Mechanisms Involved in Governing Adherence of *Vibrio cholerae* to Granular Starch[▽]

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Vibrio cholerae has been shown to adhere to cornstarch granules. The present work explored the mechanisms involved in this adhesion and the possibility of its occurrence *in vivo*. The findings suggest that both specific and nonspecific interactions are involved in the adhesion. Nonspecific hydrophobic interactions may play a role, since both *V. cholerae* and cornstarch granules exhibited hydrophobic properties when they were tested using a xylene-water system. In addition, the presence of bile acids reduced the adhesion. The adhesion also involves some specific carbohydrate-binding moieties on the cell surface, as reflected by reduced adhesion following pretreatment of the bacteria with proteinase K and sodium *m*-periodate. Further investigations supported these observations and showed that media containing low-molecular-weight carbohydrates had a significant inhibitory effect. Binding cell lysate to starch granules and removing the adhered proteins using either glucose or bile acids led to identification (by liquid chromatography-tandem mass spectrometry analysis) of several candidate *V. cholerae* outer membrane-associated starch-binding proteins. Different sets of proteins were isolated by removal in a glucose solution or bile acids. When the upper gastrointestinal tract conditions were simulated *in vitro*, both bile salts and the amylolytic activity of the pancreatic juices were found to have an inhibitory effect on the adherence of *V. cholerae* to starch. However, during acute diarrhea, this inhibitory effect may be significantly reduced due to dilution, suggesting that adhesion does occur *in vivo*. Such adhesion may contribute to the beneficial effects observed following administration of granular starch-based oral rehydration solutions to cholera patients.

Cholera is a severe diarrheal disease that kills thousands of people each year and affects the lives of millions. This disease is caused by specific serogroups of *Vibrio cholerae* that are pathogenic to humans (20). Infection by *V. cholerae* usually starts after consumption of contaminated water or food. The severity of symptoms varies among patients, and in the severe form (cholera gravis) the rate of diarrhea may quickly reach 500 to 1,000 ml h⁻¹, which leads to severe dehydration and, without appropriate treatment, death (20, 40, 41). Death in cholera patients is caused by loss of fluids and salts; therefore, the key to therapy is sufficient rehydration. Furthermore, the rehydration solution should have an electrolyte composition similar to that of the lost fluids (16, 20). This understanding led to what is considered to be one of the most important medical advances in the 20th century, oral rehydration therapy (ORT) (16).

The life-saving effect of oral rehydration solutions (ORS) is achieved primarily by maintaining the electrolyte balance (e.g., by stimulating absorption of sodium from the small intestine) (16, 20). However, these solutions do not prevent or reduce to any significant extent the symptoms of cholera. Although in controlled studies ORT is very effective at reducing mortality (4, 29), its use remains low in both developing and developed

countries. Despite extensive health education efforts (4, 48), a common perception is that oral rehydration is not effective since it does not reduce the manifestations of diarrhea, such as loss of fluid in the feces, or the duration of the illness (12). Moreover, the glucose-based ORS recommended by the World Health Organization (WHO) may paradoxically increase fecal fluid loss. Because of these limitations, there has been a substantial impetus to develop improved ORS (48).

Beneficial effects of starch-based ORS have been shown for the treatment of cholera. Clinical trials with starch-based ORS showed that there was a marked improvement in symptom manifestation, in addition to a life-saving effect (38). Ramakrishna et al. (38) hypothesized that part of the beneficial effect of ORS containing high-amylose cornstarch is due to short-chain fatty acid (SCFA) formation by the colon microbiota, which changes the fluid balance in the colon. The massive loss of fluid reported during cholera episodes (500 to 1,000 ml h⁻¹ in the severe form) has raised the question of whether significant amounts of SCFA are indeed formed under these conditions by colonic microbiota or if an alternative mechanism is responsible for the improvement in symptoms.

In search of an explanation, we previously demonstrated that *V. cholerae* strongly adheres to starch granules (15) and suggested that this may explain, at least in part, the beneficial effect of starch-containing ORS in the treatment of cholera compared to treatment with regular ORS. This study was aimed at understanding the mechanisms involved in adhesion of *V. cholerae* to starch granules.

