

Environmental Determinants of *Vibrio cholerae* Biofilm Development

Katharine Kierek and Paula I. Watnick*

Division of Geographic Medicine and Infectious Diseases, New England Medical Center, Boston, Massachusetts 02111

Received 14 March 2003/Accepted 3 June 2003

***Vibrio cholerae* is a versatile bacterium that flourishes in diverse environments, including the human intestine, rivers, lakes, estuaries, and the ocean. Surface attachment is believed to be essential for colonization of all of these natural environments. Previous studies have demonstrated that the *vps* genes, which encode proteins required for exopolysaccharide synthesis and transport, are required for *V. cholerae* biofilm development in Luria-Bertani broth. In this work, we showed that *V. cholerae* forms *vps*-dependent biofilms and *vps*-independent biofilms. The *vps*-dependent and -independent biofilms differ in their environmental activators and in architecture. Our results suggest that environmental activators of *vps*-dependent biofilm development are present in freshwater, while environmental activators of *vps*-independent biofilm development are present in seawater. The distinct environmental requirements for the two modes of biofilm development suggest that *vps*-dependent biofilm development and *vps*-independent biofilm development may play distinct roles in the natural environment.**

Vibrio cholerae is the agent of the dreaded waterborne disease cholera. Cholera is characterized by massive diarrhea, resulting in severe dehydration and even death. When *V. cholerae* is not wreaking havoc in the human intestine, it may be found in diverse aquatic environments, such as the ocean, estuaries, rivers, and lakes (5, 10, 26, 30, 36). Adhesion to surfaces both in the human intestine and in the aquatic environment plays an important role in *V. cholerae*'s success as a pathogen and an environmental organism.

Bacteria attached to a surface, which are collectively termed a bacterial biofilm, have been a subject of intense interest in recent years due to the predominance of biofilm-associated bacteria in natural environments, the complex developmental pathway that bacteria follow in forming a biofilm, and the role of biofilm formation in antibiotic-resistant bacterial infections (7, 22). Many gram-negative bacteria use flagella and/or pili to associate with surfaces (2, 9, 15, 16, 25, 28, 31, 32, 40, 41). The subsequent accumulation of bacteria on surfaces with the characteristic biofilm architecture of pillars of bacteria surrounded by water channels is thought to require exopolysaccharide synthesis (6, 40–43). Surface adhesion and exopolysaccharide production by bacteria have been shown to depend on the inorganic and organic compositions of the bathing medium. In particular, high concentrations of Na⁺ and Ca²⁺ in growth media have been shown to enhance biofilm development by diverse bacteria (17, 18, 34, 35). Furthermore, the carbohydrate composition of the growth medium influences exopolysaccharide production and biofilm development by many bacterial species (1, 3, 4, 21, 24, 35).

Exopolysaccharide-dependent and -independent biofilms have been described for both *Pseudomonas aeruginosa* and *Escherichia coli*. In a rich growth medium, wild-type *P. aeruginosa* and *E. coli* form thick biofilms with pillars and channels. When quorum sensing is eliminated in *P. aeruginosa* by muta-

tion of the *lasI* gene, however, a flat, densely packed biofilm is formed. This biofilm exhibits increased susceptibility to detergents (8). In *E. coli*, a similar flat biofilm is observed when genes for synthesis of the exopolysaccharide colanic acid are disrupted (6).

V. cholerae biofilm development has previously been studied in Luria-Bertani (LB) broth, a complex, undefined medium containing 100 mM NaCl, 1.0% yeast extract, and 1.5% tryptone (12, 27, 37, 39, 40, 43). In this medium, *V. cholerae* forms a thick biofilm containing pillars of bacteria surrounded by water channels (41). The VPS exopolysaccharide is required for formation of this biofilm structure. This exopolysaccharide is synthesized and exported by proteins encoded by genes both in the *vps* locus and in other loci (27, 43). In the absence of the *vps* genes, cells form only a monolayer on the surface (40, 43). Furthermore, *vps* transcription is greater in biofilm-associated cells than in planktonic cells, suggesting that *vps* gene transcription is activated by surface association (12).

In this study, we explored the environmental activators of *V. cholerae* biofilm development in the context of a defined growth medium. We obtained qualitative and quantitative evidence that *V. cholerae* forms *vps*-dependent biofilms and *vps*-independent biofilms in response to distinct environmental signals. *V. cholerae* is found in both freshwater and seawater environments. Our findings suggest that only *vps*-dependent biofilm development is possible in freshwater environments, while both *vps*-dependent biofilm development and *vps*-independent biofilm development are possible in seawater environments. Our results suggest that *vps*-dependent surface adhesion and *vps*-independent surface adhesion play different roles in aquatic environments.

MATERIALS AND METHODS

Bacterial strains and media. The bacterial strains used in our experiments are described in Table 1. Biofilms were formed in a medium supplemented with either 1% yeast extract (Difco) and 1.5% tryptone (Difco) (YT) or 1% vitamin assay Casamino Acids (CAA) (Difco). Various salt mixtures were added to this base, as follows: 10 mM NaCl, 100 mM NaCl, commercial artificial seawater (Instant Ocean; Aquarium Systems), a defined mixture of the salts reported to be in Instant Ocean (defined seawater [DSW], containing 468 mM NaCl, 55 mM

* Corresponding author. Mailing address: Division of Geographic Medicine and Infectious Disease, New England Medical Center, 750 Washington St., Box 041, Boston, MA 02111. Phone: (617) 636-2545. Fax: (617) 636-3216. E-mail: pwatnick@tufts-nemc.org.

TABLE 1. Bacterial strains and plasmid used

Strain or plasmid	Characteristics	Reference
Strains		
<i>V. cholerae</i> MO10	1992 clinical isolate of <i>V. cholerae</i> O139 from India; Sm ^r	38
PW328	MO10 $\Delta vpsL$, Sm ^r	12
PW357	MO10 $lacZ::vpsLp \rightarrow lacZ$, Sm ^r	12
PW396	MO10 $\Delta vpsA$, Sm ^r	This study
PW397	MO10 $\Delta vpsL \Delta vpsA$, Sm ^r	This study
Plasmid pAJH9	pWM91 carrying a fragment of the <i>vpsA</i> operon harboring an internal, unmarked deletion	This study

MgSO₄ · 7H₂O, 3.0 mM NaHCO₃, 9.9 mM CaCl₂ · 2H₂O, 10.3 mM KCl, 0.14 mM Na₂B₄O₇ · 10H₂O, 0.1 mM SrCl₂ · 6H₂O, 0.03 mM NaBr, 0.002 mM NaI, and 0.026 mM LiCl), autoclaved river water (Charles River, Newton, Mass.), and autoclaved real seawater (Crane Beach, Mass.). Where indicated below, 0.16% monosaccharides (0.015% glucose, 0.048% galactose, 0.098% mannose) was added to the growth medium. This monosaccharide mixture was determined to be equivalent to that found in a mixture of 1% yeast extract and 1.5% tryptone based on a monosaccharide analysis performed on the reagents used in our laboratory.

Construction of a *vpsA* deletion mutant. A large operon required for VPS synthesis extends from VC0917 to VC0927 (*vpsA* operon). A 407-bp fragment located between positions 979148 and 979555 on chromosome 1 and a 462-bp fragment located between positions 990697 and 991099 on chromosome 1 were amplified by PCR. The internal primers included a complementary 15-bp sequence at the 3' and 5' ends, respectively, as previously described (12). These two fragments were joined by using the technique of splicing by overlap extension, which resulted in construction of a fragment with an 11,141-bp deletion in the *vpsA* operon (14). The fragment containing the deletion was gel purified and ligated into pCR2.1TOPO (Invitrogen). This fragment was then removed from pCR2.1TOPO by digestion with *SpeI* and *XhoI* and ligated into pWM91 to create a suicide plasmid, pAJH9. This plasmid was used to create *vpsA* operon deletions in the relevant strains by double homologous recombination and sucrose selection as previously described (12).

