The *Vibrio cholerae* O139 O-antigen polysaccharide is essential for Ca^{2+}-dependent biofilm development in sea water

Katharine Kierek and Paula I. Watnick*

Division of Geographic Medicine and Infectious Diseases, Tufts–New England Medical Center, Boston, MA 02111

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***Vibrio cholerae* is both an inhabitant of estuarine environments and the etiologic agent of the diarrheal disease cholera. Previous work has demonstrated that *V. cholerae* forms both an exopolysaccharide-dependent biofilm and a Ca^{2+}-dependent biofilm. In this work, we demonstrate a role for the O-antigen polysaccharide of *V. cholerae* in Ca^{2+}-dependent biofilm development in model and true sea water. Interestingly, *V. cholerae* biofilms, as well as the biofilms of several other *Vibrio* species, disintegrate when Ca^{2+} is removed from the bathing medium, suggesting that Ca^{2+} is interacting directly with the O-antigen polysaccharide. In the Bay of Bengal, cholera incidence has been correlated with increased sea surface height. Because of the low altitude of this region, increases in sea surface height are likely to lead to transport of sea water, marine particulates, and marine biofilms into fresh water environments. Because fresh water is Ca^{2+}-poor, our results suggest that one potential outcome of an increase is sea surface height is the dispersal of marine biofilms with an attendant increase in planktonic marine bacteria such as *V. cholerae*. Such a phenomenon may contribute to the correlation of increased sea surface height with cholera.**

Vibrio cholerae, a Gram-negative rod-shaped bacterium, is both a natural inhabitant of estuarine environments and the agent of the severe diarrheal disease cholera. It has acquired great fame and infamy as the perpetrator of seven pandemics. During each pandemic, cholera has struck coastal regions before spreading inland. More recently, a correlation has been reported between sea surface height in the Bay of Bengal and cholera (1). This finding has led researchers to suggest that estuaries are the reservoirs of *V. cholerae* and that changes in estuarine conditions may precipitate cholera epidemics (2).

In aquatic environments, bacteria are generally found in association with surfaces. In fact, *V. cholerae* has been detected on the surfaces of aquatic organisms such as zooplankton, phytoplankton, insects, crustaceans, and plants (3–5), and we hypothesize that the ability of *V. cholerae* to attach to biotic and abiotic surfaces in what is termed a biofilm may be relevant to environmental survival.

The lipopolysaccharide (LPS) of Gram-negative bacteria consists of three domains: (*i*) lipid A, a hydrophobic domain that associates with the outer membrane, (*ii*) an oligosaccharide core, and (*iii*) a terminal, immunogenic polysaccharide domain termed the O antigen. The O-antigen serogroup provides a rational basis for the classification of environmental and clinical strains of *V. cholerae.* While environmental strains of *V. cholerae* fall into many different O-antigen serogroups, only the O1 and O139 serogroups have been associated with the disease cholera. The monosaccharide compositions of the *V. cholerae* O1 and O139 O antigens differ considerably. In particular, the O1 polysaccharide contains perosamine (6, 7), whereas the O139 polysaccharide is distinguished by a D-galactose 4,6-cyclophosphate moiety (8). Furthermore, *V. cholerae* in the O139 serogroup are encapsulated, whereas pathogenic bacteria in the O1 serogroup are not. Several genes are required for the synthesis of both the *V. cholerae* O139 O antigen and capsule (9, 10). Furthermore, the *V. cholerae* O139 O antigen consists of a single oligosaccharide unit that is identical to the repeating unit of the capsule (8). Thus, both the O antigen and capsule are composed of O-antigen polysaccharide.

In LB broth, the *V. cholerae* O1 El Tor strain N16961 requires the mannose-sensitive hemagglutinin (MSHA), a type IV pilus, and the flagellum to associate with abiotic surfaces (11–13), whereas the *V. cholerae* O139 strain MO10 depends only on the flagellum for surface association (14). In both strains, subsequent development of a three-dimensional biofilm requires the *vps* genes, which are responsible for the synthesis of an exopolysaccharide-based adhesive extracellular matrix (11, 14, 15).

Spontaneous rugose variants of *V. cholerae* have been reported (16, 17, †). Whereas many clinical isolates of *V. cholerae* form smooth colonies on LB agar plates, these variants form colonies with rough surfaces. The rugose colony morphology is the result of increased synthesis of the VPS exopolysaccharide (15, 19, 20), and transcriptional regulation of the *vps* genes, which are required for synthesis of the VPS exopolysaccharide, is altered in these strains (21). Thus, these variants rapidly form biofilms in LB broth that are much thicker than those formed by smooth-colony variants of *V. cholerae*.

