

# VpsR, a Member of the Response Regulators of the Two-Component Regulatory Systems, Is Required for Expression of *vps* Biosynthesis Genes and EPS<sup>ETr</sup>-Associated Phenotypes in *Vibrio cholerae* O1 El Tor

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**The rugose colonial variant of *Vibrio cholerae* O1 El Tor produces an exopolysaccharide (EPS<sup>ETr</sup>) that enables the organism to form a biofilm and to resist oxidative stress and the bactericidal action of chlorine. Transposon mutagenesis of the rugose variant led to the identification of *vpsR*, which codes for a homologue of the NtrC subclass of response regulators. Targeted disruption of *vpsR* in the rugose colony genetic background yielded a nonreverting smooth-colony morphotype that produced no detectable EPS<sup>ETr</sup> and did not form an architecturally mature biofilm. Analysis of two genes, *vpsA* and *vpsL*, within the *vps* cluster of EPS<sup>ETr</sup> biosynthesis genes revealed that their expression is induced above basal levels in the rugose variant, compared to the smooth colonial variant, and requires *vpsR*. These results show that VpsR functions as a positive regulator of *vpsA* and *vpsL* and thus acts to positively regulate EPS<sup>ETr</sup> production and biofilm formation.**

*Vibrio cholerae* O1, the causative agent of Asiatic cholera, has been isolated from coastal and estuarine environmental samples, both as free-living bacteria and in association with phytoplankton, zooplankton, crustacea, and mollusks (15–17). These distribution studies and its capacity to adaptively respond to changes in salinity, temperature, and the availability of nutrients (30, 31) have led to the idea that *V. cholerae* O1 can successfully occupy one or more ecological niches in a variety of aquatic habitats.

Laboratory microcosm studies conducted with *V. cholerae* O1 have shown that the duration of its survival in seawater is decreased if particulate matter is removed by filtration before inoculation of the filtrate with the organism. One interpretation of this result is that attachment to surfaces is important for long-term survival of the organism in marine environments. Growth on surfaces is believed to be advantageous because surfaces adsorb and thus concentrate scarce nutrients in the fluid phase. In addition, biotic surfaces, such as chitin, can be degraded by attached bacteria releasing assimilable sources of carbon and nitrogen (9). Thus, the surface mode-of-growth is likely to be preferred by *V. cholerae* in natural aquatic habitats.

Biofilms are a specialized and highly adapted form of surface growth characterized by assemblages of bacteria that form pillars or mushroom-like structures separated by fluid-filled channels. Pillars, in turn, consist of an extracellular polysaccharide (EPS) matrix and the bacteria that secrete it. Since EPS accounts for about 85% of biofilm depth, the production of EPS is critical for the development of a mature biofilm (4, 21). *V. cholerae* O1, biotype El Tor, in common with many aquatic bacterial species, forms a typical three-dimensional

biofilm on a variety of abiotic surfaces (36, 39). Investigation of this phenotype showed that the rugose colonial variant forms a thicker and more differentiated biofilm than the smooth colonial variant (39). This capacity was found to be associated with production of a glucose- and galactose-rich EPS by the rugose form. Designated EPS<sup>ETr</sup>, this compound was also shown to inactivate chlorine (39) and protect the organism from the bactericidal action of hydrogen peroxide (35). Open reading frames (ORFs) required for EPS<sup>ETr</sup> synthesis are clustered in a 30.7-kb segment on the *V. cholerae* O1 chromosome. Because their putative protein products are homologous to capsular or EPS biosynthetic enzymes of other species, the corresponding *Vibrio* genes were designated *vps*, for *Vibrio* polysaccharide (39).

Phase transition occurs between the rugose and smooth colonial variants of *V. cholerae* O1 El Tor. Unlike the rugose form, the smooth-colony type of the strain A1552 does not produce EPS, forms only low-profile biofilms, and is rapidly killed by chlorine (39). Phenotype differences of this kind between the two colonial variants suggest that the rugose form may be better adapted for growth and survival in natural aquatic habitats and that transition frequencies between the two types may be governed by environmental signals.

Here we describe the identification, cloning, and characterization of *vpsR*, a gene that regulates the expression of the *vps* biosynthetic gene cluster. VpsR exhibits homology to the response element of two-component regulatory systems, which are involved in responding to environmental stimuli. Construction of a *vpsR* knockout mutation in a *V. cholerae* O1 El Tor rugose genetic background disclosed the role of this gene in colony morphology, EPS production, and biofilm formation. Studies of the smooth-colony type of a second strain of *V. cholerae* O1 El Tor (N16961) showed that the expression of *vpsR* and the genes it controls within the *vps* biosynthetic clus-

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ter exhibit interstrain differences that correspond to differences in biofilm forming capacity.

## MATERIALS AND METHODS

**Media and growth conditions.** All strains were maintained at  $-80^{\circ}\text{C}$  in Luria-Bertani (LB) broth supplemented with glycerol (15%, vol/vol). For the experiments described below, the cells were grown aerobically at  $30^{\circ}\text{C}$  in LB broth, unless specified otherwise. The following antibiotics were added as appropriate: ampicillin (100  $\mu\text{g}/\text{ml}$ ) and chloramphenicol (5  $\mu\text{g}/\text{ml}$ ).

**Bacterial strains.** *Escherichia coli* strains DH5 $\alpha$  and S17-1 were used for standard DNA manipulations and mating, respectively. The *V. cholerae* strains used were smooth and rugose variants of 92A1552 (wild type, El Tor, Inaba, and Rif<sup>r</sup>) and *vpsR* and *rpoN* mutants of these strains, as well as strain N16961 (wild type, El Tor, and Inaba).

**Scanning electron microscopy.** Pieces of agar containing a number of colonies were excised and processed sequentially as follows: 3% glutaraldehyde in 0.1 M sodium cacodylate for 60 min, 0.1 M sodium cacodylate for 5 min, 2% osmium tetroxide in 0.1 M sodium cacodylate for 45 min, and 0.1 M sodium cacodylate for 5 min. These samples were incubated with increasing concentrations of ethanol (15, 30, 50, 70, 90, and 100%) each for 15 min and then subjected to critical point drying, sputter coated, and analyzed by scanning electron microscopy (Philips XL40).

**Transmission electron microscopy.** Bacterial cells were grown on a sterile dialysis membrane placed on the surface of LB plates, and the plates were incubated at  $30^{\circ}\text{C}$  for 24 h. Membrane-attached bacteria were processed sequentially as follows: 1% glutaraldehyde in 0.05 M sodium cacodylate containing 0.375% ruthenium red for 60 min, 0.05 M sodium cacodylate for 15 min, 2% osmium tetroxide in 0.2 M sodium cacodylate containing 1.5% ruthenium red for 60 min, and 0.2 M sodium cacodylate for 5 min. The cells were then incubated with ascending concentrations of ethanol (15, 30, 50, 70, 90, and 100%) each for 15 min and embedded according to standard procedures. The thin sections were stained with 1% uranyl acetate and then with lead citrate and then visualized using a Philips CM12 electron microscope.

**CSLM.** Biofilms were formed by incubating 200  $\mu\text{l}$  of a 1/100 dilution of an overnight LB broth culture grown on borosilicate cover chambers. The chambers were then incubated at  $30^{\circ}\text{C}$  for 24 h, rinsed with phosphate-buffered saline and analyzed by confocal scanning laser microscopy (CSLM) (MultiProbe 2010; Molecular Dynamics) using 488- and 510-nm excitation and emission wavelengths, respectively. The horizontal (xy) sections were obtained and the images were reconstructed using a maximum intensity algorithm.

**Quantitative biofilm assay.** Biofilm formation was quantitatively monitored by incubating 200  $\mu\text{l}$  of a 1/100 dilution of an overnight LB broth culture in microtiter plates (Falcon 3911). The microtiter plates were then incubated at  $30^{\circ}\text{C}$  for 24 h, and biofilm formation was quantified by crystal violet staining and ethanol solubilization as described elsewhere (27).