Biofilm assays. Borosilicate glass tubes were filled with 300 μ l of the growth medium as indicated below and inoculated with the strain under study. For reproducibility, experiments were initiated at an optical density at 595 nm (OD₅₉₅) of 0.02. Unless specifically indicated otherwise, cells were allowed to adhere to the surface during 18 h of incubation at 27°C. Biofilms were visualized by incubation with a 1-mg/ml aqueous solution of crystal violet as previously described (12). Surface-adherent cells were quantified as follows. Following formation of the biofilm, planktonic cells were removed, and the tubes were rinsed and filled with 300 μ l of fresh medium. An equivalent volume of 1-mm-diameter borosilicate glass beads (Biospec) was added to each tube. Biofilm-associated cells were mechanically removed and dispersed by vortexing in the presence of these beads for approximately 10 s. Finally, the OD₅₉₅ of the planktonic and biofilm cell suspensions were measured. This rapid method of quantifying biofilm-associated cells was quite reproducible, and the results compared favorably with measurements based on total protein and viable cell counts. Furthermore, in these assays, this method was more accurate than quantification of biofilm-associated crystal violet by solubilization with dimethyl sulfoxide.

Fluorescence microscopy and quantitative analysis of biofilm structure. Fluorescence microscopy was used to evaluate the three-dimensional biofilm structure. The biofilms used for fluorescence microscopy were formed on borosilicate glass coverslips that were placed in 50-ml Falcon tubes containing 7.0 ml of medium. Bacteria were inoculated into the medium to obtain an initial OD₅₉₅ of 0.05, and biofilms were formed over a 24-h period. 4',6-Diamidino-2-phenylindole (DAPI), which penetrates bacterial cell membranes to form a fluorescent complex with DNA, was used to visualize biofilms. Seven microliters of a 1-mg/ml aqueous solution of DAPI was added directly to each culture following formation of the biofilm. Then the coverslip, with its associated biofilm, was incubated with DAPI for 10 min.

The coverslip was removed, rinsed with fresh medium, and placed on a concave microscope slide filled with sterile medium. An Eclipse TE-200 inverted fluorescence microscope (Nikon) equipped with an Orca digital charge-coupled device camera (Hamamatsu), VVM-D1 shutter drivers (Uniblitz), and a focus motor was used to collect a stack of evenly spaced transverse sections through the biofilm. The image stacks were subjected to nearest-neighbor deconvolution and three-dimensional image reconstruction by using the Metamorph Imaging software (Universal Imaging). Various attributes of the biofilm structure were then

quantified by using the COMSTAT software developed by Heydorn and colleagues (13). In this study, total biofilm biomass, substratum coverage, average biofilm thickness, and maximum biofilm thickness were used to quantitatively describe the biofilm architecture. The total biofilm biomass was determined by dividing the volume of cells in the three-dimensional biofilm by the substratum surface area analyzed. The substratum coverage was the proportion of the total substratum surface area that was covered by cells. The values reported below are averages for six image stacks (three image stacks collected in two independent experiments).

β -Galactosidase measurements. β -Galactosidase assays were performed as previously described (12), with the following modifications. For measurements of *vpsL* promoter activity in both planktonic and biofilm-associated cells, wild-type *V. cholerae* was incubated at 27°C for 24 h in 50-ml Falcon tubes containing 3 ml of growth medium. After transfer of planktonic cells to new tubes, the biofilms were rinsed with sterile growth medium and removed from the surface by agitation with 1-mm-diameter borosilicate beads (BioSpec) as described above. The OD₅₉₅ of each resulting suspension was measured, and biofilm-associated cells were then gently pelleted and resuspended in 1 ml of Z buffer. The cells were then lysed by three freeze-thaw cycles and centrifuged to remove the cell debris. A 100- μ l aliquot of the lysate was set aside for protein determination with a Bradford assay kit (Pierce), and *o*-nitrophenyl- β -D-galactopyranoside (Sigma) was added to the remaining lysate. Samples were incubated for 18 h at 37°C to allow a yellow color to develop, and the A_{405} of each sample was measured. To obtain a relative β -galactosidase activity measurement for each sample, the A_{405} was multiplied by 1,000 and then divided by the calculated protein concentration or the OD₅₉₅. All experiments were performed in triplicate.

Biofilm matrix isolation and analysis. *V. cholerae* biofilms were formed in LB broth or commercial artificial seawater supplemented with CAA over a period of 72 h. *V. cholerae* $\Delta vpsL$, $\Delta vpsA$, or $\Delta vpsLA$ mutant biofilms were formed under similar conditions in commercial artificial seawater supplemented with CAA. In all experiments, planktonic cells were removed, and biofilm cells were dispersed and separated from the biofilm matrix by bead beating at 5,000 rpm for 10 s with 1/3 volume of 1-mm-diameter borosilicate beads (BioSpec). Intact *V. cholerae* cells were removed by centrifugation at 4,000 \times g. Three volumes of 95% ethanol was added to the supernatant, and exopolysaccharide and other components of the biofilm matrix were then allowed to precipitate overnight at 4°C. The precipitate was collected by centrifugation and sent to the Glycobiology Core at the University of San Diego for monosaccharide analysis.

Analysis of river and seawater compositions. Elemental and osmolarity analyses were performed on aliquots of Charles River water and Massachusetts seawater. An elemental analysis was performed by a commercial laboratory (Alpha Analytical Laboratories, Westborough, Mass.). Osmolarity was measured with a freezing point osmometer (model 3300; Advanced Instruments).

RESULTS AND DISCUSSION

V. cholerae is a human pathogen and a natural inhabitant of riverine, estuarine, and marine environments. In these varied environments, bacteria are principally found in association with a surface. We hypothesized that the ionic and nutrient compositions of the bathing medium might determine the nature of the biofilm formed. In particular, we were interested in identifying the environmental determinants of *V. cholerae* biofilm development, with the ultimate goal of relating these findings to freshwater and marine environments.

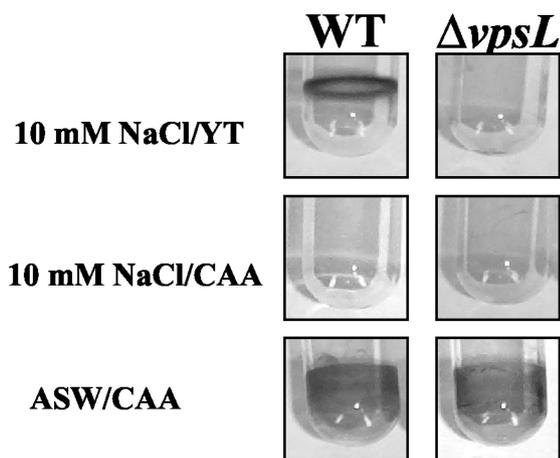


FIG. 1. Representative wild-type *V. cholerae* strain MO10 (WT) and $\Delta vpsL$ mutant PW328 biofilms formed over 18 h in 10 mM NaCl or commercial artificial seawater (ASW) supplemented with either YT or CAA. Biofilms were visualized by crystal violet staining.

***V. cholerae* biofilm development is *vps* dependent in 10 mM NaCl–YT and *vps* independent in artificial seawater.** It has been demonstrated previously that the wild-type *V. cholerae* O139 biofilm formed in LB broth depends on the *vpsL* gene product (12). LB broth, however, is a complex, poorly defined medium consisting of 100 mM NaCl, the products of a tryptic digest of casein, and the products of an ethanol extract of *Saccharomyces cerevisiae*. In order to understand the environmental enhancers of biofilm development, our goal was to develop a better defined growth medium that activated *V. cholerae* biofilm development. Because *V. cholerae* is found in both freshwater and seawater environments, we first compared *V. cholerae* biofilm development in growth media consisting of an inorganic base, either 10 mM NaCl or commercial artificial seawater, supplemented with either YT or CAA. Crystal violet-stained biofilms resulting from culture of wild-type *V. cholerae* and a $\Delta vpsL$ mutant in these media are shown in Fig. 1. Wild-type *V. cholerae*, but not the $\Delta vpsL$ mutant, was able to form a robust biofilm in 10 mM NaCl supplemented with YT. These results are similar to the results of previous studies of *V. cholerae* biofilm development in LB broth (40, 41). Amino acids in the form of peptides and proteins are a major component of yeast extract and tryptone. Thus, we tested whether addition of CAA alone to 10 mM NaCl supported wild-type *V. cholerae* biofilm development. In this medium, however, no biofilm was observed. Thus, supplementation with amino acids alone was not sufficient to induce wild-type *V. cholerae* biofilm development in 10 mM NaCl. However, biofilm development by wild-type *V. cholerae* and the $\Delta vpsL$ mutant in artificial seawater medium supplemented with CAA was surprisingly different. Commercial artificial seawater supplemented with CAA induced robust biofilm development by both wild-type *V. cholerae* and the $\Delta vpsL$ mutant. The same result was observed for biofilms formed in commercial artificial seawater supplemented with YT (results not shown). The bacterial growth in all media containing YT as a nutrient source was approximately four times that in media containing CAA. This probably