Recently, we have reported the formation of a *vps*-independent *V. cholerae* biofilm in model sea water (22). Whereas monosaccharides are required for formation of the *vps*-dependent biofilm, Ca^{2+} is required for development of the *vps*-independent biofilm. Ca^{2+} has been reported to play various roles in biofilm formation by diverse bacterial species. In particular, it has been suggested that Ca^{2+} directly stabilizes intercellular interactions in *Pseudomonas aeruginosa* and *Streptococcus downei* biofilms, presumably by the formation of intercellular salt bridges (23–25). The interactions of surface-associated lectins of oral streptococci with galactose and *N*-acetylgalactose moieties are enhanced by addition of Ca^{2+} (23). Finally, it has been suggested that the enhanced intercellular cohesion of the myxobacterium *Stigmatella aurantiaca* in response to Ca^{2+} is correlated with the altered expression and cellular localization of a number of proteins (26). The mechanisms underlying the Ca^{2+} dependence of *vps*-independent *V. cholerae* biofilm formation have not yet been established.

In this work, our goal was to delineate the genetic requirements for Ca^{2+}-dependent biofilm development, to test Ca^{2+}-dependent biofilm development as a paradigm for biofilm development by estuarine pathogens in natural environments, and to investigate the implications of Ca^{2+}-dependent biofilm de-

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Abbreviation: MSHA, mannose-sensitive hemagglutinin.

*To whom correspondence should be addressed at: Division of Geographic Medicine and Infectious Disease, New England Medical Center, 750 Washington Street, Box 041, Boston, MA 02111. E-mail: pwatnick@tufts-nemc.org.

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velopment for surface-attached bacteria in the variable estuarine environment.

Materials and Methods

Bacterial Strains, Plasmids, and Media. MO10, a *V. cholerae* O139 clinical isolate (27), and mutants derived from this strain were used in all experiments except those examining biofilms formed by a *V. cholerae* O1 El Tor clinical isolate (N1961), environmental *V. cholerae* isolates displaying various O antigens (gifts of M. K. Waldor, New England Medical Center), *Vibrio parahaemolyticus*, *Vibrio fluvialis*, *Vibrio alginolyticus*, and *Vibrio vulnificus* (gifts of S. B. Calderwood, Massachusetts General Hospital, Boston). The *V. cholerae* Δ*flaA* and Δ*mshA* mutants used in these studies have previously been described (11). Biofilms were formed in an inorganic base of either commercial artificial sea water (Instant Ocean; Aquarium Systems, Mentor, OH) or DSW medium, a defined mixture of salts based on the composition of artificial sea water as follows: 468 mM NaCl, 55 mM $MgSO_4$, 3.0 mM $NaHCO_3$, 9.9 mM $CaCl_2$, 10.3 mM KCl, 0.0014 mM $Na_2B_4O_7$, 0.1 mM $SrCl_2$, 0.03 mM NaBr, 0.002 mM NaI, and 0.026 mM LiCl. Both inorganic mixtures were supplemented with 1% vitamin assay Casamino acids (Difco). True fresh water and sea water were obtained from the Charles River (Newton, MA) and the Massachusetts coast, respectively. The Ca^{2+} ion concentrations of our true fresh water and sea water samples were 0.47 mM and 9.95 mM, respectively. In biofilm assays, true fresh water and sea water were supplemented with 1% vitamin assay Casamino acids.

Transposon-Insertion Mutagenesis and Screening. A previously generated *V. cholerae* O139 transposon-insertion mutant library was used as follows (22, 28). Mutant biofilms were formed in commercial artificial sea water medium and evaluated by crystal violet staining as previously described (28). All biofilm-altered mutants were evaluated for hemagglutination and flagellar motility as previously described (14).

Construction of Δ*wbfF*, Δ*wbfR*, and Δ*waaL* Mutants. Δ*wbfF* and Δ*wbfR* in-frame deletion mutants were constructed by double homologous recombination using the suicide plasmids pKKΔ*wbfF* and pKKΔ*wbfR*, which were constructed for this study. Each plasmid harbored a large in-frame deletion of the relevant gene. The Δ*waaL* mutant was constructed by using plasmid pKEKΔ*waaL* generously provided by J. Reidl (29). The presence or absence of the lipopolysaccharide O antigen and capsule in wild-type *V. cholerae* and mutants was confirmed by immunoblot analysis as previously described by using whole cell antiserum generously provided by M. Waldor (9, 29). As expected, immunoblots of wild-type *V. cholerae*, the Δ*wbfF* mutant, the Δ*waaL* mutant, and the Δ*wbfR* mutant demonstrated the presence of both O antigen and capsule, the O antigen alone, the capsule alone, and neither the O antigen nor the capsule, respectively (data not shown). In liquid culture, the growth rates of all mutants were indistinguishable from the growth rate of wild-type *V. cholerae.*