**Transposon mutagenesis.** Tn5 mutagenesis of *V. cholerae* O1 El Tor, strain 92A1552, was performed by conjugation between the donor *E. coli* S-17 $\lambda$ pir containing a pool of  $\sim 40,000$  signature-tagged Tn5Km2 DNAs on plasmids encoding resistance to ampicillin and the recipient, a rifampin-resistant *V. cholerae* O1 El Tor 92A1552 rugose variant. Conjugation was performed by mixing equal volumes of the donor and recipient, followed by filtration through a 0.45- $\mu\text{m}$ -pore-size filter (Nalgene). The filters were then placed on LB agar plates, incubated for 5 h at  $37^{\circ}\text{C}$ , and suspended in LB, and the bacteria were recovered by vortexing. The exconjugants were selected by plating the suspension onto LB plates supplemented with 100  $\mu\text{g}$  of rifampin and 150  $\mu\text{g}$  of kanamycin per ml. A total of 15,000 exconjugants were screened visually to isolate mutants exhibiting smooth colonial morphology.

**DNA manipulation and analysis.** Plasmid DNA and chromosomal DNA preparation, DNA ligation, bacterial transformation, agarose gel electrophoresis, PCR, and Southern blotting were carried out by the standard techniques described by Sambrook et al. (29). Restriction enzymes and DNA modification enzymes were purchased from New England Biolabs, Inc.

**Cloning of the transposon insertion site.** Chromosomal DNA was isolated from the mutants using standard procedures; digested singly with *EcoRI*, *SalI*, *KpnI*, and *PstI*, the fragments resolved on an agarose gel; transferred to Hybond N membranes; and hybridized to the kanamycin-resistance gene of pUT mini-Tn5-Km2. Restriction enzymes that yielded hybridizing fragments of between 3 and 8 kb were used to digest chromosomal DNA, the resulting fragments were ligated into pBluescript KS that had been digested with the corresponding enzyme, and the plasmids were transformed into DH5 $\alpha$ , followed by selection for kanamycin- and ampicillin-resistant transformants.

**Construction of cosmid library.** A *V. cholerae* O1 El Tor 92A1552 gene bank was constructed by partially digesting wild-type genomic DNA with 0.016 U of *Sau3A* per  $\mu\text{g}$  for 5 min, which yielded mainly 25- to 40-kb fragments. These partially digested, dephosphorylated fragments were ligated into the vector SuperCos/Mob-II that had been digested with *XbaI* and *BamHI*. This vector had been constructed by introducing a 1,700-bp *BamHI* mobilization fragment (Mob) from GP704 into the *BglII* site of pSuperCos (Stratagene). The ligation mixture was packaged into bacteriophage lambda particles using the Gigapack III gold packaging extract (Stratagene). The titer of the library was determined, and the library was amplified using *E. coli* XL1-Blue MR as a host cell and stored at  $-80^{\circ}\text{C}$  in 25% glycerol.

**PCR amplification of *vpsR*.** A 2,890-bp fragment containing *vpsR* was amplified using the following primers: *VpsR2-F* (GTTCTATGATGCCGACTACA) and *VpsR2-R* (ACGCTTCTCACGCTACTTT). The amplified fragment includes 431 nucleotides of upstream sequence, the entire *vpsR* coding sequence, and 1,130 nucleotides of sequence downstream of *vpsR*. *BamHI* and *SalI* restriction sites were added to forward and reverse primers, respectively, to facilitate cloning into pACYC184. The PCR products were generated using a high-fidelity PCR kit (Clontech), digested with *BamHI* and *SalI*, and the resulting fragment was ligated into pACYC184 that had been digested with the corresponding enzymes to generate p1. The ligation mix was then transformed into DH5 $\alpha$ , and the transformants were selected on LB plates supplemented with chloramphenicol. The plasmids were introduced into *V. cholerae* mutant strains by electroporation. The ability of the plasmid to complement the mutations was initially tested by a change in the colony morphology from smooth to rugose and then tested by expression analysis.

**Generation of null mutants.** Mutants with insertions into the *vpsR* and *rpoN* genes were regenerated in a new rugose strain background. For *rpoN*, primers *rpoN1-F* (CCCTTTAGCGATGTGGAT) and *rpoN1-R* (CAGCCATTTGCGCTCTTG) were used to amplify an internal 558-nucleotide region of *rpoN* corresponding to amino acids 144 to 339. For *vpsR*, primers *vpsR1-F* (GGGAATC TATGCCTATGAAG) and *vpsR1-R* (CGTCTCCACAGTCCCTTCTTG) were utilized to generate a 351-nucleotide internal fragment corresponding to amino acids 148 to 264. *XbaI* and *EcoRI* restriction sites were added to forward and reverse primers, respectively, to facilitate the cloning into pGP704. The plasmids were introduced to *V. cholerae* O1 El Tor wild-type rugose and smooth colonial variants by biparental mating, and exconjugants were selected on LB plates supplemented with 100  $\mu\text{g}$  of ampicillin and rifampin per ml. Insertion into the correct site was confirmed by Southern and PCR analysis.

**RNA preparation.** Overnight cultures of *V. cholerae* in LB medium at  $37^{\circ}\text{C}$  were diluted 1:100 in fresh LB medium. At the mid-log-phase growth point, 10-ml aliquots of culture were collected. Cell pellets were frozen with liquid nitrogen and stored at  $-80^{\circ}\text{C}$ . RNA was isolated from frozen pellets using Trizol reagent (Gibco-BRL). For biofilm RNA isolation, overnight LB culture was diluted 1:100, and 25 ml of this culture was incubated in petri plates at  $30^{\circ}\text{C}$  for 24 h. The plates were rinsed with LB three times, the attached bacteria were resuspended in 2 ml of Trizol reagent, and the total RNA was isolated according to the manufacturer's instructions. To remove contaminating DNA, 50  $\mu\text{g}$  of total RNA was incubated with 4 U of RNase-free DNase I (Ambion) for 30 min at  $37^{\circ}\text{C}$ . The RNeasy Mini Kit (Qiagen) was used to clean up RNA after DNase digestion, and the RNA was stored at  $-80^{\circ}\text{C}$ .

**cDNA synthesis.** Reverse transcription (RT) of RNA was performed with TaqMan RT reagents (Perkin-Elmer) and nuclease-free water (Ambion). Five hundred nanograms of total RNA was used for RT in a final volume of 50  $\mu\text{l}$  containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 5.5 mM MgCl<sub>2</sub>, 500  $\mu\text{M}$  concentrations of each deoxynucleotide triphosphate (dNTP), 2.5  $\mu\text{M}$  concentrations of random hexamers, 20 U of RNase inhibitor, and 62.5 U of Multiscribe Reverse Transcriptase. The RT-negative control reactions (without RT) were performed to determine how much contaminating genomic DNA was present in each total RNA sample. The reactions were incubated in GeneAmp PCR System 9700 (Perkin-Elmer) by using parameters of  $25^{\circ}\text{C}$  for 10 min,  $48^{\circ}\text{C}$  for 30 min,  $95^{\circ}\text{C}$  for 5 min, and cooling at  $4^{\circ}\text{C}$ . The cDNA samples were stored at  $-20^{\circ}\text{C}$ .

**Primers and probes for real-time RT-PCR.** Primers and probes for the three genes of interest, *vpsR*, *vpsA*, and *vpsL*, and for rRNA were chosen using Primer Express software (Perkin-Elmer). Fluorogenic probes were used to monitor PCR product formation continuously during PCR. The probe is an oligonucleotide, dually labeled with a reporter dye (FAM; 6-carboxyfluorescein) covalently attached at the 5' end and a quencher dye (TAMRA; 6-carboxytetramethylrhodamine) covalently attached at the 3' end. Primers and probes were synthesized and purified by Biosearch Technologies. The primers and probes used for real-time PCR were as follows: *vpsR-F* (21-mer), GTCTCAGCTCGATCTTCCCAA; *vpsR-R* (20-mer), CGTTCGCCGAATGCTTTTCAG; *vpsA-F* (19-mer), TTCCCC TTGGCCTGAAGAG; *vpsA-R* (21-mer) AGGTGCAAAGTGGTACTGCGT;

*vpsL*-F (21-mer), ATCGCACCATAGTGAATCGCT; *vpsL*-R (21-mer), TCTGT GCCATCCAGTAATGC; rDNA-F (ribosomal DNA [rDNA]; 18-mer), GAGC GGACGACACAGGA; rDNA-R (21-mer), TTTCCAGGCATTACTCACCC; *vpsR* TaqMan probe (20-mer), 5'-FAM-CTGCGACGGCCATCACTGCG-TA MRAP3'; *vpsA* TaqMan probe (20-mer), 5'-FAM-CCGCAAACCTACGGCCGCA C-TAMRAp3'; *vpsL* TaqMan probe (25-mer), 5'-FAM-CATGCTGCGTCACAA AGTGAAGCCC-TAMRAp3'; and rDNA TaqMan probe (22-mer), 5'-FAM-CG CTCGCCACCAAGGAACAAG-TAMRAp3'. The amplicon lengths generated using these primers were 67, 69, 63, and 67 bp for *vpsA*, *vpsL*, *vpsR*, and rDNA, respectively.