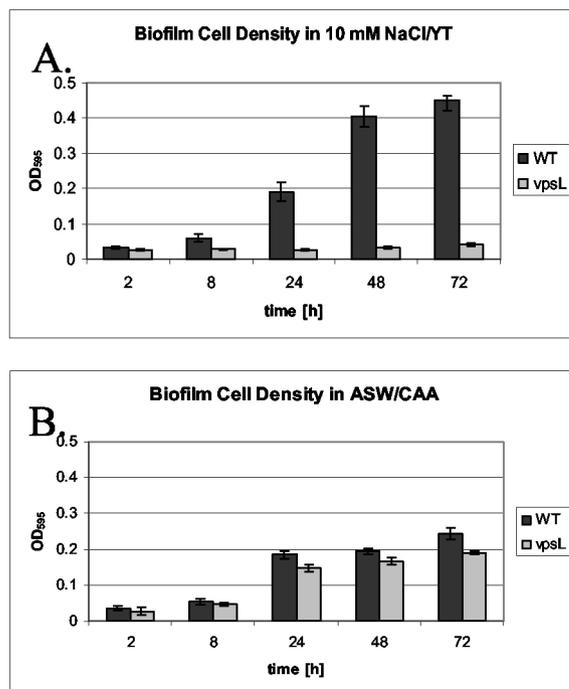


FIG. 2. Accumulation over time of wild-type *V. cholerae* strain MO10 (WT) and a $\Delta vpsL$ mutant (PW328) on a borosilicate surface in two types of media, 10 mM NaCl–YT (A) and commercial artificial seawater (ASW) supplemented with CAA (B).

reflects the B vitamins, iron, and other nutrients that are abundant in YT but not in vitamin assay CAA.

In order to quantify surface attachment, we harvested biofilms from borosilicate tubes at various times after inoculation. The increases in surface-associated cells over time in the various media are shown in Fig. 2. Wild-type *V. cholerae* was able to accumulate on borosilicate surfaces bathed in 10 mM NaCl supplemented with YT, but the $\Delta vpsL$ mutant was not (Fig. 2A). In 10 mM NaCl supplemented with CAA, neither wild-type *V. cholerae* nor $\Delta vpsL$ mutant cells accumulated on the borosilicate surface in significant numbers. After 72 h, the OD₅₉₅ of surface-associated cells did not exceed 0.05 (data not shown). In contrast, when commercial artificial seawater was supplemented with CAA, the numbers of surface-associated wild-type *V. cholerae* and $\Delta vpsL$ mutant cells were similar (Fig. 2B). It should be noted that the amount of surface-associated cells in the biofilm formed in commercial artificial seawater supplemented with CAA was only one-half the amount of surface-associated cells in the biofilm formed in 10 mM NaCl supplemented with YT. This suggests that there was either an increased rate of cell attachment to or a decreased rate of cell detachment from the biofilm formed in 10 mM NaCl supplemented with YT compared with the rate for the biofilm formed in commercial artificial seawater.

From these experiments, we formulated the following three hypotheses: (i) biofilm development in 10 mM NaCl supplemented with YT requires the *vps* genes and, therefore, is *vps* dependent, (ii) biofilm development in artificial seawater does not require the *vps* genes and, therefore, is *vps* independent,

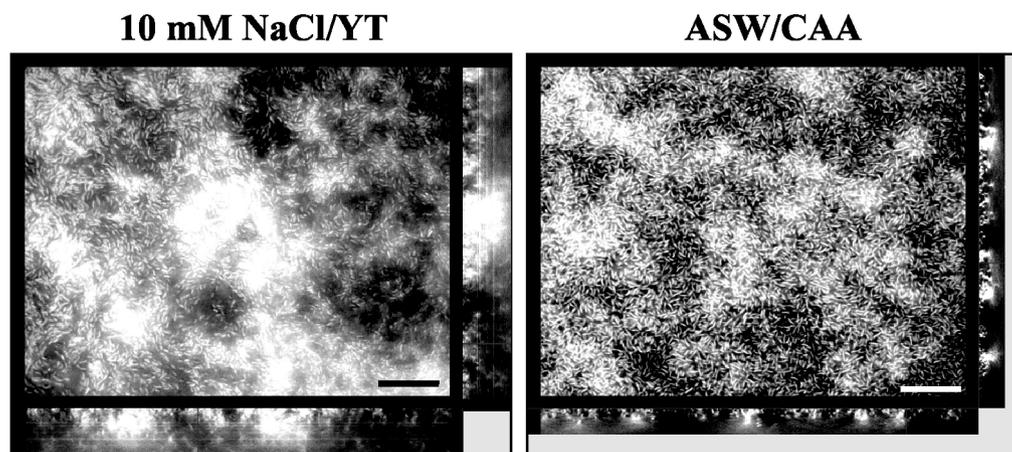


FIG. 3. Transverse and vertical cross sections through DAPI-stained wild-type *V. cholerae* strain MO10 biofilms formed in 10 mM NaCl–YT and commercial artificial seawater (ASW) supplemented with CAA. Transverse cross sections were obtained at the level of the substratum. Bars = 10 μm .

and (iii) *vps*-dependent biofilm development requires a component of YT that is not present in CAA.

The *vps*-dependent and -independent biofilms differ in architecture. Exopolysaccharide is thought to be required for the prototypical biofilm structure consisting of pillars of bacteria surrounded by water channels. Thus, we hypothesized that the structure of the *vps*-independent biofilm might differ from that of the *vps*-dependent biofilm (6). In order to test this hypothesis, deconvolution fluorescence microscopy was used to visualize biofilms formed in 10 mM NaCl–YT and artificial seawater supplemented with CAA. As shown in Fig. 3, substantial *V. cholerae* surface accumulation was observed in both growth media. While the biofilm formed in 10 mM NaCl supplemented with YT consisted of tall pillars interspersed with water channels, the biofilm formed in commercial artificial seawater supplemented with CAA had smaller, more closely spaced pillars.

In order to more objectively assess our qualitative impressions of the *vps*-dependent and -independent biofilm architectures, we used the Comstat program designed by Heydorn et al. (13). Biomass, substratum coverage, average biofilm thickness, and maximum biofilm thickness were used to characterize each biofilm (Tables 2 and 3). When normalized to surface area, the total biomass of the wild-type *V. cholerae* biofilm formed in 10 mM NaCl supplemented with YT was 2.5 times that of the biofilm formed in commercial artificial seawater supplemented with CAA. Although the surface coverage val-

ues were similar for the two biofilms, the average biofilm thickness and the maximum biofilm thickness of the biofilm formed in 10 mM NaCl supplemented with YT were approximately three and two times, respectively, greater than those of the biofilm formed in commercial artificial seawater supplemented with CAA. These results substantiated our qualitative impressions based on microscopy.