MATERIALS AND METHODS

Bacterial strains and growth media. *V. cholerae* O1 Inaba was a kind gift from E. Arakawa (NIID, Tokyo, Japan); *V. cholerae* O139 was a kind gift from T.

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TABLE 1. Treatments used to study the adhesion mechanisms

Effect	Treatment	Description
Medium composition	Fresh medium	No cell pretreatment; the adhesion expt was performed in fresh LB medium (pH 6.8) ^a
	Spent medium	No cell pretreatment; the adhesion expt was performed in filter-sterilized spent LB medium from a 16- to 18-h culture of the bacteria (pH 7.2) ^a
	M9 minimal medium	No cell pretreatment; the adhesion expt was performed in M9 minimal medium (no carbon source or Casamino Acids added, pH 7.0)
	M9 minimal medium containing Casamino Acids	No cell pretreatment; the adhesion expt was performed in M9 minimal medium containing 0.1% Casamino Acids (no carbon source added, pH 7.0)
Hydrophobic interaction	Polysorbate 80	No cell pretreatment; the adhesion expt was performed in 0.1 M phosphate buffer (pH 7.0) containing 3 g · liter ⁻¹ polysorbate 80 (Sigma, Israel) ^a
	Polysorbate 60	No cell pretreatment; the adhesion expt was performed in 0.1 M phosphate buffer (pH 7.0) containing 3 g · liter ⁻¹ polysorbate 60 (Sigma, Israel)
Ion effect	NaCl	No cell pretreatment; the adhesion expt was performed in 0.1 M phosphate buffer (pH 7.0) containing 0.2 M, 0.5 M, or 0.8 M NaCl ^a
	EDTA	No cell pretreatment; the adhesion expt was performed in modified ORS (pH 6.7) containing 2 mM EDTA
Proteinaceous factor	Pronase E	Cells (~10 ⁹ CFU · ml ⁻¹) were pretreated with 100 µg · ml ⁻¹ pronase E (Sigma, Israel) at 37°C for 1 h; the cells were then washed twice with modified ORS; the adhesion expt was performed in modified ORS (pH 6.7) ^b
	Proteinase K	Cells (~10 ⁸ CFU · ml ⁻¹) were pretreated with 10 U · ml ⁻¹ proteinase K (Biosolve, the Netherlands) at 37°C for 1 h; the adhesion expt was performed in modified ORS (pH 6.7) ^a
	M6-ANDS (12)	No cell pretreatment; the adhesion expt was performed in 0.1 M phosphate buffer (pH 7.0) containing 0.1 mg · ml ⁻¹ M6-ANDS (maltohexaose modified at the reducing end with 3-amino-naphthalene-2,7-disulfonic acid)
Bacterial polysaccharides	Sodium <i>m</i> -periodate	Cells (~10 ⁷ CFU · ml ⁻¹) were pretreated with 0.01 mM sodium <i>m</i> -periodate (Sigma, Israel) at room temperature for 10 min; the adhesion expt was performed in 0.1 M phosphate buffer (pH 7.0)
Starch granule treatments	Proteinase K	No cell pretreatment; starch granules were pretreated with 10 U · ml ⁻¹ proteinase K at 37°C for 1 h; then the granules were washed twice with modified ORS; the adhesion expt was performed in modified ORS (pH 6.7)
	Sodium <i>m</i> -periodate	No cell pretreatment; starch granules were pretreated with 10 mM sodium <i>m</i> -periodate (Sigma, Israel) for 30 min at room temperature; then the granules were washed twice with modified ORS; the adhesion expt was performed in modified ORS (pH 6.7)

^a See reference 10.^b See reference 36.

Ramarmurthy (National Institute of Cholera and Enteric Diseases, Kolkata, India); and *V. cholerae* O9 was isolated from chironomid egg masses (6). The other bacterial species tested were obtained from local stocks and included *Aeromonas hydrophila* ATCC 33654, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella enterica* serovar Typhimurium ATCC 14028, and *Escherichia coli* ATCC 43895.

The following growth media were used: Luria-Bertani (LB) broth containing 5 g · liter⁻¹ NaCl, 10 g · liter⁻¹ tryptone, and 5 g · liter⁻¹ yeast extract; and M9 minimal medium consisting of 6 g · liter⁻¹ Na₂HPO₄, 2.5 g · liter⁻¹ KH₂PO₄, 0.83 g · liter⁻¹ NH₄Cl, 0.42 g · liter⁻¹ NaCl, 1 mM MgSO₄, 0.2% (wt/vol) carbon source, and 0.1% (wt/vol) Casamino Acids (2). All chemicals were analytical grade.