**PCR amplification conditions for RT-PCR analysis.** PCR conditions were identical for all reactions. For each PCR run, a master mix was prepared on ice with the following components: 1× TaqMan Universal Master Mix containing AmpliTaq Gold DNA polymerase, Amp Erase UNG, dNTPs with UTP, passive reference dye, and optimized buffer components (Perkin-Elmer). To this were added 200 nM of the fluorogenic probe and 900 nM of the forward and reverse primers. Then, 5 µl of each appropriately diluted (250 pg, unless specified) RT sample was added to 20 µl of the PCR master mix. Experiments were performed with three replicates for each datum point, and negative-RT and no-template controls were run for each reaction. Reactions were performed in sealed Micro-Amp Optical 96-well reaction plates (Perkin-Elmer) using an ABI Prism 7700 Sequence Detection System (Perkin-Elmer) for PCR amplification and the following cycle parameters: 2 min for 50°C and 10 min for 95°C, followed by 40 cycles of 15 s for 95°C and 1 min for 60°C.

**Quantitative analysis of RT-PCR data.** Real-time PCR measures the degradation of a fluorescent oligonucleotide probe in real time concurrent with PCR amplification. The method uses a dually labeled probe with a reporter dye FAM at the 5' end and the quencher dye TAMRA at the 3' end. During PCR the 5' nucleolytic activity of *Taq* polymerase separates the 5' reporter dye from the 3' quencher dye, resulting in an increase in the fluorescence of the reporter dye, which can be detected by the laser detector. In real-time PCR analysis, quantitation is based on cycle threshold (Ct) calculations (10, 12). Ct is defined as the number of cycles required for reporter dye fluorescence, resulting from the synthesis of PCR products, to become significantly higher than background fluorescence. There is an inverse exponential relationship between the logarithm of initial target copy number in the reaction and the corresponding Ct determinations by the model 7700 instrument. Validation experiments were performed for each PCR primer-probe set, and these yielded a linear relationship when Ct was plotted against the logarithm of a varying amount of cDNA reverse-transcribed from total RNA in the range of 10 pg to 1 ng. Twofold serial dilutions of cesium chloride-purified chromosomal DNA of *V. cholerae*, corresponding to 10<sup>1</sup> to 10<sup>6</sup> theoretical copies (the molecular weight of *V. cholerae* genomic DNA was estimated to be 4.1 Mbp), were used in real-time PCR. For each set of TaqMan amplicons, the standard curve was constructed by using the equation  $y = a + b \log x$ , where  $x$  is the starting DNA copy number,  $y$  is the Ct obtained by amplifying  $x$  copies,  $a$  is the  $y$  intercept of the standard curve line, and  $b$  is the slope of the standard curve line. The results of this calculation were used to estimate the number of cDNA copies present in the different samples.

## RESULTS

**Isolation of the *vpsR* regulatory mutant and cloning of the corresponding gene.** To identify genes required for the rugose colony type, EPS production, and biofilm formation, the rugose colonial variant of *V. cholerae* O1 El Tor was subjected to transposon mutagenesis; transconjugants that exhibited smooth colonial morphology were selected for subsequent analysis. Chromosomal genes interrupted by transposon insertion were isolated by marker rescue, and DNA sequences flanking these insertion sites were determined. The majority of genes identified by this method were located within the aforementioned 30.7-kb segment of the chromosome that contains the *vps* biosynthetic cluster. However, sequence analysis of other tagged genes also disclosed two independent insertions in another locus situated elsewhere on the chromosome; analysis of these sequences revealed an ORF predicted to encode a protein homologous to the NtrC subclass of response regulators. This ORF was denoted VpsR. A sequence tag corresponding to this locus was used as a probe to isolate a complete copy of *vpsR* from a cosmid library of the smooth colonial

variant. Two such clones were obtained, returned to the mutant, and found to cause conversion from the smooth colonial morphology of the mutant to the rugose colonial morphology of the original wild-type parent.

**DNA sequence analysis of the *vpsR* region.** Sequence analysis of the *vpsR* region was undertaken with specifically designed oligonucleotides using pCos-*vpsR* as a template. A 2.897-kb region encompassing the insertion site and the flanking regions was sequenced and revealed an apparently full-length *vpsR* spanning 1,335 nucleotides and located between nucleotides 431 and 1765 of the cloned segment (Fig. 1A). An incomplete ORF was identified 274 bp upstream of the *vpsR* start codon, in the same orientation, that would specify the carboxy-terminal 51 amino acids of a protein with 87.7% identity and 91.8% similarity to *lysS*, which encodes an *E. coli* lysyl-tRNA synthetase (23). Downstream of *vpsR* and in the same orientation, a third ORF was found between nucleotides 2343 and 2594 of the cloned sequence; it is predicted to encode an 83-amino-acid protein without identifiable homologous sequences in the protein database. Directly downstream of the third ORF, in the opposite orientation, an incomplete ORF was identified; it is predicted to encode the carboxy-terminal 87 amino acids of a protein that exhibits 47.6% identity and 58.1% similarity to the *E. coli* TAS (tyrosine auxotrophy suppressor) protein (34).

*vpsR* is predicted to encode a 444-amino-acid, 49.76-kDa protein homologous to HydG (51% similarity, 35.7% identity) from *Salmonella enterica* serovar Typhimurium (3), AlgB (43.2% similarity, 34.3% identity) from *Pseudomonas aeruginosa* (11, 37), and NtrC (55.8% similarity, 44.1% identity) from serovar Typhimurium (8) (Fig. 1B). Each of these proteins belongs to the response regulator component of two-component signal transduction systems and possesses an N-terminal domain that can be phosphorylated by a sensor kinase, the other component of this signal transduction system (33). The conserved aspartate residue of VpsR (Asp-59) that is postulated to be phosphorylated is indicated by a star (Fig. 1B). Two other conserved residues, Asp and Lys, typically present within the N-terminal domain of response regulators, are not present in the corresponding region of VpsR.

The central region of VpsR harbors the following consensus sequences shown or postulated to be important for interaction with the alternative sigma-54 factor (Fig. 1B): 1, (L,I,V,M,F,Y) 3xG(D,E,Q)(S,T,E)G(S,T,A,V)GKx2(L,I,V,M,F,Y); and 2, (G,S) x(L,I,V,M,F)x2A(D,N,E,Q,A,S,H)(G,N,E,K)G(S,T,I,M)(L,I,V, M,F,Y)3(D,E)(E,K)(L,I,V,M) (7). These are ATP binding motifs and are located in the N-terminal segment of the central domain. ATP binding promotes open complex formation by a sigma-54 containing RNA polymerase (22, 26). A segment of the *vpsR* sequence, which would code for amino acids 214 to 356, had been previously identified by Klose et al. as a sigma-54-dependent transcriptional activator (*s54act5*) through their use of degenerate primers designed to amplify genes coding for this class of transcriptional activators. However, these investigators did not determine the function of the *s54act5* gene product (20).

Like other response regulators, VpsR harbors a helix-turn-helix DNA-binding domain (28), located between amino acids 415 and 434 near the carboxy terminus of the protein (Fig. 1B).