Monosaccharides are critical activators of *vps*-dependent biofilm formation. We observed that supplementation of 10 mM NaCl with CAA did not activate *vps*-dependent biofilm development by wild-type *V. cholerae*, while addition of YT did. Thus, we hypothesized that a nonproteinaceous component of YT must be an activator of and/or a substrate for VPS synthesis. In an attempt to activate *vps*-dependent biofilm development, B vitamins, choline, iron, and nucleic acids were added to 10 mM NaCl supplemented with CAA at concentrations similar to those reported for YT. However, no *V. cholerae* biofilm was observed in the presence of these supplements (data not shown). Monosaccharide analysis of YT revealed the presence of mannose, glucose, and galactose. Because the presence of monosaccharides in growth medium has been shown to influence exopolysaccharide production and biofilm formation by a wide variety of bacteria, we tested whether supplementation of 10 mM NaCl–CAA with monosaccharides activated *vps*-dependent biofilm formation by *V. cholerae*. In fact, when wild-type *V. cholerae* was cultured in 10 mM NaCl supplemented with CAA, 0.098% mannose, 0.015% glucose,

TABLE 2. Quantitative comparison of the architectures of wild-type *V. cholerae* biofilms formed in various model freshwater media^a

Medium	Biomass ($\mu\text{m}^3/\mu\text{m}^2$)	Fraction of substratum covered by cells	Avg thickness (μm)	Maximum thickness (μm)
10 mM NaCl/YT	1.93 ± 0.66	0.58 ± 0.06	2.83 ± 1.02	10.19 ± 1.15
10 mM NaCl/CAA	0.11 ± 0.02	0.08 ± 0.01	0.16 ± 0.02	1.49 ± 0.31
10 mM NaCl/CAA/CRB	1.85 ± 0.27	0.42 ± 0.05	2.17 ± 0.31	11.38 ± 0.69
CRW/CAA	0.4 ± 0.06	0.37 ± 0.04	0.45 ± 0.06	2.64 ± 0.84

^a Biofilms were formed in either 10 mM NaCl supplemented with YT (10 mM NaCl/YT), 10 mM NaCl supplemented with CAA (10 mM NaCl/CAA), 10 mM NaCl supplemented with CAA and monosaccharides (10 mM NaCl/CAA/CRB), or Charles River water supplemented with CAA (CRW/CAA). Values were calculated by using the Comstat program (13).

TABLE 3. Quantitative comparison of the architectures of wild-type *V. cholerae* and $\Delta vpsL$ mutant biofilms formed in three model seawater media^a

Medium	Strain	Biomass ($\mu\text{m}^3/\mu\text{m}^2$)	Fraction of substratum covered by cells	Avg thickness (μm)	Maximum thickness (μm)
ASW/CAA	Wild type	0.76 ± 0.01	0.59 ± 0.06	0.96 ± 0.07	5.39 ± 0.24
	$\Delta vpsL$	0.45 ± 0.01	0.62 ± 0.02	0.48 ± 0.04	2.62 ± 0.03
DSW/CAA	Wild type	0.29 ± 0.02	0.44 ± 0.03	0.41 ± 0.05	2.0 ± 0.48
	$\Delta vpsL$	0.23 ± 0.05	0.41 ± 0.05	0.30 ± 0.05	1.76 ± 0.25
RSW/CAA	Wild type	0.41 ± 0.08	0.42 ± 0.05	0.55 ± 0.08	6.66 ± 0.4
	$\Delta vpsL$	0.40 ± 0.02	0.45 ± 0.08	0.51 ± 0.05	5.64 ± 1.77

^a Biofilms were formed in either commercial artificial seawater supplemented with CAA (ASW/CAA), seawater prepared from defined components supplemented with CAA (DSW/CAA), or seawater from the Massachusetts coast supplemented with CAA (RSW/CAA). Values were calculated by using the Comstat program (13).

and 0.048% galactose, a biofilm formed (Fig. 4). The *vps* mutant, however, formed no biofilm in 10 mM NaCl supplemented with CAA and monosaccharides. Both glucose alone and mannose alone were able to activate *vps*-dependent biofilm development. Galactose, however, augmented *vps*-dependent biofilm development only in the presence of glucose or mannose (data not shown).

We also used quantitative biofilm parameters to compare the structures of wild-type *V. cholerae* biofilms formed in 10 mM NaCl supplemented with YT, with CAA, or with CAA and monosaccharides (Table 2). The biomass of the biofilm formed

in 10 mM NaCl supplemented with CAA was 17-fold less than that of the biofilm formed in 10 mM NaCl supplemented with YT. Substratum coverage, average thickness, and maximum thickness were also severalfold less. However, the quantitative parameters describing the biofilm formed in 10 mM NaCl supplemented with CAA and monosaccharides were remarkably similar to those describing the biofilm formed in 10 mM NaCl supplemented with YT. These results support the hypothesis that the polysaccharide component of YT induces *vps*-dependent biofilm development by *V. cholerae*.

Because monosaccharides are the substrates for exopolysac-

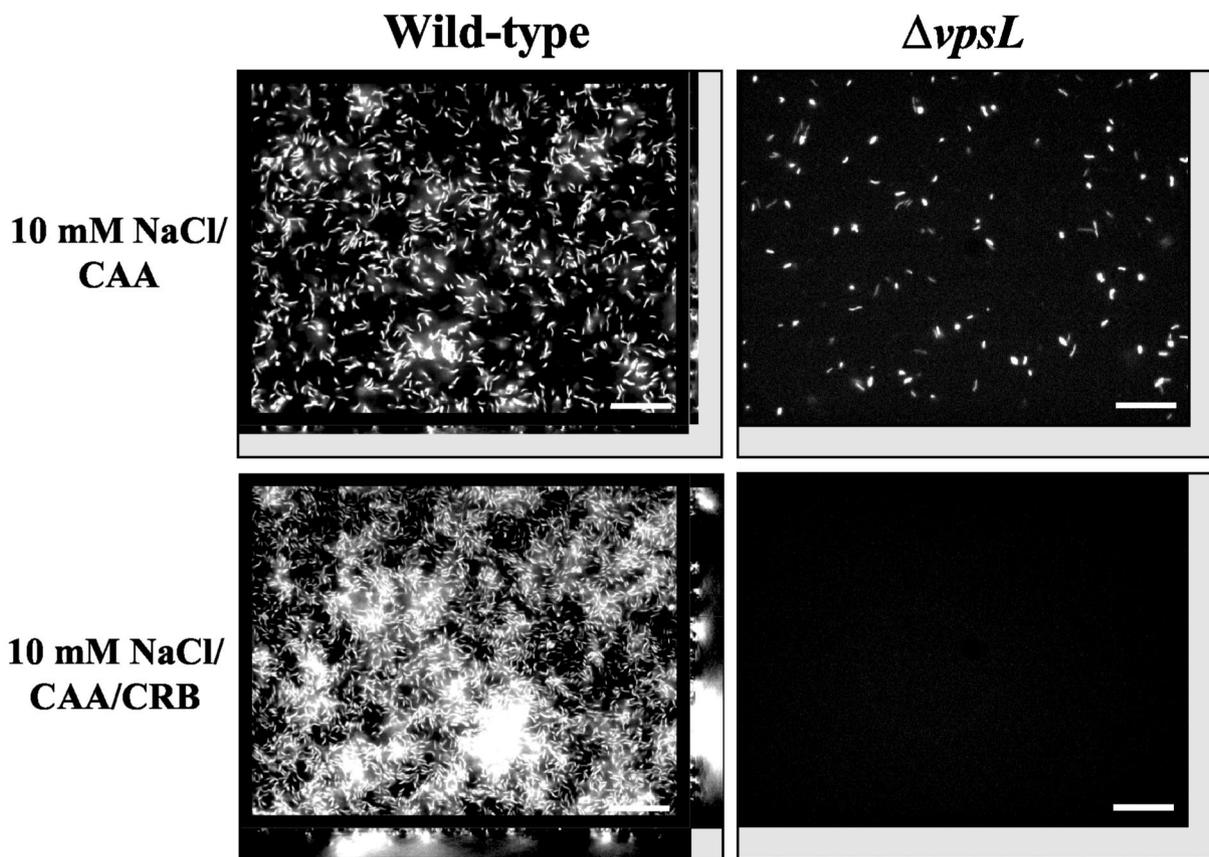


FIG. 4. Architecture of *V. cholerae* biofilms formed in 10 mM NaCl supplemented with monosaccharides and/or CAA: transverse and vertical cross sections through DAPI-stained wild-type *V. cholerae* strain MO10 and $\Delta vpsL$ mutant PW328 biofilms in 10 mM NaCl supplemented with CAA and 10 mM NaCl supplemented with CAA and monosaccharides (CRB). Transverse sections were obtained at the level of the substratum. Bars = 10 μm .