Biofilm Quantifications. Quantification of surface association was performed as described previously (22). Briefly, borosilicate glass tubes were filled with 300 μl of DSW medium and inoculated with the strain under study. After incubation for 24 h, tubes were rinsed and filled with 300 μl of fresh medium. Biofilm-associated cells were dispersed by mixing in a Vortex apparatus for 10 sec in the presence of borosilicate glass beads (Biospec). Finally, the optical density of the biofilm cell suspension was measured.

Phase-Contrast Microscopy, Fluorescence Microscopy, and Quantitative Analysis of Biofilm Structure. For phase-contrast microscopy, biofilms were formed in sterile 24-well polystyrene microtiter dishes containing 300 μl of DSW medium. After 24 h, biofilms were washed three times with fresh medium and visualized with an Eclipse TE-200 microscope (Nikon) equipped with an Orca digital charge-coupled device camera (Hamamatsu). The bathing medium was then removed and replaced with complete DSW medium, DSW medium lacking Ca^{2+}, DSW medium lacking Mg^{2+}, or DSW medium lacking Ca^{2+} but containing an equivalent concentration of Mn^{2+}. Biofilms were incubated with the relevant fresh medium for the length of time indicated, rinsed with fresh medium, and then revisualized by microscopy.

Biofilms were prepared and visualized by fluorescence microscopy as previously described (22). Various attributes of the biofilm structure were then quantified by using the COMSTAT software developed by Heydorn and colleagues (30). Reported values represent the average of six image stacks (three image stacks collected in two independent experiments).

Results and Discussion

Structures Identified in the Genetic Screen for Biofilm-Altered Mutants. The *vps*-independent biofilms of 6,000 transposon-insertion mutants were evaluated, and 86 biofilm-altered mutants were identified. Flagellar motility and the MSHA type IV pilus have previously been implicated in *V. cholerae* O1 El Tor biofilm development. Thus, all biofilm-defective mutants were tested for hemagglutination and motility. Ten biofilm-defective mutants displayed decreased or absent motility, and seven mutants were unable to agglutinate red blood cells. The transposon-insertion junctions of 8 motile, hemagglutination-competent biofilm-altered mutants were successfully sequenced after amplification by the arbitrary PCR technique (14, 31, 32). These mutants were found to have transposon insertions in genes located in operons responsible for either O-antigen polysaccharide synthesis, such as the putative asparagine synthetase *wbfR*, or export of capsular precursors, such as *wbfF*. Crystal violet staining of mutant biofilms suggested that surface adherence of mutants lacking the capsule was increased, whereas surface adherence of mutants lacking both the O antigen and capsule was markedly defective.

MSHA and the Flagellum Are Required for *vps*-Independent Biofilm Development. The results of our genetic screen suggested that MSHA and the flagellum are required for *vps*-independent *V. cholerae* biofilm development. To confirm these results, previously constructed *V. cholerae* O139 strains harboring deletions in *mshA* and *flaA* were assayed for *vps*-independent biofilm development over time (11). As shown in Fig. 1, both mutants displayed a defect in biofilm development. However, the Δ*mshA* mutant was able to accumulate on the surface at a rate just slightly less than that of wild-type *V. cholerae*, whereas the Δ*flaA* mutant displayed little surface accumulation. To visualize the three-dimensional structures of the Δ*mshA* and Δ*flaA* mutant biofilms, we used fluorescence microscopy. Vertical and horizontal cross sections through wild-type and mutant biofilms formed over 24 h are shown in Fig. 2. Analysis of these images by using the COMSTAT software of Heydorn *et al.* (30) is shown in Table 1. This analysis demonstrated that the total biomass and substratum coverage of Δ*mshA* biofilms was not significantly different from that of wild-type *V. cholerae*. These biofilms differed primarily in average and maximum biofilm thickness, suggesting that intercellular contacts were less efficiently established in the Δ*mshA* biofilm. In contrast, all parameters of the Δ*flaA* mutant biofilm were significantly decreased compared with those of the wild-type *V. cholerae* biofilm.