Modified ORS composition. A modified ORS containing 3.5 g · liter⁻¹ NaCl, 2.5 g · liter⁻¹ NaHCO₃, and 1.5 g · liter⁻¹ KCl (WHO formula for ORS without glucose [31]) was used in the basic adhesion experiments.

Basic adhesion procedure. To examine their adhesion, the strains tested were first reared from frozen stocks (in 25% [wt/vol] glycerol kept at -80°C) by inoculating 50 ml of LB medium with the contents of one aliquot (500 µl) (with antibiotic supplementation when required). The cultures were grown overnight in Erlenmeyer flasks at 37°C shaken at 200 rpm. The bacteria were then har-

vested by centrifugation (16,060 × *g*, 5 min) (Biofuge pico; Heraeus, United States) and washed three times with 1 ml of the modified ORS or 0.1 M phosphate buffer (pH 7.0). The washed bacterial cells were then suspended in 1 ml of modified ORS (or phosphate buffer) containing starch to obtain an estimated final concentration of 10⁶ cells · ml⁻¹ along with 10% (wt/vol) cornstarch granules. After the starch and cells were mixed for short periods (2 min or 15 min overall), the starch granules were removed by centrifugation (320 × *g* for 30 s). The bacteria remaining in the supernatant were enumerated by plating on LB agar, with the appropriate antibiotic when necessary. Controls were subjected to the same procedure using modified ORS or 0.1 M phosphate buffer without starch. All experiments were repeated at least three times with fresh bacteria, starch, and ORS (or phosphate buffer). It should be noted that there was no difference between adhesion in ORS and adhesion in phosphate buffer; for both preparations there was over 90% adhesion.

Study of adhesion mechanism. Different aspects that may affect the adhesion process were examined, including growth media, hydrophobic interactions, ions, proteins, and polysaccharides (Table 1). To test each of these effects, the bacteria or starch was first pretreated, and then an adhesion assay was performed. Each experiment was repeated at least three times. For each treatment, a control

TABLE 2. *In vitro* experimental procedures simulating physiological conditions in the upper gastrointestinal tract

Simulated conditions	Exptl procedure ^a
pH.....	No cell pretreatment; the adhesion assay was performed in modified ORS at pH 4.0, 5.0, 6.0, 6.7 (ORS), and 8.0
Stomach (acid and pepsin).....	No cell pretreatment; the starch granules were incubated in 0.2 M HCl-KCl buffer (pH 2.0) containing pepsin (30 U · ml ⁻¹) for different periods of time (30 min to 2 h at 37°C); then the starch was washed twice with the 0.1 M phosphate buffer (pH 7.0), mixed, and incubated with the bacteria, as in the basic adhesion procedure
Small intestine (bile).....	No cell pretreatment; the adhesion assay was performed in 0.1 M phosphate buffer (pH 7.0) containing 3 g · liter ⁻¹ bile extract
Small intestine (pancreatic enzymes).....	No cell pretreatment; starch granules were incubated in 0.1 M phosphate buffer (pH 7.0) containing 10 mg · ml ⁻¹ pancreatin for 1 to 4 h at 37°C; the starch was then washed twice with 0.1 M phosphate buffer (pH 7.0), mixed, and incubated with the bacteria, as in the basic adhesion procedure

^a Each experimental procedure was tested separately and was based on the method of Crittenden et al. (10). All incubations of bacteria with starch granules were performed at 37°C.

experiment was performed with either 0.1 M phosphate buffer (pH 7.0) or modified ORS.

Adhesion under simulated GIT conditions. To test the relevance of the adhesion, adhesion experiments were performed *in vitro* under conditions simulating the physiological environment of the upper gastrointestinal tract (GIT). We tested the effects of changes in the pH, stomach acidity, and bile and pancreatic enzymes secreted into the small intestine. The test conditions are described in Table 2.

