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VpsT Is a Transcriptional Regulator Required for Expression of *vps* Biosynthesis Genes and the Development of Rugose Colonial Morphology in *Vibrio cholerae* O1 El Tor

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***Vibrio cholerae* switches between smooth and rugose colonial variants. The rugose variant produces more vibrio polysaccharides ($VPS^{El\ Tor}$) and forms well-developed biofilms. Both phenotypes depend on expression of *vps* biosynthesis genes. We identified a positive transcriptional regulator of *vps* gene expression, VpsT, which is homologous to response regulators of two-component regulatory systems. Disruption of *vpsT* in the rugose variant yields smooth colonies, prevents formation of mature biofilms, and decreases *vps* gene expression. The interaction between VpsT and VpsR, a previously identified positive regulator of *vps* genes, was also investigated.**

Vibrio cholerae, the causative agent of the disease cholera, is a natural inhabitant of aquatic ecosystems. The pathogen causes periodic, seasonal cholera outbreaks in regions where the disease is endemic and can spread worldwide in pandemics (10). The ability of *V. cholerae* to cause epidemics is linked to its survival in aquatic habitats.

During its life cycle, *V. cholerae* undergoes phase variation which results in the generation of two morphologically different variants termed smooth and rugose (23). Compared to smooth variants, rugose variants have increased capacity to produce exopolysaccharide $VPS^{El\ Tor}$, which enables them to form well-developed biofilms and to better resist environmental stresses (14, 21–23, 27). Exopolysaccharide $VPS^{El\ Tor}$ production depends on transcription of the vibrio polysaccharide synthesis (*vps*) genes (27). *vps* genes are clustered in two regions in the *V. cholerae* chromosome. One cluster harbors genes *vpsA* through *vpsK*, and the other one harbors genes *vpsL* through *vpsQ*. Rugose variants lacking *vpsA* or *vpsL* do not produce $VPS^{El\ Tor}$ and exhibit a smooth colonial morphology (27). One positive regulator of *vps* genes, VpsR (25), and two negative regulators, HapR (5, 8, 28) and CytR (6), have been identified. In this communication, we report the identification of a second positive regulator of *vps* genes, designated VpsT, which is required for the formation of a corrugated colonial morphology, biofilm formation, and *vps* gene expression in the rugose variant. We further show that VpsT and VpsR positively autoregulate their own expression and also form a complex regulatory network by positively regulating each other's expression.

Identification of *vpsT*. Whole-genome expression profiling of exponentially grown smooth and rugose variants revealed that expression of gene VCA0952 (TIGR annotation; now named *vpsT*) was fourfold elevated in the rugose variant compared to that in the smooth variant (F. H. Yildiz et al., submitted for publication). The *vpsT* gene product is 671 bp long and is predicted to encode a 224-amino-acid, 25.8-kDa protein that is similar to proteins that belong to the UhpA (FixJ) family of transcriptional response regulators (12, 15). VpsT is homologous (44% homology and 65% similarity) to the transcriptional regulators CsgD and AgfD from *Escherichia coli* and *Salmonella enterica*, respectively. CsgD and AgfD are required for the production of extracellular matrix components, cellulose and curli fimbriae, which are important for the development of wrinkled colonies and biofilm formation in these bacteria (3, 4, 16, 17).

VpsT affects colony morphology. In order to determine the role of *vpsT* in maintaining the rugose colonial morphology and associated phenotypes, we deleted *vpsT* in the *V. cholerae* O1 El Tor rugose variant (FY_Vc_0004) (Table 2), designated the RΔ*vpsT* mutant (FY_Vc_0005). Deletions were done according to the modified method of Horton (2, 7, 9), using the VCA0952 primer set listed in Table 1. The RΔ*vpsT* mutant exhibited a smooth colonial morphology on Luria-Bertani (LB) agar plates (Fig. 1), indicating that the *vpsT* gene product is required for the formation of a rugose colonial morphology. To verify that the smooth colony phenotype of the RΔ*vpsT* mutant was caused by the *vpsT* deletion, we amplified the wild-type copy of *vpsT*, including 559 bp upstream and 380 bp downstream, and cloned it into the low-copy-number plasmid pACYC177, generating pCC17. Introduction of pCC17 into the RΔ*vpsT* mutant resulted in conversion of the smooth colonial morphology to the wild-type rugose colonial morphology (Fig. 1). We also found that *vpsT* cloned from either the smooth (pCC14) or the rugose (pCC16) variant complemented the RΔ*vpsT* mutant, indicating that this gene is not physically altered during phase variation. Introduction of the cloning vector alone did not result in complementation. Deletion of the previously identified transcriptional regulator VpsR (using primer set VC0665) (Table 1) in the rugose variant also resulted in formation of a smooth colonial morphology (25). To determine any epistasis between the two positive transcriptional regulators, we generated a Δ*vpsR* Δ*vpsT* double mutant