The defects of the *V. cholerae* O139 Δ*mshA* and Δ*flaA* mutants in *vps*-independent biofilm development differ from those pre-

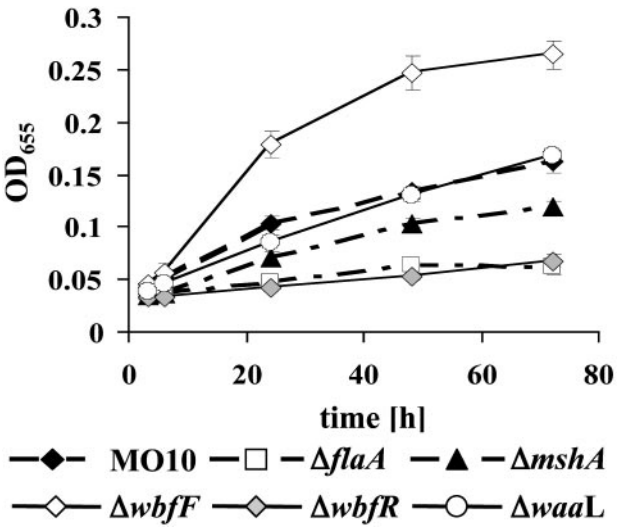

Fig. 1. Quantification of time-dependent surface association in defined sea water medium (DSW) by wild-type *V. cholerae* (MO10) and Δ*flaA*, Δ*mshA*, Δ*wbfF*, Δ*wbfR*, and Δ*waaL* mutants.

viously reported for *vps*-dependent biofilm development in LB broth (11). In LB broth, biofilm development by the Δ*mshA* mutant was indistinguishable from that of wild-type *V. cholerae.* Furthermore, surface accumulation by the Δ*flaA* mutant was much less defective in LB broth than in DSW medium. This observation suggests that an additional mediator of surface adhesion, synthesized during growth in LB broth but not DSW medium, masks the attachment defects of the Δ*mshA* and Δ*flaA* mutants.

Both the *V. cholerae* O139 O Antigen and Capsule Promote *vps*-Independent Biofilm Development. *V. cholerae* biofilms formed in artificial sea water are independent of the *vps* genes. Our genetic screen suggested that the capsule and/or O antigen of *V. cholerae* O139 might provide an alternative adhesive polysaccharide for biofilm development in sea water. In particular, transposon insertion in *wbfF*, which blocks capsule transport, was found to increase surface association by *V. cholerae*, whereas transposon insertion in *wbfR*, which abolishes both O-antigen and capsule synthesis, was found to decrease surface association (33).

To confirm the phenotypes of these transposon-insertion mutants, we engineered mutants carrying in-frame deletions in *wbfF* or *wbfR.* Biofilm development by these mutants was compared with that by wild-type *V. cholerae* in both sea water medium and LB broth. In LB broth, biofilm development by both mutants was quite similar to that developed by wild-type *V. cholerae* (data not shown). In contrast, in DSW medium, biofilm development by the Δ*wbfF* mutant was markedly increased, whereas that by the Δ*wbfR* mutant was markedly defective.

Surface association by wild-type *V. cholerae*, Δ*wbfF*, and Δ*wbfR* mutants was quantified over time. As shown in Fig. 1, the Δ*wbfF* mutant biofilm accumulated almost 70% more cells than that of wild-type *V. cholerae*, whereas surface accumulation by the Δ*wbfR* mutant was negligible. Biofilms formed over 24 h were also visualized by fluorescence microscopy (Fig. 2) and then quantified by using COMSTAT analysis (Table 1). The biomass, substratum coverage, and average thickness of the Δ*wbfF* mutant biofilm were ≈30% greater than that of wild-type *V. cholerae*, whereas the maximum thickness was not significantly different. Thus, the Δ*wbfF* mutant biofilm consisted of more closely packed pillars of cells. In contrast, the Δ*wbfR* mutant biofilm consisted of scattered attached cells.

Because the Δ*wbfF* mutant displayed increased surface adhesion, we hypothesized that either the *V. cholerae* O139 capsule interfered with *vps*-independent biofilm development by masking the O antigen or that the capsule itself was a mediator, albeit a weaker one, of biofilm development. To distinguish between these two possibilities, we constructed and studied a *V. cholerae* Δ*waaL* mutant. WaaL is a putative O-antigen ligase that attaches

