, however, support a model in which VpsS

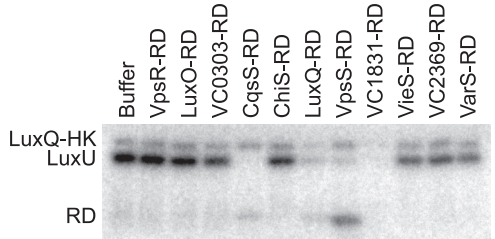

FIG. 7. LuxU phosphotransfers to the RDs of VpsS, CqsS and LuxQ. In vitro phosphotransfer analyses showed transfer of phosphoryl groups from LuxU~P to the RDs of VpsS, CqsS, LuxQ, and VC1831. Each phosphotransfer reaction mixture containing LuxQ-HK, LuxQ-RD, and LuxU was incubated with radiolabeled ATP before dilution and addition of the RDs indicated for 1 or 5 min at room temperature. Incubation for 1 min and incubation for 5 min produced similar results; only data for 1 min of incubation are shown. Buffer was used as a negative control.

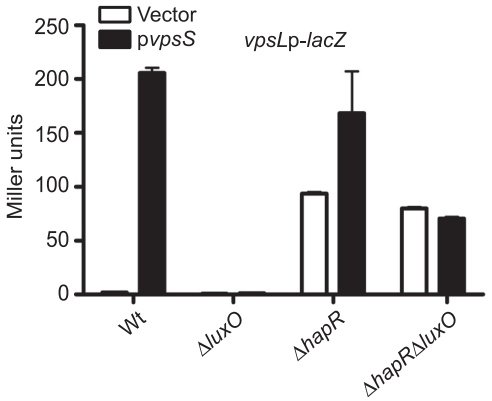

FIG. 8. VpsS requires LuxO, but not HapR, to activate *vps*. Expression of *vpsL* from a *vpsLp-lacZ* reporter in wild-type (Wt), Δ*luxO*, Δ*hapR*, and Δ*luxO* Δ*hapR* strains harboring the vector (open bars) or p*vpsS* (filled bars) was examined. *vpsS* was overexpressed in LB medium supplemented with ampicillin and 0.2% arabinose. The error bars indicate standard deviations of eight technical replicates. The results of one representative experiment of four biological replicates are shown.

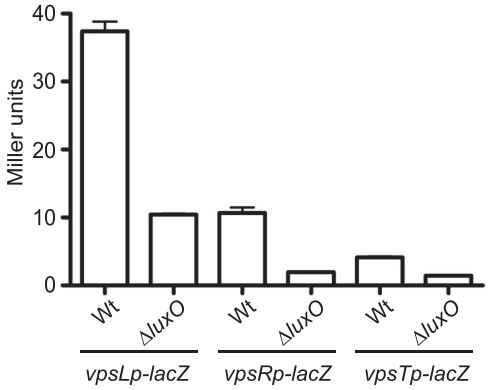

FIG. 9. LuxO positively regulates *vpsL*, *vpsR*, and *vpsT* expression. Expression of *vpsL*, *vpsR*, and *vpsT* in wild-type (Wt) and Δ*luxO* strains harboring single-copy chromosomal *vpsLp-lacZ*, *vpsRp-lacZ*, and *vpsTp-lacZ* reporters and grown to exponential phase in LB medium was examined. The error bars indicate standard deviations of eight technical replicates. The results of one representative experiment of four biological replicates are shown.

can drive the phosphorylation of LuxU, as it does with LuxQ and CqsS.

VpsS requires LuxO for induction of *vps* expression. LuxU relays a phosphate signal from the hybrid HKs CqsS and LuxQ to the RR LuxO, leading to the regulation of HapR (32). Since VpsS requires LuxU to activate *vps* expression, we hypothesized that VpsS could positively regulate *vps* expression through the known quorum-sensing pathway involving LuxU, LuxO, and HapR.