**Generation and analysis of *vpsR* knockout mutants.** To test if *vpsR* is required for rugose colonial morphology, EPS pro-

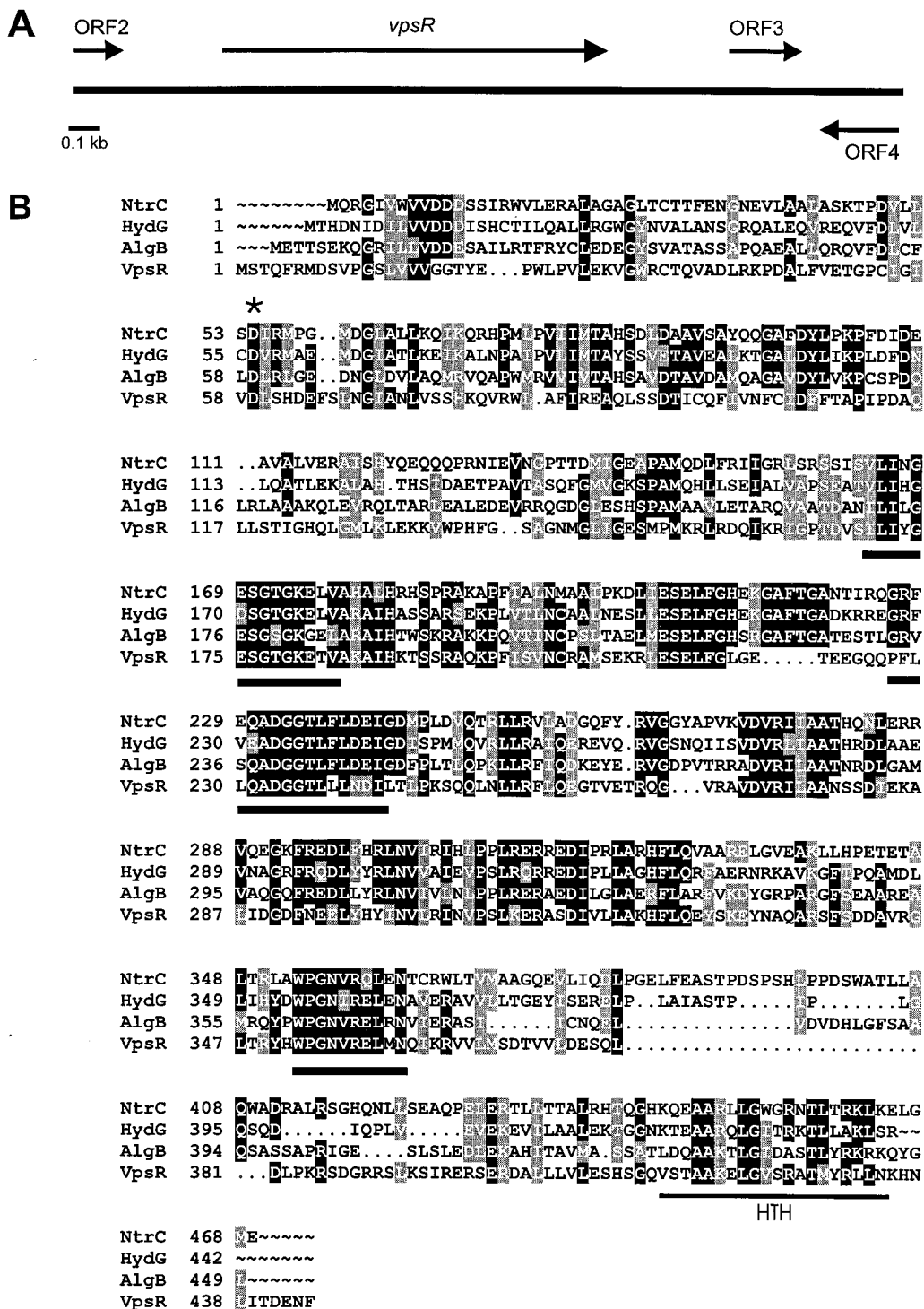


FIG. 1. VpsR homologues and functional motifs. (A) Map of the *vpsR* region on the *V. cholerae* O1 El Tor chromosome. (B) Amino acid sequence alignment of VpsR from *V. cholerae* O1, NtrC from serovar Typhimurium (P41789), HydG from serovar Typhimurium (P25852), and AlgB from *P. aeruginosa* (P23747). Amino acids identical in all four sequences are shaded in black. The star denotes a conserved aspartate residue and a putative phosphorylation site. Predicted RpoN interaction sites are indicated by heavy lines, and a C-terminally located helix-turn-helix (HTH) motif is shown by the thin line.

duction, and biofilm formation, it was independently inactivated in the rugose genetic background by using pGP704 harboring a 351-nucleotide internal fragment, which corresponds to the coding sequence for amino acids 148 to 264. Knockouts

carrying this mutation, designated *vpsR::pGP704*, were examined for colonial morphology, EPS production and localization, and biofilm formation.

The capacity of *V. cholerae* O1 to naturally oscillate between

two distinctive colony morphotypes—the rugose and smooth colonial variants—led to the identification of the rugose-associated phenotypes described above (39). If *vpsR* is required for the rugose colonial morphotype, then disruption of this gene in the rugose background should yield phenotypically “smooth” colonies that do not revert to the rugose colonial variant. The nonreverting nature of the mutation was tested by cultivating *vpsR*::pGP704 under conditions of nutrient limitation that favor reversion of the wild-type smooth variant to the rugose variant (35, 39). This growth condition did not yield any rugose colonies of *vpsR*::pGP704, indicating that the smooth colonial phenotype of this mutant is stable. To determine if disruption of *vpsR* in the rugose genetic background resulted in the same colonial morphology as the wild-type smooth variant, colonies formed by *vpsR*::pGP704 and by the original smooth and rugose colonial variants of the wild-type parent strain were compared by scanning electron microscopy. Figure 2A depicts such a comparison. The colonial morphology of *vpsR*::pGP704 (denoted R-*vpsR* in this figure) is dramatically different from the corrugated colonial morphology of the wild-type rugose parent, even though this mutation was made in the genetic background of the wild-type rugose variant. Instead, colonies formed by *vpsR*::pGP704 resemble the featureless, flat colonies of the wild-type smooth variant.

One explanation for differences between the colony types depicted in Fig. 2A might be qualitative or quantitative differences in the EPS<sup>ETr</sup> produced by each. To compare the abundance, distribution, and tinctorial qualities of EPS produced by *vpsR*::pGP704 and the wild-type rugose and smooth variants of the same strain, each bacterial type was grown on a cellophane dialysis membrane placed on the surface of LB agar plates, and the resulting bacterial lawn was subjected to thin-section transmission electron microscopy of ruthenium red-stained samples, a method that preferentially stains acidic polysaccharides. This method led to the comparative features depicted in Fig. 2B. The wild-type rugose variant produces a ruthenium red-positive matrix between well-separated bacteria. In contrast, the wild-type smooth variant and the *vpsR*::pGP704 mutant were identical; neither produces the ruthenium red-staining interbacterial matrix typical of the rugose variant. EPS<sup>ETr</sup> production by *vpsR*::pGP704 was also tested by enzyme-linked immunosorbent assay using an EPS<sup>ETr</sup>-specific antiserum, and the result showed that *vpsR*::pGP704 was EPS<sup>ETr</sup> antigen negative (data not shown). Taken together, these results provide compelling evidence that *vpsR* is required for EPS<sup>ETr</sup> production.

**Biofilm formation by *vpsR*::pGP704.** The capacity of the rugose colonial variant to form high-profile biofilms is the rugose-associated property most likely to be important for the environmental persistence of this species in natural aquatic habitats. To determine if biofilm formation by the rugose variant is affected by the *vpsR*::pGP704 mutation of the rugose parent strain, two assays were performed that monitor different aspects of the biofilm-forming phenotype. Quantitative differences in biofilm-forming capacity were measured by monitoring the intensity of crystal violet staining of a biofilm that formed on the surface of a polyvinyl chloride microtiter well during 24 h of growth in LB medium (27). This assay was used to examine the wild-type rugose and smooth colonial variant, the *vpsR*::pGP704 mutant, and this mutant complemented with

pACYC184 containing a cloned copy of *vpsR*. To control for possible plasmid effects, each of the other tested strains also contained pACYC184 without the *vpsR* insert. The biofilm-forming capacity of the wild-type rugose variant was found to be 17-fold greater than the wild-type smooth variant (Fig. 3A). In contrast, the biofilm-forming capacity of the noncomplemented *vpsR*::pGP704 mutant of the rugose variant was intermediate between the wild-type rugose and smooth variants, being 3-fold less than that of the rugose variant but 5.5-fold greater than that of the smooth variant (Fig. 3A). Furthermore, a time course of biofilm development by the *vpsR*::pGP704 mutant also exhibited an intermediate biofilm formation phenotype, where the biofilm-forming capability of the *vpsR* mutant is less than that of the wild-type rugose and more than that of the wild-type smooth strain (data not shown).