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TABLE 1. Primers used in this study

Primer	Sequence[a]
VC2338_A	CATCACCCTAAGCAGCAG
VC2338_B	*TTTACTCCTCGGCTTG*CCACAATAAGCCAGAGAGC
VC2338_C	*ATTGTGG*CAAGCCGAGGAGTAAAGAAG
VC2338_D	GCCAGACCACCACGATGATAACCAATC
VCA0952_A	GTCCCATGGCGATATTGTGGGTGGACGC
VCA0952_B	*TGTTAGGA*GTCTCTTCGAGTTTTAGTTCAATAGGC
VCA0952_C	*CTCGAAGAGA*CTCCTAACACATCAAGGCTAACATG
VCA0952_D	CTCGAGCTCGATACGCTATGAATAAATTGAGTGGTAAG
VCA0952_rev_prom	GCGCTATCTTTTGTTTACTTGACGC
VC0665_AII	CCGCCATGGGCTCCTGACCAATACTCACACTATCCG
VC0665_BII	*GTTGCACGCG*CAGTCTCGACAAATTACGCATCGG
VC0665_C	*TTCCGTATGG*CGCGTGCAACCATGTATCGC
VC0665_D	CTGGAGCTCGCTCAAATCATTGCCCATTGCG
*vpsL*_prom_5′	GGATCCTGAGTGAGTGACATATTGTTCTGTTTTTCCT
*vpsL*_prom_3′	GGATCCTTAGTTAGTTAGTACTGAATCCATACGGAAT
*vpsA*_prom_5′	GGATCCTGAGTGAGTGATTTCTTAGCAAGGCGAAT
*vpsA*_prom_3′	GGATCCTTAGTTAGTTACAGAGGTGCCATTTTGAT
*vpsR*_prom_5′	GGATCCTGAGTGAGTGAACGATGCTGAAGACCAAGAT
*vpsR*_prom_3′	GGATCCTTAGTTAGTTAGTACTGAATCCATACGGAAT
lacZrev4	GCCAGTGAATCCGTAATCATGGTC

[a] Restriction sites are underlined and additional primer sequences that anneal with another primer sequence are shown in italics.

TABLE 2. Plasmids and strains used in this study

Plasmid or strain	Properties	Reference or source
Plasmid		
pCC2	pGP704sac-28Δ*lacZ*	This study
pCC9	pGP704sac-28Δ*vpsT*	This study
pCC27	pGP704sac-28Δ*vpsR*	This study
pGP704sac-28		Laboratory collection
pRS415		19
pCC11	pRS415 *vpsA* promoter	This study
pCC12	pRS415 *vpsL* promoter	This study
pCC25	pRS415 *vpsT* promoter	This study
pCC10	pRS415 *vpsR* promoter	This study
pCC14	pCR 2.1-TOPO S*vpsT*[a]	This study
pCC16	pCR 2.1-TOPO R*vpsT*	This study
pACYC177		Fermentas
pCC17	pACYC177 R*vpsT*	This study
GFP	pv25.1	20
Strain		
FY_Vc_0001	O1 El Tor, Inaba, smooth, Rif^r	26
FY_Vc_0002	O1 El Tor, Inaba, rugose, Rif^r	25
FY_Vc_0003	smoothΔ*lacZ*	This study
FY_Vc_0004	rugoseΔ*lacZ*	This study
FY_Vc_0005	RΔ*vpsT* Δ*lacZ*	This study
FY_Vc_0006	RΔ*vpsR* Δ*lacZ*	This study
FY_Vc_0007	RΔ*vpsR* Δ*vpsT* Δ*lacZ*	This study

[a] S denotes the gene that originated from a smooth variant; R denotes the gene that originated from a rugose variant.

in the rugose variant and observed that the colonial morphologies of the single and double mutants did not differ in their characteristics (Fig. 1). Taken together, these results indicate that both VpsT and VpsR are involved in the formation of corrugated colonies.