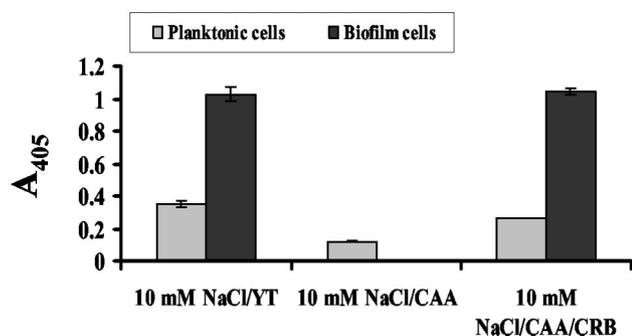


FIG. 5. Normalized β -galactosidase activities of biofilm-associated and planktonic wild-type *V. cholerae* strain PW357 cells grown in 10 mM NaCl–YT, 10 mM NaCl–CAA, and 10 mM NaCl supplemented with CAA and carbohydrates (CRB). This strain carries a chromosomal fusion of the *vpsL* promoter to *lacZ*.

charide synthesis, we questioned whether monosaccharide-rich growth medium activated transcription of *vps* genes or merely accelerated exopolysaccharide synthesis by providing an abundance of monosaccharide building blocks. To determine this, we measured *vpsL* transcription in biofilm-associated and planktonic cells grown in 10 mM NaCl supplemented with CAA in the presence and absence of monosaccharides and compared the results to the results obtained for *vpsL* transcription of planktonic and biofilm-associated cells grown in 10 mM

NaCl supplemented with YT. As shown in Fig. 5, *vpsL* transcription in biofilms formed in 10 mM NaCl supplemented with YT was 3-fold higher than *vpsL* transcription in the neighboring planktonic cells and 10-fold higher than *vpsL* transcription in planktonic cells grown in 10 mM NaCl supplemented with CAA. However, when the monosaccharide mixture was added to 10 mM NaCl supplemented with CAA, the pattern of *vpsL* transcription observed for planktonic and biofilm-associated cells was similar to the pattern observed for planktonic and biofilm-associated cells grown in 10 mM NaCl supplemented with YT. These data suggest that in addition to serving as the building blocks of VPS, monosaccharides activate *vps* transcription in both planktonic and biofilm-associated cells.

Calcium is required for formation of the *vps*-independent biofilm. We hypothesized that one or several components of artificial seawater were responsible for induction of the *vps*-independent biofilm. We initially used commercial artificial seawater in our experiments. Commercial artificial seawater, however, is intended for salt water aquariums rather than for laboratory experiments. In order to create a better defined medium whose composition could be altered, we purchased high-purity inorganic components and combined them in the concentrations reported to be in commercial artificial seawater to obtain DSW. The biofilms formed by wild-type *V. cholerae* and a $\Delta vpsL$ mutant in DSW supplemented with CAA are shown in Fig. 6. The biofilms formed in DSW exhibited the *vps* independence observed for biofilms formed in commercial ar-

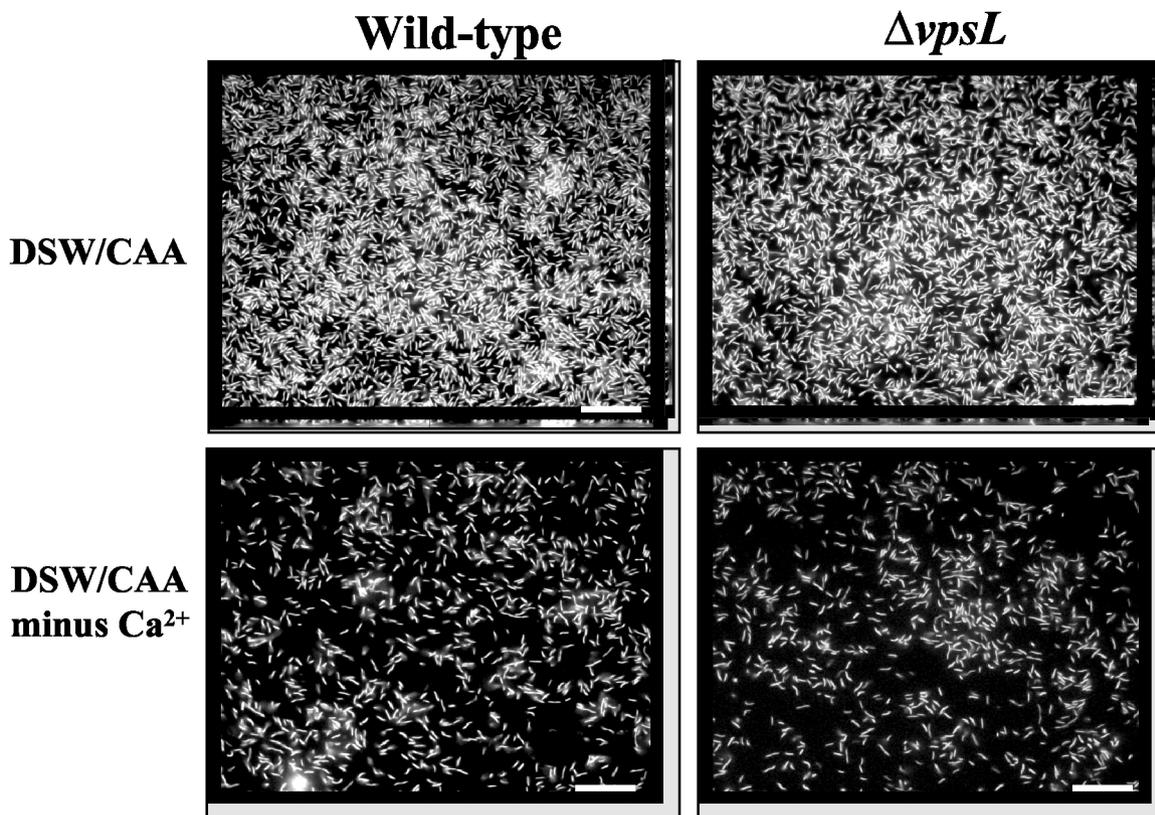


FIG. 6. Architecture of biofilms formed in DSW supplemented with CAA with and without Ca^{2+} ; transverse and vertical cross sections through DAPI-stained wild-type *V. cholerae* strain MO10 and $\Delta vpsL$ mutant PW328 biofilms. Transverse sections were obtained at the level of the substratum. Bars = 10 μ m.

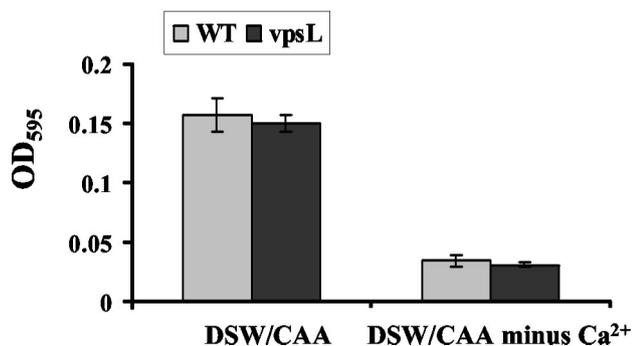


FIG. 7. Quantification of wild-type *V. cholerae* strain MO10 and $\Delta vpsL$ mutant PW328 surface-associated cells in DSW supplemented with CAA with and without Ca^{2+} .

tificial seawater. We also used quantitative parameters to compare wild-type *V. cholerae* and $\Delta vpsL$ mutant biofilms formed in commercial artificial seawater or DSW supplemented with CAA (Table 3). Interestingly, this analysis underscored differences between the biofilms formed in commercial artificial seawater and DSW. The biofilm formed in commercial artificial seawater was 2.7-fold thicker and contained 2.6-fold more biomass than the biofilm formed in DSW. Furthermore, the wild-type *V. cholerae* biofilm formed in commercial artificial seawater contained 1.7-fold more biomass than the biofilm formed by the $\Delta vpsL$ mutant, suggesting that there was moderate *vps* dependence. No *vps* dependence was observed in the structure of the biofilm formed in DSW. Quantification of the cells associated with wild-type *V. cholerae* and $\Delta vpsL$ mutant biofilms formed in DSW supplemented with CAA confirmed the *vps* independence (Fig. 7). We attributed the *vps* dependence of the biofilm formed in commercial artificial seawater to unreported organic components of this preparation. Furthermore, we noted that the $\Delta vpsL$ mutant biofilm formed in commercial artificial seawater contained almost twice as much biomass as the $\Delta vpsL$ mutant biofilm formed in DSW. We hypothesized that unreported components of artificial seawater enhance *vps*-independent biofilm development, as well as *vps*-dependent biofilm development.