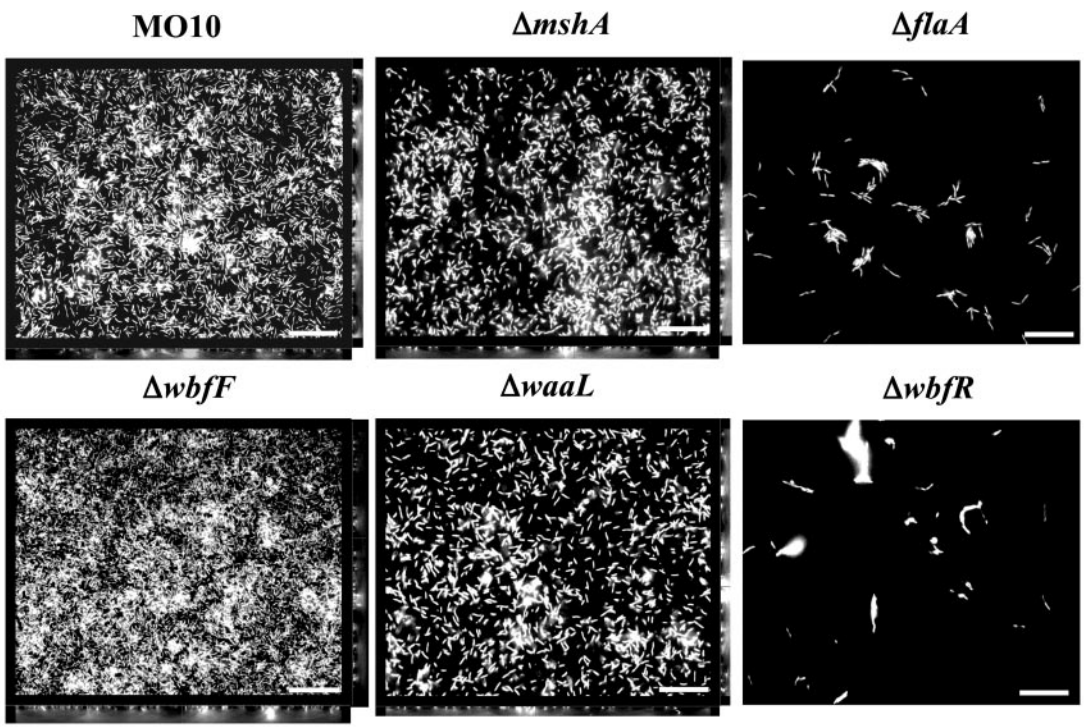

Fig. 2. Transverse and vertical cross sections obtained by fluorescence microscopy through DAPI-stained wild-type *V. cholerae* MO10 and Δ*flaA*, Δ*mshA*, Δ*wbfF*, Δ*wbfR*, and Δ*waaL* mutant biofilms in DSW medium. Transverse sections were taken at the level of the substratum. (Bar = 10 μm.)

Table 1. Quantitative comparison of the architectures of wild-type *V. cholerae* and Δ*mshA*, Δ*flaA*, Δ*wbfF*, Δ*waaL*, and Δ*wbfR* mutant biofilms formed in DSW medium

V. cholerae	Biomass, $\mu m^3/\mu m^2$	Fraction of substratum covered by cells	Thickness, μm	
			Mean	Maximum
Wild type	0.18 ± 0.03	0.23 ± 0.04	0.23 ± 0.03	2.45 ± 0.25
Δ*mshA*	0.12 ± 0.02	0.17 ± 0.01	0.14 ± 0.02	1.8 ± 0.48
Δ*flaA*	0.002 ± 0.001	0.019 ± 0.005	0	1
Δ*wbfF*	0.28 ± 0.05	0.33 ± 0.05	0.32 ± 0.05	3.03 ± 0.25
Δ*waaL*	0.13 ± 0.04	0.16 ± 0.05	0.17 ± 0.04	2.03 ± 0.29
Δ*wbfR*	0.002 ± 0.001	0.02 ± 0.01	0	1

Values were calculated by using the COMSTAT software (30). Results are expressed as the mean ± SD. Each value represents the mean of six image stacks in two independent experiments.

the O antigen to the lipid A core. Previous studies, as well as our own immunoblots, demonstrated that the *V. cholerae* O139 Δ*waaL* mutant synthesizes a capsule but no lipopolysaccharide-associated O antigen (29). We predicted that, if the capsule itself had no adhesive properties, this mutant would not form a biofilm. As shown in Fig. 1, this mutant did indeed form a biofilm. Furthermore, quantitation of the Δ*waaL* biofilm parameters demonstrated that this biofilm was not significantly different from that of wild-type *V. cholerae* (Table 1). These results suggest that the *V. cholerae* O139 capsule itself is able to mediate *vps*-independent biofilm development.