Complementation of this mutant with an intact copy of *vpsR* increased its biofilm-forming capacity to nearly the same level as the wild-type rugose parent strain (Fig. 3A). These results demonstrated that *vpsR* is required for some but not all of the biofilm-forming capacity of the rugose variant. However, to determine if differences in the biofilm-forming capacity of the *vpsR*::pGP704 and the wild-type strains were due to differences in the growth rate, both were cultivated in liquid medium. No differences in the growth rate or final cell density were observed (data not shown). Thus, the decreased capacity of *vpsR*::pGP704 strain to form a biofilm is not due to an altered growth rate.

The same strains were studied by a second assay that provided comparative information about the topographic and architectural features of the biofilms produced by these variants on a glass surface. To this end, a plasmid carrying a gene that constitutively expresses the green fluorescent protein (GFP) was introduced into each of these variants. The biofilms they formed after 24 h of incubation at 30°C were compared using scanning confocal laser microscopy to obtain horizontal (*xy*) and sagittal (*xz*) images (Fig. 3B). Under these conditions, the rugose variant produces a well-developed biofilm containing multicellular, 75- $\mu$ m high pillars and fluid-filled columns. Horizontal projections of the rugose strain showed prominent islands of bacterial aggregates. In contrast, the smooth variant produces a low-profile, 6- $\mu$ m biofilm lacking pillars, columns, or islands of bacterial aggregates. The *vpsR*::pGP704 mutant of the rugose variant also formed a low-profile 7- to 10- $\mu$ m biofilm, similar in depth to the biofilm formed by the wild-type smooth colonial variant. However, in contrast to the smooth variant, the *vpsR*::pGP704 biofilm contained more bacteria per unit area, and in some areas the bacteria coalesced into poorly developed or temporally delayed islands (Fig. 3B). Taken together, these results show that *vpsR* is required for normal biofilm development by the rugose colonial variant, but they also point to the probable existence of genes that are independent of *vpsR* which contribute to the complete biofilm phenotype by this colonial variant.

**Transcriptional regulation of *vps* biosynthesis genes *vpsA* and *vpsL* by *vpsR*.** Sequence analysis of the 30.7-kb region that contains *vps* biosynthesis genes showed that these genes are organized into two clusters of 11.4 and 6.6 kb harboring 11 and 6 *vps* genes, respectively (unpublished data). Because *VpsR* was found to be homologous to several previously described response regulators (Fig. 1B), we reasoned that it might con-

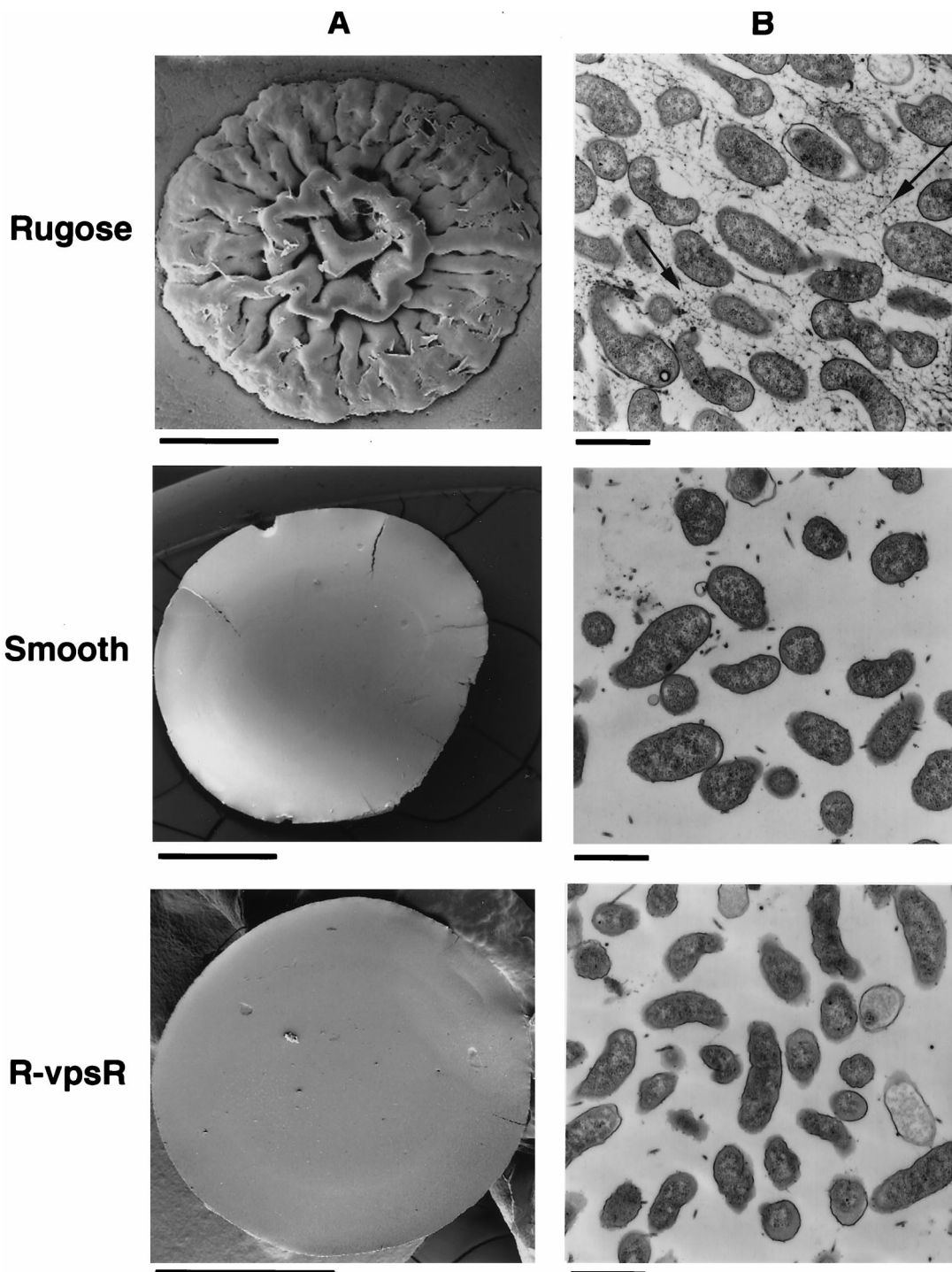
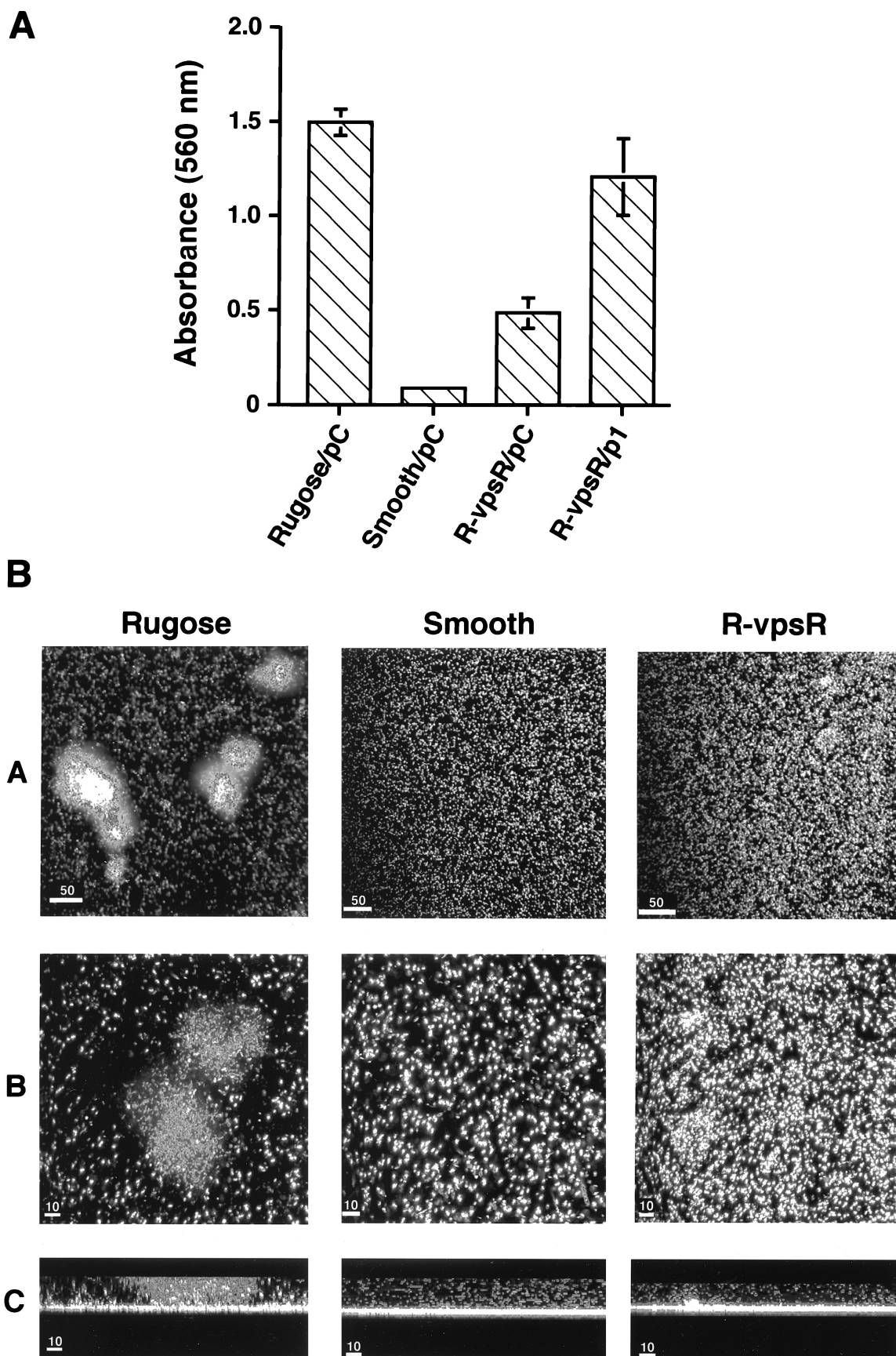