In order to identify the component(s) of defined seawater that were required for *vps*-independent biofilm development, we first combined every additional component of DSW singly with a solution containing 468 mM NaCl and 10.3 mM KCl supplemented with 1% CAA. We found, however, that no single component of DSW, when combined with 468 mM NaCl, 10.3 mM KCl, and 1% CAA, restored surface attachment by wild-type *V. cholerae* (data not shown). We then hypothesized that several components of DSW and rich medium might be required for biofilm development but might not be sufficient to activate a *vps*-independent biofilm. To test this hypothesis, we added each component of DSW to LB broth. Biofilm development by wild-type *V. cholerae* and a $\Delta vpsL$ mutant was tested in all these media. We observed that Ca^{2+} was required for *vps*-independent biofilm formation in LB broth. We then grew wild-type *V. cholerae* in a medium containing all elements present in defined seawater except Ca^{2+} . In this medium, very few attached cells were observed, and these cells were arranged in a flat monolayer (Fig. 6). Further-

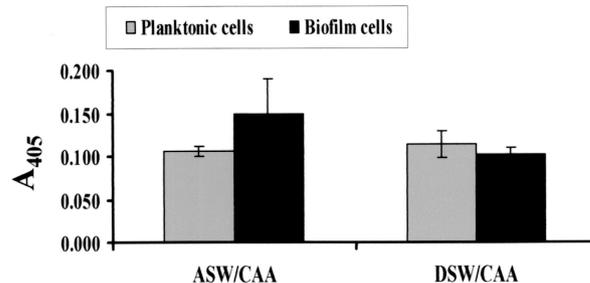


FIG. 8. Normalized β -galactosidase activities of biofilm-associated and planktonic wild-type *V. cholerae* strain PW357 cells grown in commercial artificial seawater (ASW) supplemented with CAA and DSW supplemented with CAA. This strain carries a chromosomal fusion of the *vpsL* promoter to *lacZ*.

more, quantification of surface association demonstrated that there were very few attached cells (Fig. 7). Thus, we concluded that Ca^{2+} is the component of DSW that enables *vps*-independent biofilm formation.

Transcription of *vps* genes is low in artificial seawater supplemented with CAA. Because biofilms formed in artificial seawater and defined seawater supplemented with CAA displayed *vps* independence, we predicted that the *vpsL* genes would not be highly transcribed by planktonic cells or biofilm cells grown in these media. To test this prediction, we measured *vpsL* transcription by planktonic and surface-associated wild-type *V. cholerae* and $\Delta vpsL$ mutant cells. As shown in Fig. 8, *vpsL* transcription was quite low in cells associated with the *vpsL*-independent biofilms formed in both artificial seawater and DSW supplemented with CAA.

A distinct *vps*-independent exopolysaccharide is not observed in the extracellular matrix of *V. cholerae* biofilms formed in artificial seawater. We observed that the *V. cholerae* $\Delta vpsL$ mutant formed a biofilm in commercial artificial seawater supplemented with CAA. Because biofilm formation often requires an extracellular matrix containing exopolysaccharide, we hypothesized that the extracellular matrix of the Δvps mutant biofilm might contain a novel exopolysaccharide that was independent of the *vps* genes. To test this hypothesis, we isolated the extracellular matrices of a wild-type *V. cholerae* biofilm formed in LB broth and wild-type *V. cholerae* and Δvps mutant biofilms formed in commercial artificial seawater supplemented with CAA and determined their monosaccharide compositions. The *V. cholerae* biofilm matrix formed in LB broth contained monosaccharides in the following molar ratio: *N*-acetylglucosamine, 0.49; galactose, 0.13; glucose, 0.23; and mannose, 0.15. This composition is similar to that reported for the biofilm matrix of a rugose variant of *V. cholerae* El Tor, although our biofilm matrix contained considerably more glucose (43). The wild-type *V. cholerae* biofilm formed in commercial artificial seawater supplemented with CAA had a monosaccharide composition similar to that of the biofilm formed in LB broth, except that significant amounts of *N*-acetylgalactosamine were present. The exact molar ratio of monosaccharides for this biofilm matrix was as follows: *N*-acetylglucosamine, 0.25; galactose, 0.08; glucose, 0.19; mannose, 0.11; and *N*-acetylgalactosamine, 0.26. Because *N*-acetylgalactosamine was not present in the matrix of the wild-type *V.*

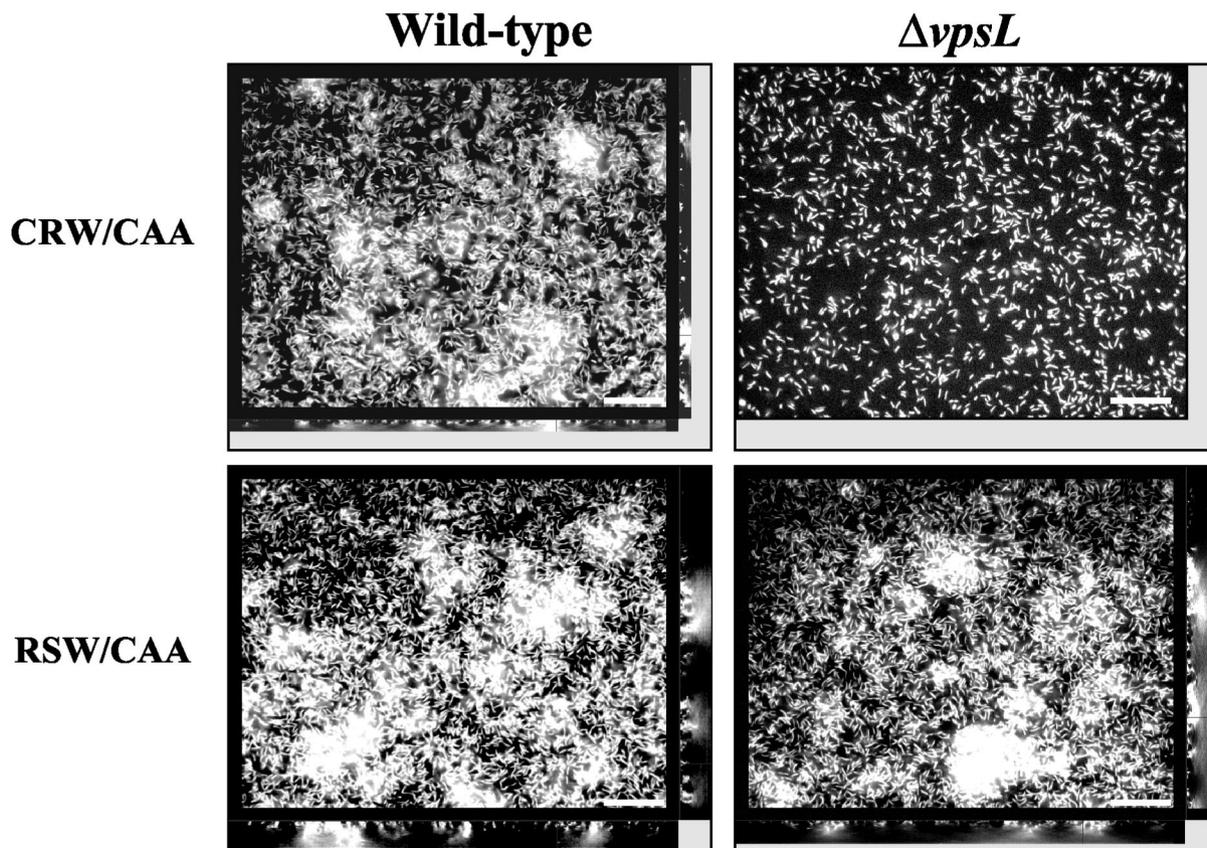


FIG. 9. Architecture of wild-type *V. cholerae* and $\Delta vpsL$ mutant biofilms formed in water from the Charles River in Massachusetts (CRW) and in water from the Massachusetts coast (RSW) supplemented with CAA: transverse and vertical cross sections through DAPI-stained biofilms. Transverse sections were obtained at the level of the substratum. Bars = 10 μm .

cholerae biofilm formed in artificial seawater supplemented with YT, we hypothesized that this component is the result of the nutrient source provided rather than the result of the components of artificial seawater (data not shown). The presence of polysaccharide in the matrix of the wild-type *V. cholerae* biofilm formed in artificial seawater is consistent with the *vps* dependence noted by Comstat analysis of this biofilm. Analysis of the Δvps mutant biofilm matrix formed in commercial artificial seawater did not suggest that there was an alternative *vps*-independent exopolysaccharide matrix. Rather, the number of moles of monosaccharide per microgram of the extracellular matrix isolated from the Δvps mutant biofilms was approximately 20-fold less than that of the extracellular matrix isolated from the wild-type *V. cholerae* biofilm formed in seawater. *N*-Acetylgalactosamine was always absent from the matrix of the Δvps mutant biofilm, suggesting that this component was dependent on the *vps* genes as well. Thus, we found no evidence that there was a novel *vps*-independent exopolysaccharide in the extracellular matrix of the Δvps mutant biofilm formed in commercial artificial seawater.