Because the composition of the O antigen and the repeating unit of the capsule are identical, one might have predicted that biofilm development by a capsule-defective mutant would be indistinguishable from that of the wild-type *V. cholerae* strain. We postulate that structural constraints imposed by multimerization of the O-antigen polysaccharide destabilizes Ca^{2+}-mediated intercellular contacts. Alternatively, the presence of additional surface-associated structures, exposed only when the capsule is absent, may enhance biofilm development by capsule-deficient mutants.

Spontaneous Unencapsulated Variants of *V. cholerae* O139 also Exhibit Markedly Increased Surface Association. Spontaneous unencapsulated phase variants of *V. cholerae* O139 have previously been described and isolated (9, 34). Because our studies suggested that unencapsulated mutants display increased *vps*-independent biofilm development, we chose one such spontaneous mutant, MO10-T4, for further study. In fact, both biofilm quantification and microscopy confirmed that this spontaneous variant displayed increased Ca^{2+}-dependent surface adhesion (see Fig. 5).

Spontaneous rugose phase variants of *V. cholerae* have previously been described (16, 17, 19, 20, †). These variants display increased *vps*-dependent surface adhesion in polysaccharide-rich environments (15). We propose that spontaneous capsule-negative variants of *V. cholerae* O139 may represent an analogous type of phase variation that results in increased surface association in Ca^{2+}-rich environments such as the sea.

Ca^{2+} Is an Integral Component of the *vps*-Independent Extracellular Biofilm Matrix. Previous experiments demonstrated that a *vps*-independent biofilm would not form in the absence of Ca^{2+} (22). On the basis of these results, we hypothesized that Ca^{2+} either was involved in activation of gene transcription or was a direct mediator of cell–cell and/or cell–surface interactions in the biofilm. The *V. cholerae* O-antigen polysaccharide is present on cells cultured in both DSW medium and LB broth, a Ca^{2+}-poor medium (9, 29). Thus, the discovery that the O-antigen polysaccharide was required for *vps*-independent biofilm development suggested to us that Ca^{2+} might serve as an integral component of the *vps*-independent extracellular matrix. To test this idea, we formed a *vps*-independent biofilm in DSW medium over 24 h, removed Ca^{2+} from the bathing medium either by adding the chelator EDTA or by replacing DSW medium with fresh medium lacking Ca^{2+}, and then observed the response of the *vps*-independent biofilm over time. As shown in Fig. 3, biofilms formed by wild-type *V. cholerae* as well as the Δ*mshA* and Δ*wbfF* mutants disintegrated rapidly after removal of Ca^{2+} from the medium. In contrast, replacement of the bathing medium with fresh DSW medium containing Ca^{2+} did not significantly alter the biofilm structure (data not shown).

We questioned whether the requirement for Ca^{2+} in the bathing medium was specific, or whether removal of any divalent cation would have the same effect. Interestingly, replacement of the bathing medium with fresh DSW medium lacking Mg^{2+} did not result in biofilm dissolution, even though DSW medium contains 5 times more Mg^{2+} than Ca^{2+}. Furthermore, replacement of DSW medium with DSW containing an equivalent concentration of Mn^{2+} in place of Ca^{2+} slowed biofilm dissolution but did not prevent it. These results strongly suggest that Ca^{2+} is interacting directly and specifically with the O-antigen polysaccharide to maintain biofilm structure. This observation, however, does not preclude a dual role for Ca^{2+} in activating the synthesis of the O-antigen polysaccharide or other requisite adhesive structures and then forming salt bridges between these structures.

Association of Ca^{2+} with several bacterial polysaccharides has been demonstrated (35–37). Our results suggest that Ca^{2+} associates with the O antigen and capsule of *V. cholerae* O139. These polysaccharides possess a common repeating polysaccharide unit that is negatively charged because of a cyclic phosphate group (8, 38). We hypothesize that Ca^{2+} interacts with this negatively charged phosphate group to form salt bridges between O-antigen moieties on the same bacterium or on different bacteria.