FIG. 2. Colonial morphology and EPS production by the *vpsR* mutant. (A) The appearance of agar grown colonies was determined by scanning electron microscopy for the wild-type rugose and smooth variants of *V. cholerae* O1 El Tor and for the *vpsR* mutant of the rugose variant (designated R-*vpsR*). Disruption of *vpsR* is associated with a change from the rugose to the smooth-colony type. Bars, 1 mm. (B) EPS production by bacteria growing on a cellulose membrane atop nutrient agar was determined by transmission electron microscopic examination of thin sections stained with ruthenium red. A stained matrix, evident between rugose-type bacteria (arrow), is not produced by the *vpsR* mutant of the rugose variant (designated R-*vpsR*) or by the wild-type smooth variant. Bars, 1  $\mu$ m.

trol the expression of *vps* biosynthesis genes within one or both of these clusters. To investigate this possibility, the first gene of each cluster was selected for gene expression analysis, since each had been shown to be required for EPS<sup>ETr</sup> production by

transposon insertion mutagenesis (39). These genes are described below.

*vpsA*, the first gene in the first *vps* region, is predicted to encode a 356-amino-acid, 39.2-kDa protein with 73.8% simi-



larity and 66.0% identity to *E. coli* WecB, which is required for bacteriophage N4 adsorption and encodes UDP-*N*-acetylglucosamine 2-epimerase function (19). It is also highly homologous (71.2% similarity and 64.6% identity) to EpsC of *Pseudomonas solanacearum*, which is required for the production of the exopolysaccharide 1 (EPS1) virulence factor (14). *vpsL*, the first gene in the second *vps* cluster, is predicted to encode a 464-amino-acid, 52.9-kDa protein that is homologous to members of a large enzyme family that catalyze the transfer of glucosyl-1-phosphate from UDP glucose to polyprenolphosphate—the first step in the biosynthesis of lipid intermediates for polysaccharide synthesis. Homologous family members of VpsL include: WcaJ of *E. coli* (32) (56.0% similarity and 45.6% identity), AmsG (2) of *Erwinia amylovora* (39.7 and 30.5%), RfbP (18) of serovar Typhimurium (39.5 and 28.5%), and ExoY (25) of *Rhizobium meliloti* (48.5 and 38.8%).

To estimate the abundance of *vpsA*, *vpsL*, and *vpsR* mRNA in smooth and rugose colonial variants, total RNA was isolated from exponentially growing smooth and rugose cultures and reverse transcribed using random hexamers, and the resulting cDNAs were used for real-time PCR using the corresponding TaqMan PCR primer-probe set. A standard curve for each of the genes was generated using different amounts of chromosomal DNA and the respective TaqMan PCR primer-probe set; this standard curve was then used to measure message abundance by reference to the Ct value for each gene. Figure 4A, which depicts the calculated message abundance for the each of the three tested genes in exponentially growing rugose and smooth variants, shows low levels of *vpsA* mRNA ( $1.5 \times 10^3$  cDNA copies/250 pg of RNA) and *vpsL* mRNA ( $2.3 \times 10^3$  cDNA copies/250 pg of RNA) in the smooth colonial variant. Even though these levels were low, samples subjected to RT contained higher amounts of template molecules than the untreated samples. Thus, *vpsA* and *vpsL* are transcribed during exponential growth of the smooth variant, although at low “basal levels.” In comparison, the message abundances of *vpsA* and *vpsL* in the rugose variant were dramatically greater,  $2.3 \times 10^5$  and  $1.3 \times 10^5$  copies/250 pg of RNA, respectively. Therefore, high levels of *vpsA* and *vpsL* mRNA are correlated with the rugose colonial morphotype and accordingly with EPS<sup>ETr</sup> production and biofilm formation. In contrast, the abundance

of the *vpsR* message was similar in both colonial variants (Fig. 4A).

These results show that changes in *vpsR* expression, at the transcriptional level, are not involved in the regulated expression of *vpsA* and *vpsL*. However, this finding is still compatible with the possibility that *vpsR* is required for the expression of these genes because this class of response regulators is activated by a posttranslational event, namely, phosphorylation of a critical Asp residue by the sensor kinase. Therefore, to further test if *vpsR* is required for the transcriptional regulation of the EPS<sup>ETr</sup> biosynthetic gene cluster, the abundance of *vpsA* mRNA and *vpsL* mRNA was determined for the *vpsR* mutant of the rugose colonial variant. As shown in Fig. 4B, the expression of *vpsA* and *vpsL* is dramatically lower in the *vpsR* regulatory mutant. However, expression levels were partially restored upon complementation. The partial complementation could be due to the fact that the expression of the normal chromosomal copy of *vpsR* might differ from the expression of an episomal copy of the same gene. These results indicate that *vpsR* likely functions as a positive transcriptional regulator of *vpsA* and *vpsL* and therefore acts to positively regulate EPS<sup>ETr</sup> production and biofilm formation as well.