***vps*-dependent biofilm development occurs in a freshwater-based medium, while *vps*-independent biofilm development occurs in a seawater-based medium.** Because calcium is scarce in freshwater and abundant in seawater, we hypothesized that only *vps*-dependent surface adhesion might be available to *V.*

cholerae in freshwater environments, while both *vps*-dependent and *vps*-independent biofilm development would be possible in marine environments. We collected local freshwater and seawater from the Charles River and from the Massachusetts coast, respectively. In order to test our hypothesis, we formed wild-type *V. cholerae* and $\Delta vpsL$ mutant biofilms in true freshwater and seawater supplemented with CAA and studied their *vps* dependence. As shown in Fig. 9, the wild-type *V. cholerae* biofilm formed in freshwater had pillars, while the $\Delta vpsL$ mutant biofilm consisted only of a monolayer of cells. Thus, formation of the three-dimensional structure of the *V. cholerae* biofilm formed in this freshwater medium required the *vps* genes. In contrast, both wild-type *V. cholerae* and a $\Delta vpsL$ mutant formed robust biofilms in true seawater (Fig. 9). Thus, a *vps*-independent biofilm was formed in true seawater. We performed Comstat analysis on the wild-type *V. cholerae* and $\Delta vpsL$ mutant biofilms formed in true freshwater and seawater (Tables 2 and 3). Although the freshwater biofilm exhibited *vps* dependence, the biomass and average thickness of the wild-type *V. cholerae* biofilm formed in freshwater supplemented with CAA were 4.8- and 6.2-fold less, respectively, than those of the wild-type *V. cholerae* biofilm formed in 10 mM NaCl supplemented with CAA and monosaccharides. This most likely reflected the relative paucity of monosaccharides present in our freshwater sample. In contrast, the quantitative mea-

TABLE 4. Inorganic components of true river water, DSW, and true seawater

Water	Concn (mM) of:			Osmolarity (mosM)
	Na ⁺	K ⁺	Ca ²⁺	
Charles River	8.7	0.23	0.47	45
DSW	468	10.31	9.95	990
Massachusetts coast	435	15.64	9.5	968

surements of the wild-type *V. cholerae* and $\Delta vpsL$ mutant biofilms formed in true seawater supplemented with CAA suggested that there was no *vps* dependence (Table 3). The structure of the *V. cholerae* biofilm formed in true seawater differed significantly from the structure of the biofilm formed in DSW. Although the overall biomass of the biofilm formed in true seawater was only 1.4-fold greater than the overall biomass of the biofilm formed in DSW, the maximum thickness of the former biofilm was 3-fold greater than the maximum thickness of the latter biofilm. Thus, the biofilm formed in true seawater contained a few tall pillars. We hypothesized that true seawater contains organic or inorganic components that promote the formation of pillars without a requirement for the VPS polysaccharide.

In order to determine if the activators of *vps*-dependent and -independent biofilms were present in true freshwater and seawater, respectively, we analyzed the inorganic and organic constituents of our true freshwater and seawater. The results of the inorganic analysis are shown in Table 4. We found that the Ca²⁺ concentration of true freshwater was quite low, as required for formation of a *vps*-dependent biofilm. The Na⁺ concentration and osmolarity were quite low, as expected for freshwater. The inorganic composition and osmolarity of our DSW were very similar to those of our true seawater. In particular, the Ca²⁺ concentration was high, as required for *vps*-independent biofilm development.

Potential environmental roles for *vps*-dependent and *vps*-independent biofilm development. In this work, we identified and characterized a *vps*-dependent *V. cholerae* biofilm and a *vps*-independent *V. cholerae* biofilm. The *vps*-dependent biofilm predominated in growth media containing monosaccharides. We hypothesized, therefore, that *vps*-dependent biofilm development occurs only in nutrient-rich environments. This type of biofilm development may facilitate *V. cholerae*'s colonization of favorable aquatic environments. Conversion of environmental monosaccharides into an exopolysaccharide matrix may even serve as a form of nutrient storage. In contrast, millimolar Ca²⁺ concentrations, which are present in seawater but not in freshwater, are required for *vps*-independent biofilm development. Thus, we hypothesized that *vps*-independent biofilm development occurs primarily in marine environments. Furthermore, in contrast to the variability of organic nutrients in aquatic environments, the concentration of Ca²⁺ is uniformly high in the marine environment. Thus, we speculated that *V. cholerae* surface adhesion in marine environments may be less discriminating.

Cholera epidemics have been associated with heavy rainfall and increases in sea surface height (19, 20). Both of these conditions are predicted to alter the organic and inorganic compositions of an estuary, which is the primary interface

between humans and the marine environment (11, 23, 29, 33). We hypothesized that changes in the organic and inorganic compositions of the aquatic environment may alter the nature of *V. cholerae*'s association with surfaces. It is interesting to speculate that these types of transitions may play some role in the initiation of cholera epidemics.

ACKNOWLEDGMENTS

We thank Arne Heydorn, Soren Molin, and their colleagues for generous sharing of expertise and the Comstat software. We also thank Anne Kane of the GRASP Center and her staff for their patience with the development of the various growth media and their expert preparation of many reagents, which greatly accelerated the course of our experiments.

This work was supported by grant NIH R01 AI50032 to P.I.W. and also by pilot project grant P30 DK34928 from the New England Medical Center GRASP Center (NIH/NIDDK).