VpsT affects biofilm formation. In the rugose variant, formation of corrugated colonies and well-developed biofilms depend on VPS$^{El\ Tor}$ production. To determine whether VpsT influences biofilm formation, we compared quantitative and qualitative differences in biofilms of the RΔ*vpsT* mutant to those of the rugose and smooth variants. For quantitative analysis, biofilms were formed on polyvinyl microtiter plates. After 8 h of growth in LB medium at 30°C under static conditions, biofilms were quantified by crystal violet staining (1). The results presented in Fig. 2A show that under the conditions tested the rugose variant formed six times more biofilm than the smooth variant. The biofilm-forming capacity of the RΔ*vpsT* mutant was reduced to the level of that of the smooth variant. Complementation of the RΔ*vpsT* mutant by pCC17 restored biofilm formation to wild-type levels. The cloning vector alone did not result in complementation. The biofilm formation of the RΔ*vpsR* mutant and of the RΔ*vpsR* Δ*vpsT* double mutant was similar to that of the RΔ*vpsT* mutant and also to that of the smooth variant (Fig. 2A). The growth rates of the strains were similar (data not shown), indicating that the differences in biofilm formation were not due to different growth rates.

To compare the biofilm morphologies of the wild-type variants and the mutants, we introduced plasmid pV25, harboring a gene constitutively expressing the green fluorescent protein (20), into each strain. Biofilms were formed on a borosilicate cover glass in LB medium at 30°C under static conditions and analyzed after 12 h by confocal scanning laser microscopy. Horizontal projected views (Fig. 2B) show that the rugose variant formed biofilms with distinct islands, whereas the smooth variant mainly attached as evenly spread-out single cells. Vertical views of the same biofilms show that the rugose biofilm (approximately 60 μm) was about five times thicker

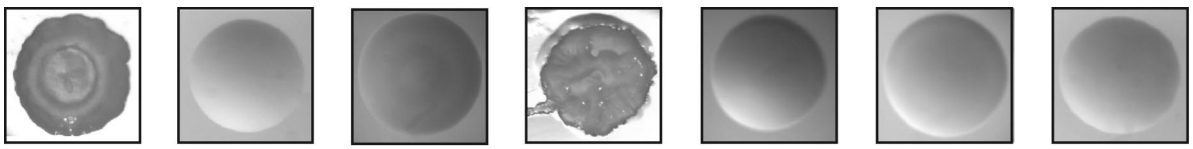

FIG. 1. VpsT is required for the rugose colonial morphology. Colonial morphology of the smooth and rugose variants and the indicated deletion mutants formed on LB plates after 2 days of incubation at 30°C is shown.