A *V. cholerae* Biofilm Formed in True Sea Water Exhibits O-Antigen Polysaccharide Dependence and Disintegrates upon Exposure to True Fresh Water. A major goal of these experiments was to formulate a testable prediction of the fate of a sea water biofilm after transport into a fresh water environment. Because it is difficult to remove components of true sea water, we developed a defined sea water medium for initial experiments. To test the accuracy of our observations in more realistic aquatic environments, we quantitated surface adhesion by wild-type *V. cholerae*, a Δ*wbfF* mutant, and a Δ*wbfR* mutant in true sea water. As shown in Fig. 4, the Δ*wbfF* mutant demonstrated increased surface association in true sea water, whereas the Δ*wbfR* mutant demonstrated markedly decreased surface association. Interestingly, the spontaneous unencapsulated mutant, MO10-T4, also demonstrated increased surface association in true sea water. Furthermore, as shown in Fig. 4, we documented dissolution of these true sea water biofilms after replacement of the bathing medium with true fresh water. These data suggest that O-antigen polysaccha-

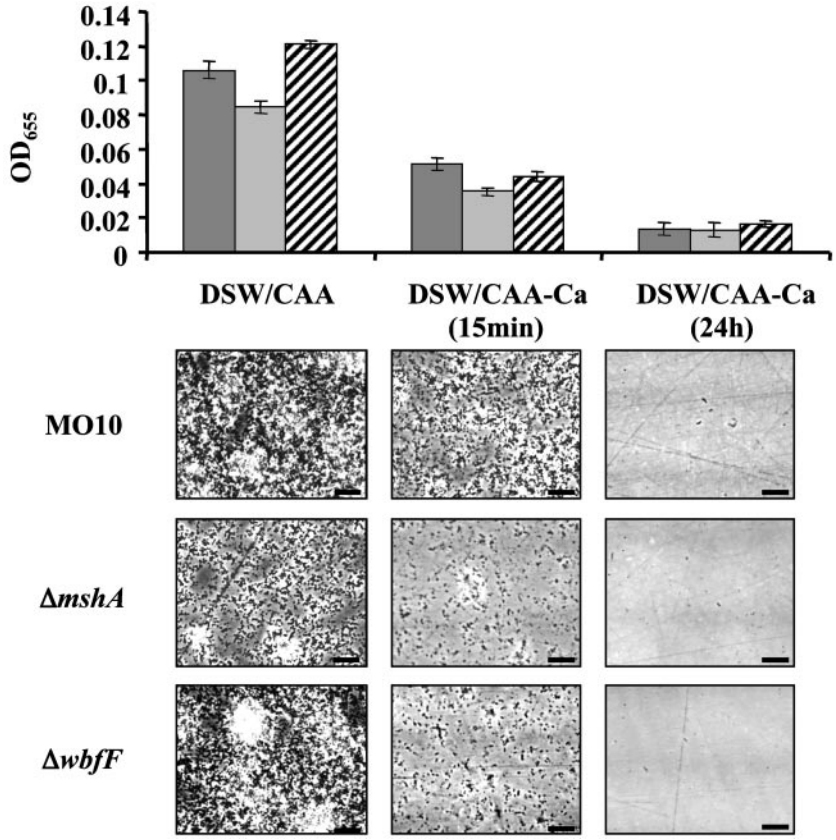

Fig. 3. Quantification (*Upper*) and phase-contrast microscopy (*Lower*) of wild-type *V. cholerae* (MO10), Δ*mshA* mutant, and Δ*wbfF* mutant biofilms after incubation in DSW medium (including Casamino acids, CAA) for 24 h and then 15 min and 24 h after replacement of bathing medium with DSW medium lacking Ca^{2+} (-Ca). Black bars, wild-type *V. cholerae*; gray bars, Δ*mshA*; striped bars, Δ*wbfF*. (Scale bars = 4 μm.)

ride-dependent biofilm development may be operative in true sea water environments. Furthermore, the transport of these sea water biofilms into fresh water environments may result in dispersal of surface-associated bacteria.

Many Environmental and Clinical *Vibrio* Strains Exhibit Ca^{2+}-Dependent Biofilm Dissolution. We were curious to determine whether Ca^{2+}-dependent biofilm dissolution was a feature of other pathogenic and environmental *Vibrio* strains. To test this possibility, biofilms formed over 24 h by a variety of *Vibrio* species were quantified before and after replacement of DSW medium with DSW medium lacking Ca^{2+}. As shown in Fig. 5, the vast majority of *Vibrio* strains exhibited biofilm dissolution upon removal of Ca^{2+} from the bathing medium. Replacement of the bathing medium with fresh medium containing Ca^{2+} did not significantly alter the biofilm structure of any of these strains (data not shown). Only one surface adhesion-competent strain, *V. cholerae* O103, did not follow this pattern. Possible explanations for the resistance of the *V. cholerae* O103 biofilms to Ca^{2+} depletion include (*i*) a requirement for much lower concentra-