To determine whether VpsS requires LuxO and/or HapR for its effect on *vpsL* expression, we generated Δ*luxO*, Δ*hapR*, and Δ*luxO* Δ*hapR* deletion mutants of the *vpsLp-lacZ* reporter strain, overexpressed *vpsS*, and analyzed *vpsL* expression. Overexpression of *vpsS* in the Δ*luxO* strain did not result in enhanced *vpsL* expression compared to the wild-type expression (Fig. 8). As expected from deletion of a negative regulator, the Δ*hapR* mutant harboring the vector alone exhibited increased expression of *vpsL* compared to the wild type harboring the vector. However, when *vpsS* was overexpressed in the Δ*hapR* background, a further increase in *vpsL* expression was observed compared to the same strain harboring the vector. This additional activation of *vpsL* expression, due to overexpression of *vpsS*, was abolished in the Δ*luxO* Δ*hapR* mutant. Therefore, VpsS requires LuxO, but not HapR, to upregulate *vpsL* expression. Furthermore, the *vpsL* expression phenotypes of the Δ*luxO* and Δ*luxO* Δ*hapR* mutants could be complemented by inserting a single copy of *luxO* into the chromosome (see Fig. S1 in the supplemental material). Together, these results indicate that VpsS activates *vpsL* expression through LuxO, and this activation is not dependent on HapR.

Our results described above suggest that LuxO regulates the expression of *vpsL* independent of HapR. To our knowledge, the only other gene shown to be regulated by LuxO independent of HapR is VCA0939 (18). VCA0939 is predicted to encode a protein containing a GGDEF domain. Proteins with GGDEF domains are known to act as diguanylate cyclases, which produce cyclic diguanylate (c-di-GMP), a secondary messenger that affects virulence, motility, VPS production, and biofilm formation in *V. cholerae* (11). The stability of VCA0939 mRNA was shown to be mediated by the Qrr sRNAs, which are positively regulated by LuxO (18). We thus tested whether VpsS was dependent on VCA0939 to regulate *vpsL*. We found that deleting VCA0939 did not alter *vpsL* expression when *vpsS* was overexpressed (see Fig. S2 in the supplemental material), suggesting that VpsS does not require VCA0939 for upregulation of *vpsL* expression and that LuxO activates *vpsL* independent of VCA0939.

LuxO positively regulates *vpsR* and *vpsT* expression. As described above, the *V. cholerae* quorum-sensing system acts through LuxO to modulate *vps* expression (17, 38). Additionally, the master quorum-sensing regulator, HapR, was shown to negatively regulate the expression of the two positive *vps* regulators, VpsR and VpsT (3). We therefore asked whether LuxO modulates the expression of *vpsR* and *vpsT*. To examine this possibility, *luxO* was deleted in reporter strains carrying chromosomal *vpsL*, *vpsR*, or *vpsT* promoter-*lacZ* fusions (*vpsLp-lacZ*, *vpsRp-lacZ*, and *vpsTp-lacZ*, respectively). When these strains were grown to mid-exponential phase and assayed for β-galactosidase activity, the expression of *vpsL*, *vpsR*, and *vpsT* was lower in the Δ*luxO* mutant than in the wild type (Fig. 9). These results link the quorum-sensing phosphotransfer cascade of LuxO to the positive regulation of both *vpsR* and *vpsT*.

We also entertained the notion that LuxO could directly regulate the expression of *vpsR* and *vpsT* and thus lead to HapR-independent regulation of *vpsL* expression. Since LuxO activation is σ^{54} dependent, we searched for possible σ^{54} binding sites (5′-TGGCAN-N_5-TTGCA/T-3′) (27) upstream of *vpsR* and *vpsT*. However, no σ^{54} binding sites were identified, suggesting that LuxO regulates *vpsR* and *vpsT* indirectly.

VpsS regulates protease activity. Our results indicate that VpsS regulates biofilm formation through the known quorum-sensing pathway involving LuxU, LuxO, and HapR. We therefore hypothesized that other quorum-sensing-dependent phenotypes would be modulated in response to *vpsS*