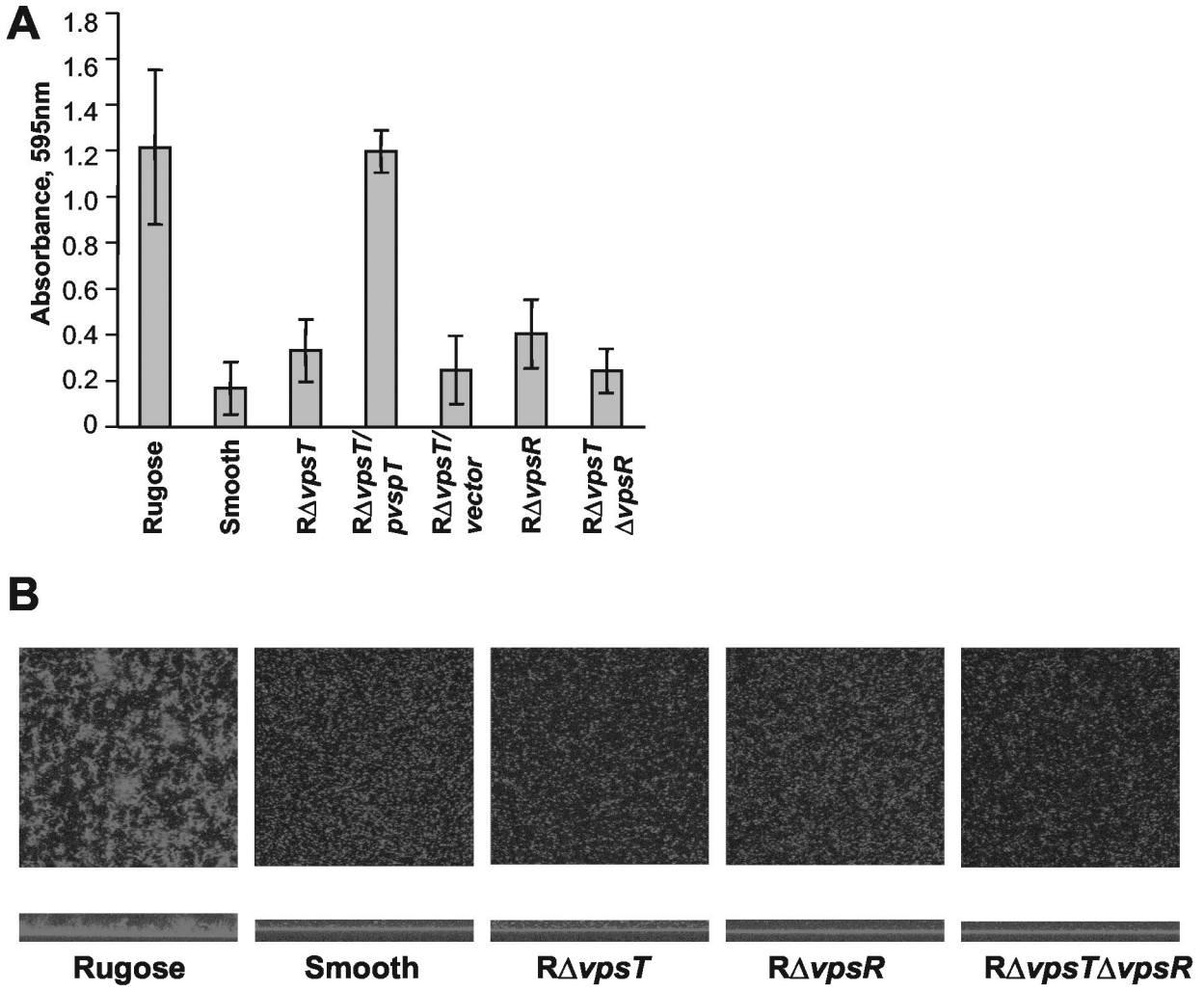

FIG. 2. Biofilm formation of *vpsT* and *vpsR* mutants. (A) Biofilm biomass measured with crystal violet-staining assay. The figure shows the average and standard deviation of three independent measurements for each mutant and for the wild-type parents. (B) Confocal scanning laser microscopic images of biofilms formed on borosilicate cover glass chambers. Top panels, horizontal projections; bottom panels, vertical projections.

than the smooth biofilm and displayed distinctive structures. Surface colonization of the RΔ*vpsR* and RΔ*vpsT* mutants and the RΔ*vpsR* Δ*vpsT* double mutant was similar to that of the smooth variant. Together these results suggest that VpsR and VpsT both contribute to biofilm formation in the rugose variant.

VpsT induces *vps* gene expression. The RΔ*vpsT* mutant is affected in its capacity to form rugose colony morphology and biofilms, which both depend on *vps* gene expression (6, 25). As VpsT is homologous to response regulators of two-component signal transduction systems, we examined whether VpsT affects *vps* gene expression. For the expression analysis, we chose *vpsA* (VC0917) and *vpsL* (VC0934), which are the first genes of the two *vps* operons (27), respectively. We constructed transcriptional fusions of the upstream regulatory sequences of *vpsA* (574 bp upstream) and *vpsL* (565 bp upstream) to the β-galactosidase (*lacZ*) gene. To this end, *vpsAp* and *vpsLp* were amplified and cloned upstream of promoterless *lacZ* in vector pRS415, yielding plasmids pCC11 and pCC12, respectively. Both plasmids and the parent vector (pRS415) (19) were introduced into the smooth, rugose RΔ*vpsT* and RΔ*vpsR* mutants and the RΔ*vpsR* Δ*vpsT* double mutant. Transcription was measured by determining β-galactosidase activity (13) of cultures grown to mid-exponential phase (optical density at 600 nm, 0.3 to 0.4) in LB medium at 30°C by shaking. Figure 3A shows that *vpsA* transcription was five times higher in the rugose variant than in the smooth variant. Deletion of the *vpsT* gene in the rugose variant resulted in a significant reduction of *vpsA* gene transcription. Deletion of *vpsR* resulted in a similar decrease in *vpsA* gene transcription. The RΔ*vpsR* Δ*vpsT* double mutant had low *vpsA* gene expression, similar to that for the individual deletion mutants. Strains transformed with vector pRS415 had no β-galactosidase activity (data not shown). *vpsL* transcription was forty times higher in the rugose variant than in the smooth variant. Transcription of *vpsL* was markedly (13-fold) decreased in the RΔ*vpsT* mutant. In contrast, in the RΔ*vpsR* mutant and the RΔ*vpsR* Δ*vpsT* double mutant, *vpsL* transcription was below the detection level. The results indi-