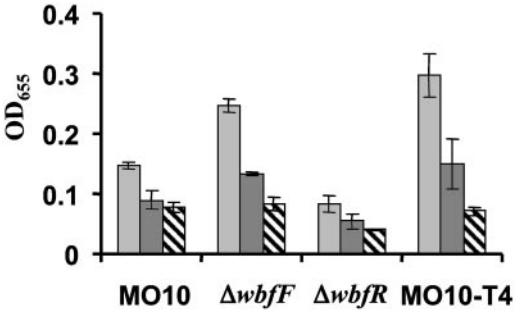

Fig. 4. Quantification of surface adhesion by wild-type *V. cholerae* (MO10), a Δ*wbfF* mutant, a Δ*wbfR* mutant, and a spontaneous unencapsulated mutant (MO10-T4) after incubation for 24 h in true sea water (gray bars) and 1 h (black bars) and 5 h (striped bars) after replacement of the bathing medium with true fresh water.

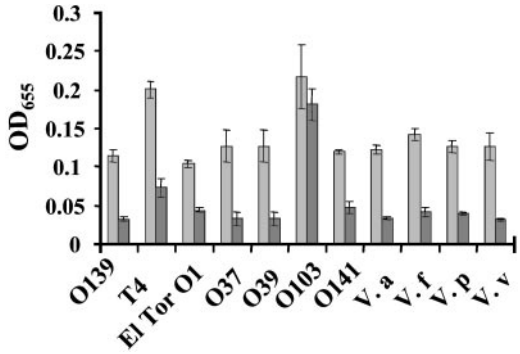

Fig. 5. Biofilm detachment after medium change from DSW medium to DSW medium lacking Ca^{2+}. A quantification of surface association by *V. cholerae* O139 (MO10), MO10-T4 (T4), *V. cholerae* O1 El Tor (N16961), *V. cholerae* O37, *V. cholerae* O39, *V. cholerae* O103, *V. cholerae* O141, *V. alginolyticus* (*V. a*), *V. fluvialis* (*V. f*), *V. parahaemolyticus* (*V. p*), and *V. vulnificus* (*V. v*) after incubation for 24 h in DSW medium (gray bars) and 5 h after replacement of the bathing medium with DSW medium lacking Ca^{2+} (black bars). *V. cholerae* strain data are labeled with the respective *V. cholerae* O-antigen designations.

tions of Ca^{2+} in maintenance of the biofilm structure, (*ii*) dependence of the biofilm structure on an alternative divalent cation, or (*iii*) dependence of the biofilm structure on a cation-independent adhesive exopolysaccharide matrix.

These results suggested to us that Ca^{2+}-dependent biofilm dissolution is not unique to *V. cholerae* O139 strain MO10, but is a feature of many *Vibrio* species. The mechanism of Ca^{2+}-dependent biofilm development and dissolution in these various *Vibrio* strains has not yet been examined. Cyclic phosphate groups, which we have suggested may play a role in Ca^{2+}-dependent biofilm formation in *V. cholerae* O139, are a component of the O antigens of a few *Vibrio* strains (18, 39). However, such a group is not known to be present in the O antigen of *V. cholerae* O1. We hypothesize that alternative negatively charged groups in the O antigen or capsule of other *Vibrio* species are able to mediate Ca^{2+}-dependent cell adhesion as well, but this must be investigated further.

Potential Impact of Increases in Sea Surface Height on Ca^{2+}-Dependent Bacterial Biofilms in Estuaries. Our results suggest that Ca^{2+}-dependent biofilm development and dissolution is a common theme among *Vibrio* species and, potentially, other marine bacteria as well. Furthermore, dissolution of Ca^{2+}-dependent biofilms may occur as the result of transport of biofilm-covered particulates from a marine or estuarine environment into a fresh water environment. We have observed that not all marine bacteria form a Ca^{2+}-dependent biofilm. Thus, we hypothesize that, during times of estuarine instability, a redistribution of the microbial populations associated with surfaces may occur.

Dissolution of Ca^{2+}-dependent biofilms during times of estuarine flux may also have implications for the epidemiology of cholera. Increases in sea surface height in the Bay of Bengal have been positively correlated with cholera (1). One potential outcome of an increase in sea surface height is transport of sea water particulates and attendant adherent *V. cholerae* into fresh water. The subsequent dissolution of Ca^{2+}-dependent biofilms might result in increased titers of planktonic *V. cholerae*, which would then be more readily ingested and potentially more virulent. The laboratory-based experiments described here suggest readily testable hypotheses regarding the impact of estuarine ion flux on the microbial ecology of the estuary. Environmental studies to evaluate these hypotheses are necessary.

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