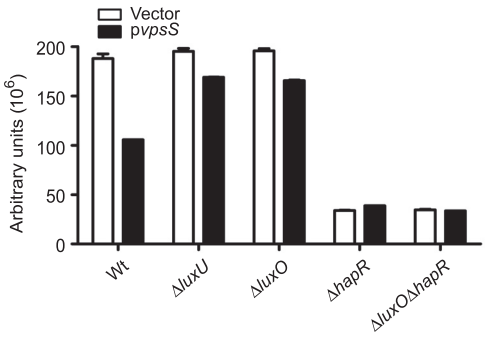

FIG. 10. VpsS negatively regulates protease activity. Protease activity in wild-type (Wt), Δ*luxU*, Δ*luxO*, Δ*hapR*, and Δ*luxO* Δ*hapR* strains harboring the vector (open bars) or p*vpsS* (filled bars) was examined. Strains were grown in LB medium supplemented with ampicillin and 0.2% arabinose to induce *vpsS* expression. The results are expressed in arbitrary units of fluorescence measured at 530 nm normalized to cell density (OD_{600}). The error bars indicate standard deviations of three technical replicates. The results of one representative experiment of four biological replicates are shown.

overexpression. Since protease production is positively regulated by quorum sensing in *V. cholerae* (23, 43), we hypothesized that overexpression of *vpsS* would repress the production of extracellular proteases. Indeed, when *vpsS* was overexpressed in the wild-type strain, the protease activity decreased compared to that in cells containing the vector alone (Fig. 10). Furthermore, overexpression of *vpsS* in Δ*luxU* and Δ*luxO* strains did not result in a significant decrease in protease activity compared to the activity in the same strains carrying the vector alone. These results are congruent with the notion that VpsS contributes to the phosphotransfer cascade involving LuxU and LuxO to modulate *hapR* expression and protease activity. Consistent with the idea that HapR is a positive regulator of protease production, Δ*hapR* and Δ*hapR* Δ*luxO* strains carrying only the vector exhibited substantially lower protease activities than the wild type carrying the vector. Furthermore, overexpression of VpsS in Δ*hapR* and Δ*hapR* Δ*luxO* mutants appeared not to affect protease activity. Taken together, these results suggest that overexpression of VpsS modulates protease activity in *V. cholerae*.

DISCUSSION

V. cholerae is an inhabitant of two very different environments, the aquatic milieu and the human digestive tract. Recent studies revealed that *V. cholerae*'s ability to form biofilms facilitates its environmental survival, transmission, and interaction with its human host (1, 14, 15). In this study, we identified a hybrid HK, VpsS, which positively regulates biofilm formation in *V. cholerae*. VpsS homologs exist in other closely related *Vibrio* species. These homologs include VF_1296 of *Vibrio fischeri* (47% identity, 70% positives, 5 gaps), VP1547 of *Vibrio parahaemolyticus* (50% identity, 69% positives, 4 gaps), and VV1_2622 of *Vibrio vulnificus* (51% identity, 72% positives, 15 gaps) (see Fig. S3 in the supplemental material), but to date, none of these homologs have been characterized. It is intriguing that the upstream genomic contexts of *vpsS* and its homologs in other *Vibrio* species are similar (see Fig. S3 in the supplemental material). It has not been determined yet if VpsS homologs are involved in quorum-sensing signal transduction in other *Vibrio* species.

VpsS is a hybrid kinase that contains both HK and RR domains but not an HPT domain. The transfer of phosphoryl groups between such a hybrid HK and a downstream RR would normally be mediated by an HPT protein. We thus speculated that VpsS does not phosphorylate its cognate RR directly but acts via a separate HPT. We determined that the HPT LuxU is required for VpsS to upregulate *vps* expression. Subsequent analysis revealed that LuxU can reverse phosphotranfer to VpsS. In vitro reverse phosphotransfer was shown to occur previously in other systems (13), and we showed that LuxU specifically transfers phosphate to the RDs of other known hybrid HK partners, LuxQ and CqsS, but not to the control RDs of five other hybrid HKs. Interestingly LuxU can also transfers phosphate to the RD of VC1831, and the importance of this hybrid HK in quorum-sensing-regulated processes is currently under investigation. Taken together, our results indicate that VpsS positively regulates biofilm formation through a phosphotransfer signaling cascade previously identified to be involved in quorum sensing.