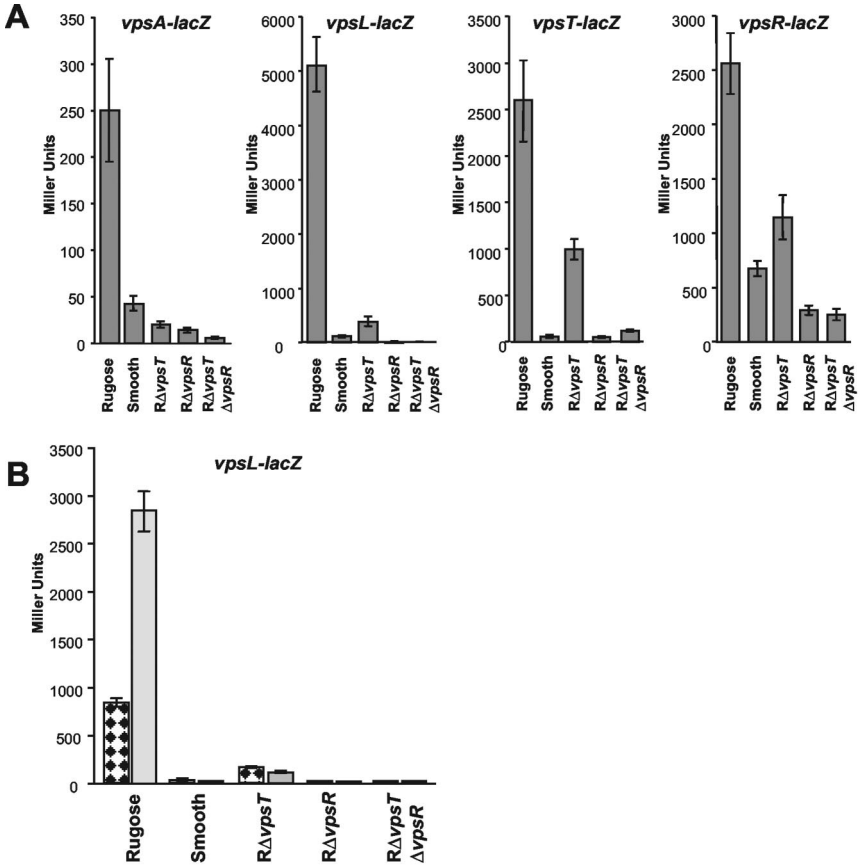

FIG. 3. *vps* gene expression in logarithmic, planktonic, and biofilm growth states. (A) Expression of *vpsA*, *vpsL*, *vpsT*, and *vpsR* in logarithmically grown wild-type variants and mutants. (B) *vpsL* transcription in planktonic cells (stippled bars) and in biofilm cells (grey bars) in the indicated mutants. Error bars indicate the standard deviation. Note the different *y* axis scales in all panels.

cate that VpsT and VpsR are both required for maximal transcription of the *vpsA* and *vpsL* promoters in the rugose variant during the logarithmic growth phase. Transcription of *vpsL* was higher than that of *vpsA* in all tested strains. The relative effects of VpsT were similar on *vpsA* and *vpsL* transcription. VpsR deletion, on the other hand, had a stronger effect on *vpsL* transcription than on *vpsA* transcription. The results suggest that the regulation of these two genes is different in the rugose variant. Interestingly, there is a computationally identified VpsR binding site upstream of *vpsL* but not upstream of *vpsA* (Yildiz et al., submitted). It remains to be determined if the actions of VpsT and VpsR are indeed mediated by the direct binding to the *vpsL* and *vpsA* promoter regions or through other regulatory proteins. Complex regulation of exopolysaccharide biosynthesis genes is a common phenomenon. In the alginate biosynthesis pathway of *Pseudomonas aeruginosa*, response regulators AlgB and AlgR are both required as positive regulators of *algD*, the first gene of the alginate biosynthetic operon (11, 24). Furthermore, activation of the *eps* operon, which harbors genes required for exopolysaccharide I production in *Ralstonia solanacearum*, is mediated by two response regulators that are themselves under the control of a complex regulatory network (18).