**Comparison of *vps* gene expression by *V. cholerae* O1 El Tor strains A1552 and N16961 during biofilm formation.** Transposon mutagenesis of *V. cholerae* O1 N16961 by Watnick and Kolter led to the identification of three classes of genes (pili, flagella, and some of the EPS<sup>ETr</sup> biosynthesis genes previously identified by Yildiz and Schoolnik [39] that contribute to the biofilm phenotype [36]). However, Watnick and colleagues used only the smooth colonial variant in their studies, yet their mutational strategy and biofilm-forming screen were able to identify genes in the EPS biosynthetic cluster. In contrast, the experiments we report here, which were conducted with strain A1552 in a liquid medium, show that *vpsR*-regulated genes of the EPS biosynthetic cluster are highly expressed in the rugose colonial variant (Fig. 4A). Three possible reasons might account for this apparent discrepancy: first, the expression levels of the *vps* genes may exhibit strain-specific differences under the same condition of growth; second, the biofilm environment, including contact with the glass or plastic surface, might induce transcription of the *vps* biosynthetic gene cluster, even

FIG. 3. Biofilm-forming phenotype of the *vpsR* mutant. (A) Quantitative comparison of biofilm-forming capacity by the wild-type rugose and smooth colonial variants of *V. cholerae* O1 El Tor and by the *vpsR* mutant of the rugose variant. Biofilms were quantified for the following four variants of the same strain: the wild-type rugose and smooth colonial morphotypes, each carrying the control plasmid pACYA184 (pC), and the *vpsR* mutant of the rugose morphotype (R-*vpsR*) harboring either the control plasmid (pC) or a complementing plasmid (p1), which contains a wild-type copy of *vpsR* on pACYC184. Biofilm quantification was carried out by growing these variants overnight in LB broth at 30°C in polyvinyl chloride microtiter plates. The wells were washed, the attached bacteria were stained with crystal violet, the stained film was solubilized in 95% ethanol, and the intensity of crystal violet staining was then monitored by absorbance spectroscopy at 595 nm. Disruption of *vpsR* in the rugose variant, denoted R-*vpsR*, caused a threefold reduction in biofilm-forming capacity compared to the wild-type rugose parent strain. Complementation of R-*vpsR* with p1 restored most of the wild-type biofilm phenotype of the rugose variant. The biofilm-forming capacity of R-*vpsR* was significantly greater than that of the wild-type smooth variant, indicating that biofilm-forming genes, which do not require *vpsR*, are expressed by the rugose variant. (B) Topographical features of biofilm development by the rugose and smooth wild-type colonial morphotypes and by the *vpsR* mutant of the rugose variant (denoted R-*vpsR*). Each of the three tested strains, carrying a plasmid that constitutively expresses the green fluorescent protein, was incubated for 24 h in borosilicate glass-bottom chambers containing LB broth. The chambers were then emptied, and the glass surface was examined with a scanning confocal microscope using 488- and 510-nm excitation and emission wavelengths, respectively. Horizontal (*xy*) projected images of each of the strains are shown at low and high magnification in rows A and B, respectively. Biofilms formed by the rugose variant contain large circumscribed aggregates of adherent bacteria. In contrast, only individual bacteria of the smooth variant interact with the glass surface. The adherence pattern of R-*vpsR* is intermediate between the rugose and smooth patterns, showing both small aggregates and individual glass-bound bacteria. Image reconstruction led to the sagittal (*xz*) view of the same biofilms shown in row C. The high-profile biofilm (>10 μm) produced by the rugose variant contains a mushroom-shaped pillar that is not present in the low-profile biofilms (<10 μm) produced by the wild-type smooth variant or the R-*vpsR* mutant of the rugose variant. Bars: 50 μm, row A; 10 μm, rows B and C.



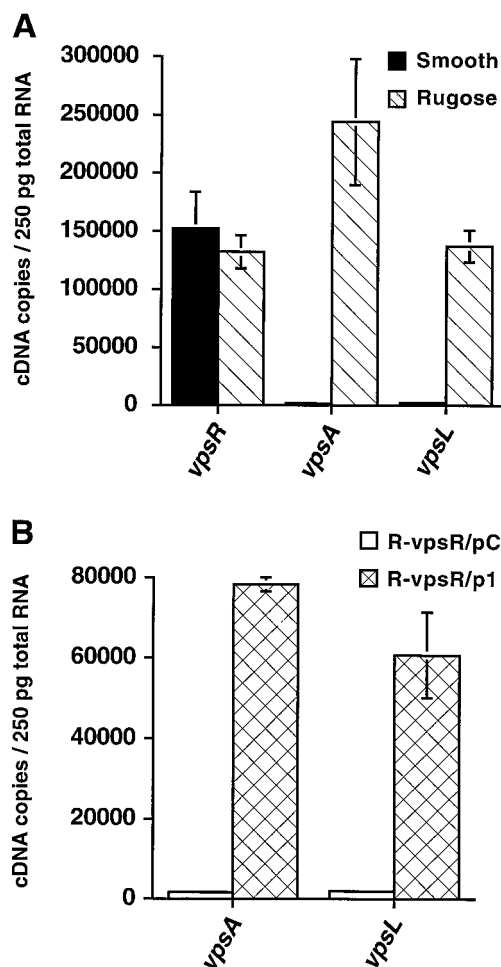


FIG. 4. Transcriptional activity of *vpsR* and of the EPS<sup>ETr</sup> biosynthetic gene cluster. (A) *vpsR*, *vpsA*, and *vpsL* mRNA abundance in wild-type smooth and rugose colonial variants. Standard curves for the *vpsR*, *vpsA*, and *vpsL* genes were generated by plotting the Ct value against the input DNA concentration, as described in Materials and Methods. Message abundance in exponentially growing wild-type smooth and rugose strains was calculated using the corresponding equations from the standard curves and the Ct values obtained after RT of total RNA, followed by real-time PCR amplification. *vpsA* and *vpsL* of the EPS<sup>ETr</sup> biosynthetic gene cluster are strongly expressed in the rugose variant, but they are also expressed at low, basal levels in the smooth variant. Smooth and rugose variants expressed *vpsR* at equivalent levels. (B) Complementation of the *vpsR* mutant restores *vpsA* and *vpsL* expression. The *vpsR* mutant of the rugose colonial variant (denoted R-*vpsR*) was complemented with either a control plasmid (pC) or the control plasmid containing a wild-type copy of *vpsR* (p1). *vpsA* and *vpsL* mRNA abundances were determined during exponential growth for the complemented (R-*vpsR*/p1) and non-complemented (R-*vpsR*/pC) mutant. Message abundance is expressed as the number of cDNA copies detected in 250 pg of total RNA.

in the smooth variant, when compared to growth in a liquid medium; and third, the biofilm microenvironment might induce phase transition from the smooth to the rugose morphotype. To address these issues, biofilms formed by the smooth colonial variants of the two strains after 24 h of incubation at 30°C were quantitatively compared using the crystal violet-based method described for Fig. 3. The results (Fig. 5A) indicate that N16961 forms an approximately twofold-greater bio-

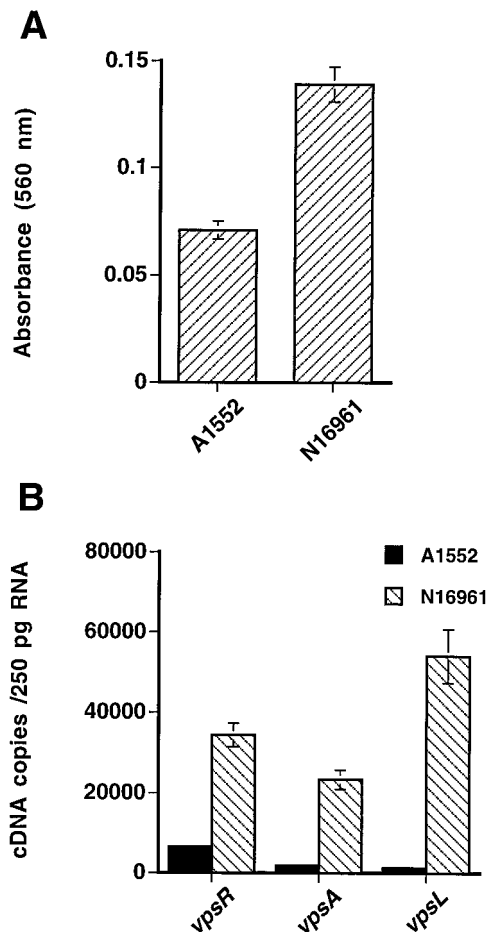


FIG. 5. Comparison of *V. cholerae* O1 El Tor strains A1552 and N16961 for biofilm phenotype and *vps* gene expression. (A) Biofilm-forming phenotypes of the smooth strains A1552 and N16961. Biofilm formation was analyzed by growing the strains in LB broth at 30°C for 24 h in polyvinyl chloride microtiter plates, and then the biofilms were quantified by crystal violet staining. The biofilm-forming capacity of the N16961 strain after 24 h of growth in polyvinyl chloride microtiter plates is twofold higher than that of the A1552 strain. (B) Expression analysis of *vps* genes in the strains A1552 and N16961. The abundance of *vpsR*, *vpsA*, and *vpsL* messages was quantified by copy number determination using real-time PCR. All three genes were expressed at a higher level in N16961 than in A1552.

film than A1552. To determine if differences between the biofilm phenotype of the two strains are correlated with differences in the expression of *vps* biosynthesis genes, the abundance of *vpsR*, *vpsA*, and *vpsL* mRNA in biofilms formed in polystyrene petri plates after 24 h of incubation at 30°C was quantified for each of the two strains using real-time PCR. As shown in Fig. 5B, the amounts of *vpsR*, *vpsA*, and *vpsL* message were 5.2-, 12.6-, and 44.7-fold higher, respectively, in N16961 biofilms compared to A1552 biofilms. The differences in expression data are therefore consistent with the differences in biofilm magnitude for the smooth variants of the two strains.