REFERENCES

- Allison, D. G., and M. J. Goldsbrogh. 1994. Polysaccharide production in *Pseudomonas cepacia*. J. Basic Microbiol. **34**:3–10.
- Bechet, M., and R. Blondeau. 2003. Factors associated with the adherence and biofilm formation by *Aeromonas caviae* on glass surfaces. J. Appl. Microbiol. **94**:1072–1078.
- Bryan, B. A., R. J. Linhardt, and L. Daniels. 1986. Variation in composition and yield of exopolysaccharides produced by *Klebsiella* sp. strain K32 and *Acinetobacter calcoaceticus* BD4. Appl. Environ. Microbiol. **51**:1304–1308.
- Burdman, S., E. Jurkevitch, B. Schwartsburd, M. Hampel, and Y. Okon. 1998. Aggregation in *Azospirillum brasilense*: effects of chemical and physical factors and involvement of extracellular components. Microbiology **144**:1989–1999.
- Colwell, R. R., and W. M. Spira. 1992. The ecology of *Vibrio cholerae*, p. 107–127. In D. Barua and W. B. I. Greenough (ed.), Cholera. Plenum, New York, N.Y.
- Danese, P. N., L. A. Pratt, and R. Kolter. 2000. Exopolysaccharide production is required for development of *Escherichia coli* K-12 biofilm architecture. J. Bacteriol. **182**:3593–3596.
- Davey, M. E., and G. A. O'Toole. 2000. Microbial biofilms: from ecology to molecular genetics. Microbiol. Mol. Biol. Rev. **64**:847–867.
- Davies, D. G., M. R. Parsek, J. P. Pearson, B. H. Iglewski, J. W. Costerton, and E. P. Greenberg. 1998. The involvement of cell-to-cell signals in the development of a bacterial biofilm. Science **280**:295–298.
- Di Martino, P., N. Cafferini, B. Joly, and A. Darfeuille-Michaud. 2003. *Klebsiella pneumoniae* type 3 pili facilitate adherence and biofilm formation on abiotic surfaces. Res. Microbiol. **154**:9–16.
- Garay, E. A., A. Arnau, and C. Amaro. 1985. Incidence of *Vibrio cholerae* and related vibrios in a coastal lagoon and seawater influenced by lake discharges along an annual cycle. Appl. Environ. Microbiol. **50**:426–430.
- Han, W. Q., and P. J. Webster. 2002. Forcing mechanisms of sea level interannual variability in the Bay of Bengal. J. Phys. Oceanogr. **32**:216–239.
- Haugo, A. J., and P. I. Watnick. 2002. *Vibrio cholerae* CytR is a repressor of biofilm development. Mol. Microbiol. **45**:471–483.
- Heydorn, A., A. T. Nielsen, M. Hentzer, C. Sternberg, M. Givskov, B. K. Ersboll, and S. Molin. 2000. Quantification of biofilm structures by the novel computer program COMSTAT. Microbiology **146**:2395–2407.
- Horton, R. M., Z. L. Cai, S. N. Ho, and L. R. Pease. 1990. Gene splicing by overlap extension: tailor-made genes using the polymerase chain reaction. BioTechniques **8**:528–535.
- Kachlany, S. C., P. J. Planet, R. DeSalle, D. H. Fine, and D. H. Figurski. 2001. Genes for tight adherence of *Actinobacillus actinomycetemcomitans*: from plaque to plague to pond scum. Trends Microbiol. **9**:429–437.
- Kang, Y., H. Liu, S. Genin, M. A. Schell, and T. P. Denny. 2002. *Ralstonia solanacearum* requires type 4 pili to adhere to multiple surfaces and for natural transformation and virulence. Mol. Microbiol. **46**:427–437.
- Kim, S. H., S. Ramaswamy, and J. Downard. 1999. Regulated exopolysaccharide production in *Myxococcus xanthus*. J. Bacteriol. **181**:1496–1507.
- Knobloch, J. K., K. Bartscht, A. Sabotitke, H. Rohde, H. H. Feucht, and D. Mack. 2001. Biofilm formation by *Staphylococcus epidermidis* depends on functional RsbU, an activator of the *sigB* operon: differential activation mechanisms due to ethanol and salt stress. J. Bacteriol. **183**:2624–2633.
- Lipp, E. K., A. Hug, and R. R. Colwell. 2002. Effects of global climate on infectious disease: the cholera model. Clin. Microbiol. Rev. **15**:757–770.
- Lobitz, B., L. Beck, A. Hug, B. Wood, G. Fuchs, A. S. G. Faruque, and R. Colwell. 2000. Climate and infectious disease: use of remote sensing for detection of *Vibrio cholerae* by indirect measurement. Proc. Natl. Acad. Sci. USA **97**:1438–1443.
- Looijesteijn, P. J., I. C. Boels, M. Kleerebezem, and J. Hugenholtz. 1999.

- Regulation of exopolysaccharide production by *Lactococcus lactis* subsp. *cremoris* by the sugar source. *Appl. Environ. Microbiol.* **65**:5003–5008.
22. Mah, T. F., and G. A. O'Toole. 2001. Mechanisms of biofilm resistance to antimicrobial agents. *Trends Microbiol.* **9**:34–39.
 23. Mahadevan, A., and B. Subramanian. 1999. Seasonal and diurnal variation of hydrobiological characters of coastal water of Chennai (Madras), Bay of Bengal. *Indian J. Mar. Sci.* **28**:429–433.
 24. Mozzi, F., G. Rollan, G. S. de Giori, and G. Font de Valdez. 2001. Effect of galactose and glucose on the exopolysaccharide production and the activities of biosynthetic enzymes in *Lactobacillus casei* CRL 87. *J. Appl. Microbiol.* **91**:160–167.
 25. Murphy, T. F., and C. Kirkham. 2002. Biofilm formation by nontypeable *Haemophilus influenzae*: strain variability, outer membrane antigen expression and role of pili. *BMC Microbiol.* **2**:7.
 26. Myatt, D. C., and G. H. Davis. 1989. Isolation of medically significant *Vibrio* species from riverine sources in south east Queensland. *Microbios* **60**:111–123.
 27. Nesper, J., C. M. Lauriano, K. E. Klose, D. Kapfhammer, A. Kraiss, and J. Reidl. 2001. Characterization of *Vibrio cholerae* O1 El tor *galU* and *galE* mutants: influence on lipopolysaccharide structure, colonization, and biofilm formation. *Infect. Immun.* **69**:435–445.
 28. O'Toole, G. A., and R. Kolter. 1998. Flagellar and twitching motility are necessary for *Pseudomonas aeruginosa* biofilm development. *Mol. Microbiol.* **30**:295–304.
 29. Padmavathi, D., and D. Satyanarayana. 1999. Distribution of nutrients and major elements in riverine, estuarine and adjoining coastal waters of Godavari, Bay of Bengal. *Indian J. Mar. Sci.* **28**:345–354.
 30. Perez-Rosas, N., and T. C. Hazen. 1989. In situ survival of *Vibrio cholerae* and *Escherichia coli* in a tropical rain forest watershed. *Appl. Environ. Microbiol.* **55**:495–499.
 31. Planet, P. J., S. C. Kachlany, D. H. Fine, R. DeSalle, and D. H. Figurski. 2003. The widespread colonization island of *Actinobacillus actinomycetem-comitans*. *Nat. Genet.*, **34**:193–198.
 32. Pratt, L. A., and R. Kolter. 1998. Genetic analysis of *Escherichia coli* biofilm formation: roles of flagella, motility, chemotaxis and type I pili. *Mol. Microbiol.* **30**:285–293.
 33. Reemtsma, T., V. Ittekkot, M. Bartsch, and R. R. Nair. 1993. River inputs and organic-matter fluxes in the northern Bay of Bengal. *Chem. Geol.* **103**:55–71.
 34. Rose, R. K., and S. J. Turner. 1998. Extracellular volume in streptococcal model biofilms: effects of pH, calcium and fluoride. *Biochim. Biophys. Acta* **1379**:185–190.
 35. Sheikh, J., S. Hicks, M. Dall'Agnol, A. D. Phillips, and J. P. Nataro. 2001. Roles for Fis and YafK in biofilm formation by enteroaggregative *Escherichia coli*. *Mol. Microbiol.* **41**:983–997.
 36. Venkateswaran, K., C. Kiiyukia, M. Takaki, H. Nakano, H. Matsuda, H. Kawakami, and H. Hashimoto. 1989. Characterization of toxigenic vibrios isolated from the freshwater environment of Hiroshima, Japan. *Appl. Environ. Microbiol.* **55**:2613–2618.
 37. Wai, S. N., Y. Mizunoe, A. Takade, S. I. Kawabata, and S. I. Yoshida. 1998. *Vibrio cholerae* O1 strain TSI-4 produces the exopolysaccharide materials that determine colony morphology, stress resistance, and biofilm formation. *Appl. Environ. Microbiol.* **64**:3648–3655.
 38. Waldor, M. K., and J. J. Mekalanos. 1994. Emergence of a new cholera pandemic: molecular analysis of virulence determinants in *Vibrio cholerae* O139 and development of a live vaccine prototype. *J. Infect. Dis.* **170**:278–283.
 39. Watnick, P. I., K. J. Fullner, and R. Kolter. 1999. A role for the mannose-sensitive hemagglutinin in biofilm formation by *Vibrio cholerae* El Tor. *J. Bacteriol.* **181**:3606–3609.
 40. Watnick, P. I., and R. Kolter. 1999. Steps in the development of a *Vibrio cholerae* biofilm. *Mol. Microbiol.* **34**:586–595.
 41. Watnick, P. I., C. M. Lauriano, K. E. Klose, L. Croal, and R. Kolter. 2001. Absence of a flagellum leads to altered colony morphology, biofilm development, and virulence in *V. cholerae* O139. *Mol. Microbiol.* **39**:223–235.
 42. Yildiz, F. H., N. A. Dolganov, and G. K. Schoolnik. 2001. VpsR, a member of the response regulators of the two-component regulatory systems, is required for expression of *vps* biosynthesis genes and EPS^{ET}-associated phenotypes in *Vibrio cholerae* O1 El Tor. *J. Bacteriol.* **183**:1716–1726.
 43. Yildiz, F. H., and G. K. Schoolnik. 1999. *Vibrio cholerae* O1 El Tor: identification of a gene cluster required for the rugose colony type, exopolysaccharide production, chlorine resistance, and biofilm formation. *Proc. Natl. Acad. Sci. USA* **96**:4028–4033.