In this study, we report that VpsS is dependent on only some components of the *V. cholerae* quorum-sensing system to activate *vps* expression. VpsS was not dependent on the quorum-sensing master regulator HapR to activate *vps* genes. We show that VpsS can affect protease activity, a HapR-regulated process, and that the ability of VpsS to upregulate *vps* expression did, however, require the HPT LuxU and the RR LuxO. We further showed that LuxO positively regulates *vpsR* and *vpsT* expression. Both LuxU and LuxO are required for the phosphorelay that responds to the abundance of the *V. cholerae* AIs CAI-1 and AI-2 (17, 32). CAI-1 [(*S*)-3-hydroxytridecan-4-one] is synthesized by CqsA and is detected by CqsS (22), while AI-2 [the furanosyl borate diester (2*S*,4*S*)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran borate] is synthesized by LuxS and is detected by the LuxQP receptor (9, 34). The nature of the signal that initiates autophosphorylation of VpsS has not been determined yet. VpsS is not predicted to contain any transmembrane domains and might therefore receive an intracellular signal, in contrast to its extracellular autoinducer-sensing counterparts, CqsS and LuxQ. Cytoplasmic HKs in other bacteria are known to sense a variety of intracellular signals, such as those involved in cellular metabolism, or environmental signals detectable in the cytoplasm (29). It is well documented that the transcription of genes involved in biofilm formation is enhanced by the cytoplasmic signaling molecule c-di-GMP via an as-yet-unknown mechanism. We hypothesized that VpsS senses c-di-GMP and its activity may be regulated by c-di-GMP. However, purified VpsS did not bind to c-di-GMP (unpublished data), suggesting that VpsS is not a c-di-GMP sensor protein. *vpsV* precedes *vpsS* in a predicted two-gene operon, and our results showed that deletion of either *vpsS* or *vpsV* leads to a decrease in *vps* expression. VpsV is annotated as a hypothetical protein and does not contain any putative transmembrane domains. VpsV does, however, contain a FIST (*F*-box and *i*ntracellular *s*ignal *t*ransduction, PF08495) domain, and such domains are often associated with other well-known signal transduction domains (7). It is therefore possible that VpsV and VpsS are both required to initiate the phosphotransfer cascade in response to a stimulus. The role of

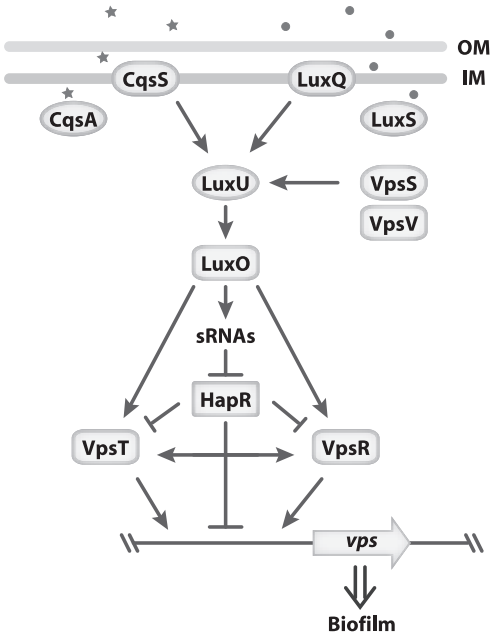

FIG. 11. Model of the VpsS phosphotransfer cascade. VpsS activates *vps* expression through LuxU and LuxO. LuxO can regulate *vps* expression through HapR, as well as independent of HapR through VpsR and/or VpsT. Stars and dots represent CAI-1 and AI-2, respectively. Details of the model are discussed in the text.

VpsV in VpsS-dependent activation of *vpsL* expression is currently under investigation.

Our current understanding of the regulatory network controlling *vps* gene expression is summarized in Fig. 11. At low cell density, phosphotransfer is initiated by the hybrid HKs CqsS, LuxQ, and VpsS to the HPT LuxU, which then transfers the phosphoryl group to the quorum-sensing RR LuxO. Phosphorylated LuxO then negatively regulates HapR via the Qrr sRNAs and positively regulates VpsR and VpsT through a yet-to-determined mechanism. HapR negatively regulates the transcription of *vpsR*, *vpsT*, and the *vps* genes, while VpsR and VpsT positively regulate the transcription of *vps* genes.

V. cholerae's ability to cause epidemics is tied to its ability to survive in aquatic habitats in biofilms. A better understanding of the signals and molecular mechanisms involved in biofilm formation is therefore critical for understanding the environmental survival and transmission of *V. cholerae.*

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