To determine if the differences could be due to a possible effect of the biofilm microenvironment that might induce transition from the smooth to the rugose morphotype and thereby be associated with increased expression of the *vps* genes, cells from the same biofilms were removed, serially diluted, and

plated onto LB agar plates. We recovered  $10^8$  to  $10^9$  cells per biofilm and observed that neither of the strains exhibited conversion from the smooth to the rugose morphotype under the conditions tested. Thus, the difference between the biofilm phenotypes of *V. cholerae* O1 El Tor A1552 and N16961 depicted in Fig. 5A is most likely due in part to strain-specific variation in the expression of *vps* genes.

## DISCUSSION

We have identified *vpsR*, a gene encoding a positive transcriptional regulator of EPS<sup>ETr</sup> biosynthetic gene expression and the biofilm-forming phenotype by the rugose colonial variant of *V. cholerae* O1 El Tor. When mutation of *vpsR* was undertaken in a rugose background, the colony formed by the resulting mutant resembled the smooth colonial variant. However, the quantitative biofilm assay results depicted in Fig. 3 showed that this mutant exhibits a biofilm-forming capacity that is intermediate between those of the wild-type rugose and smooth variants. EPS biosynthesis mutants of the rugose colonial variants, including *vpsA* and *vpsL* mutants, also exhibit an intermediate biofilm phenotype (unpublished data). In view of these results, it seems most likely that the rugose variant selectively expresses not only genes in the *vps* biosynthetic cluster but also other genes as well. These other genes are not under the control of *vpsR*, but they nonetheless contribute to the biofilm-forming phenotype of the rugose variant. Thus, the fully manifested three-dimensional biofilm of the rugose variant is likely to be the product of several separate effector genes and their cognate regulators, including *vpsR*.

VpsR was found to be highly homologous to several response regulators which, in well-characterized two-component signal transduction systems, have been shown to act in conjunction with a cognate sensor kinase protein. Activation of the response regulator typically occurs as a result of sensor kinase-mediated phosphorylation of a conserved Asp residue within the N-terminal segment of the response regulator component. VpsR lacks two conserved residues, Asp and Lys, which are critical for phosphorylation and are present at the N-terminal domain. AlgB and AlgR response regulators, which control alginate production by activating the transcription of *algD*, do not need to be phosphorylated for their roles in alginate production (24). Therefore, it will be of interest to determine if VpsR requires phosphorylation for its function and if the mode of phosphorylation differs from the typical members of the response regulators.

In well-characterized two-component signal transduction systems, response regulators have been shown to act in conjunction with a cognate sensor kinase protein. Because the response regulator and the sensor kinase are in close proximity to one another in many two-component regulatory systems, we examined ca. 2 kb of the sequence flanking *vpsR*. However, we failed to locate a gene that might encode the cognate sensor component of VpsR within this sequenced region, nor was a candidate cognate sensor kinase identified among the transposon mutants characterized thus far. Analysis of the annotated genome sequence of *V. cholerae* O1 may lead to the identification of candidate genes for this sensor kinase (13). VpsR also contains a helix-turn-helix, DNA-binding motif near the C terminus. Thus, it is possible that VpsR directly activates the

transcription of *vps* biosynthetic genes by binding upstream promoter regions within this cluster. Alternatively, VpsR may mediate its effect on *vps* gene transcription indirectly, by interacting with another positive regulator of *vps* expression. The results described here are compatible with either possibility.

VpsR is highly homologous with NtrC of serovar Typhimurium (Fig. 1B), and most such homologues act in concert with the alternative sigma factor RpoN. However, the expression of *vpsA* and *vpsL* were not altered in an *rpoN*-null mutant generated in the rugose variant (data not shown). Further, analysis of the upstream regions of *vpsA* and *vpsL* did not reveal the TGGCACn4TTTGCA sequences recognized by sigma-54 (7). Thus, like AlgB of *P. aeruginosa*, a response regulator that harbors a RpoN-binding domain (11, 37), VpsR does not require RpoN to activate *vps* gene transcription.

Complementation studies reported in this study were performed with the *vpsR* gene cloned from the smooth form of *V. cholerae* O1 El Tor. At present the mechanism of smooth-to-rugose transition and vice versa is not known. However, our observations suggest that *vpsR* is not the gene functioning as the switch. Instead, VpsR may be the target of the switch.

Biofilm formation and decay must be a tightly controlled system if it is to confer a selective advantage in natural aquatic habitats that are subject to changes in a variety of physicochemical parameters, including nutrient availability, salinity, and shear force generated by flowing water. For the system to be adequately responsive, flexible, and robust, some environmental signals should trigger biofilm formation, whereas others should inhibit this process and initiate biofilm dissolution. Thus, depending on the signal, processes would be initiated that change the ratio of the sessile to the planktonic populations of the species. We predict that VpsR will be only one of several regulators of this process as is the case for the much more thoroughly studied alginate-dependent biofilm system of *P. aeruginosa*. Alginate biosynthesis by mucoid strains of this species requires two response regulators (AlgB and AlgR) (6, 11, 37), an alternative sigma factor (AlgT) (38), and a ribbon-helix-helix DNA-binding protein (AlgZ) (1). Further, the development of a mature biofilm architecture containing pillars of alginate-embedded bacteria that are separated by water-containing channels requires quorum sensing mediated by the *lasI* product, *N*-(3-oxododecanoyl)-L-homoserine (5). The *V. cholerae* O1 biofilm system is likely to be equally complex. For example, studies by Watnick and Kolter of the complex biofilm-forming system of this species (36) led them to propose a three-step model of biofilm development: first, mannose-sensitive hemagglutinin type IV pili and flagella facilitate the attachment of free-swimming bacteria to a surface; second, the flagella cause attached bacteria to spread across the surface; and third, EPS is secreted (36), providing the extracellular matrix of the mature biofilm's three-dimensional structure. The work presented here highlights another level of complexity by pointing out that different *V. cholerae* O1 El Tor isolates can vary significantly with respect to the third proposed step of biofilm development, *vps* gene expression. *V. cholerae* O1 El Tor, strain A1552, the prototype strain used in our study, was isolated in Peru at the beginning of the recent South American outbreak, where epidemic cholera had been absent for over a century. In contrast, strain N16961, studied by Watnick and Kolter, was isolated in India, where cholera has long been

endemic. Based on the comparative analysis of the biofilm-forming phenotypes of these strains depicted in Fig. 5A, the smooth variant of N16961 is a better biofilm former than the smooth variant of A1552 and correspondingly exhibits more *vpsA* and *vpsL* expression. Strain-to-strain variation in the biofilm phenotype further compounded by intrastrain differences between the rugose and smooth-colony types. If the biofilm phenotype is important for the survival of *V. cholerae* O1 El Tor in an aquatic habitat, then differences of the kind exhibited by strains A1552 and N16961 could cause the latter to be more environmentally fit than the former. If so, then it is possible that adaptation to different geographic locations and, in turn, acclimation to distinct microenvironments could have led to the observed differences in this important phenotype.

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