To determine a possible interaction between the two positive regulators, we analyzed *vpsT* and *vpsR* transcription in the described strains (Fig. 3). To this end, we constructed *vpsRp-lacZ* and *vpsTp-lacZ* transcriptional fusions by amplifying the upstream regulatory sequences of *vpsT* (primers VCA0952_C and rev_prom) and *vpsR* (primers *vpsR*_prom_5′ and *vpsR* _prom_3′) and cloning them into pRS415. Transcription of *vpsT* and *vpsR* was determined during exponential growth (optical density at 600 nm, 0.3 to 0.4) in LB medium at 30°C by measuring β-galactosidase activity. The results revealed that *vpsT* transcription was 45 times higher in the rugose variant than in the smooth variant, confirming the trend of the initial microarray experiment (Yildiz et al., submitted). Furthermore, *vpsT* transcription was 2.5-fold lower in the RΔ*vpsT* mutant than in the rugose variant, indicating that VpsT positively regulates its own expression. Deletion of *vpsR* from the rugose variant caused a 50-fold reduction in *vpsT* expression. *vpsT* expression in the RΔ*vpsR* RΔ*vpsT* double mutant was similar to that in the RΔ*vpsR* mutant.

We also determined *vpsR* transcription in wild-type phase variants and the mutant strains. Figure 3 shows that *vpsR* transcription was fourfold higher in the rugose variant than in the smooth variant. The experiments also showed that, compared to that for the rugose variant, *vpsR* transcription was decreased twofold in the RΔ*vpsT* mutant and ninefold in the RΔ*vpsR* mutant and in the RΔ*vpsR* Δ*vpsT* double mutant. The results indicate that VpsT and VpsR positively regulate *vpsR*

expression and that VpsR had a more dramatic effect on its own expression.

Next we examined whether *vpsA* and *vpsL* transcription differ in planktonic and biofilm cells. For these measurements, overnight-grown cultures were diluted in LB medium, inoculated into polystyrene petri plates, and incubated at 30°C under static conditions for 12 h. β-Galactosidase activities of planktonic and attached bacteria were compared for each of the strains. In the rugose variant, *vpsL* transcription was three times higher in the biofilm cells than in the planktonic cells (Fig. 3B). This result is similar to measurements in the *V. cholerae* O139 strain (6). The smooth variant formed less-developed biofilms. Significantly, biofilm cells of the smooth variant did not have an increased *vpsL* transcription compared to that of planktonic cells. *vpsL* transcription in smooth planktonic and biofilm cells was 30- and 100-fold lower, respectively, than expression in the corresponding rugose cells. When *vpsT* was deleted from the rugose variant, the biofilm growth-dependent *vpsL* induction did not occur. In addition, *vpsL* transcription in the RΔ*vpsT* mutant planktonic and biofilm cells was 5- and 25-fold lower than that for the respective rugose variant cells.

Deletion of *vpsR* in the rugose variant or in the double mutant prevented *vpsL* transcription altogether, similar to the results obtained from logarithmically grown cells. *vpsA* transcription in the rugose variant was increased 25% in the biofilm cells compared to that in the planktonic cells (data not shown). In contrast, this induction was not observed in the other strains.

In summary, we have identified VpsT, a positive regulator of *vps* gene expression. VpsT and the previously identified regulator VpsR (25) are both necessary for maximal *vps* transcription in the rugose variant. VpsT and VpsR both influence *vpsA* and *vpsL* expression and positively regulate their own and each other's expression.

VpsR and VpsT are homologous to response regulators of two-component regulatory systems. Response regulators usually act together with a sensor histidine kinase. Sensor histidine kinase(s) that regulates expression of *vps* genes and in turn the development of the rugose colonial morphology of *V. cholerae* O1 El Tor has not been identified thus far and is under investigation.

V. cholerae occupies different niches during its life cycle and is likely to be exposed to fluctuating environmental conditions (26). Two-component signal transduction systems are involved in sensing and responding to environmental stimuli. Future work will focus on the identification of environmental signals sensed by signal transduction systems involving VpsR and VpsT and on the importance of the processes regulated by these two regulators in the adaptation responses of the pathogen.

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