Measurement of cell surface hydrophobicity. The cell surface hydrophobicities of the different bacterial strains were measured by using the bacterial adhesion to hydrocarbon (BATH) test (10, 26). Briefly, following overnight growth, bacteria were washed twice in 150 mM phosphate-buffered saline (PBS) (pH 7.4) and resuspended in the same buffer at an optical density at 600 nm (OD₆₀₀) of ~0.4 (*A_p*). For each species, 1 ml of either xylene or hexadecane was added to 4 ml of a bacterial suspension. Each tube was then thoroughly mixed for 20 s and equilibrated for 30 min at 37°C to allow phase separation. After the incubation, the OD₆₀₀ of the aqueous lower phase was measured (*A_p*). The cell surface hydrophobicity was calculated as follows: $(1 - A_p/A_i) \times 100$. An analysis of the correlation between cell surface hydrophobicity and bacterial adhesion to starch was performed by linear regression using the least-squares method.

Effects of growth phase and carbon source in the medium on adhesion. Following overnight growth, the bacteria were diluted 1:100 in fresh M9 minimal medium supplemented with 0.2% (wt/vol) glucose, maltose, or maltodextrin (DE19). Bacterial growth was monitored by measuring the OD₆₀₀ (WPA Bio-wave CO8000 cell density meter; Biochrom Ltd., England). At three times during growth, 1 ml of the growing culture was removed and used to measure adhesion as described above.

Removal of adhered bacteria. Bacteria were first adhered to the starch granules using the basic adhesion procedure described above. Following removal of the supernatant, 1 ml of the simulation solution, which was modified ORS, ORS (WHO formula), modified ORS containing 111 mM NaCl, 0.1 M phosphate buffer (pH 7.0), or 0.1 M phosphate buffer (pH 7.0) containing 3 g · liter⁻¹ bile extract, was added to the starch sediment. After the starch granules were suspended by gentle pipetting, the suspension was incubated at room temperature for 2 and 15 min. Then the starch granules were removed by centrifugation (320 × *g* for 30 s), and the number of bacteria in the supernatant was determined by plating on LB agar. The percentage of bacteria removed was calculated as follows: $1 - (\text{total number of bacteria in the upper fluid}/\text{calculated number of adhered bacteria})$.

Isolation of candidate starch-binding proteins. *V. cholerae* O1 was grown overnight and then centrifuged (10,000 × *g*, 15 min, 4°C) and washed three times with Dulbecco's PBS (Biological Industries, Beit Haemek, Israel). The cells were resuspended in PBS containing complete, mini, EDTA-free protease inhibitor cocktail (Roche Diagnostics GmbH, Germany) and then broken by ultrasonication (25% amplitude, 30-s pulse on ice, model 555 W ultrasonic processor). Sonication was repeated six times, with 60 s of cooling between pulses. The lysate was then centrifuged to pellet the unbroken cells (10,000 × *g*, 15 min, 4°C), and aliquots of the supernatant were added to tubes containing cornstarch granules. The mixtures were incubated at room temperature for 5 min with gentle rotation and then washed five times by centrifugation with PBS (10,000 × *g*, 5 min, 4°C) to remove the unbound proteins. To remove the starch-bound proteins, each

starch pellet was then resuspended and incubated for 5 min with PBS (control), phosphate buffer containing 111 mM glucose, or phosphate buffer containing 3 g · liter⁻¹ bile extract. The starch was removed by centrifugation, and the supernatant from each tube was separated and concentrated in a Microcon centrifugal filter device (YM-3; Millipore Corporation, United States). The concentrated samples were digested with trypsin, analyzed by liquid chromatography-tandem mass spectrometry (LC-MS-MS) using LTQ-Orbitrap (Thermo), and identified by using Pep-Miner and Sequest software with the bacterial part of the nr database (at the Smoler Proteomic Research Center, Department of Biology, Technion, Israel).

Statistical analysis. Statistical differences were analyzed by one-way and two-way analyses of variance and Student's *t* test ($\alpha = 0.05$) using JMP 7.0.1 (SAS Institute Inc., United States).

RESULTS

Factors affecting adhesion. Our first step was to estimate the contributions of a series of possible adhesion mechanisms to the overall adhesion of *V. cholerae* to starch (Table 1). Several growth media were used to test their effects on adhesion. In the presence of LB and spent LB media, adhesion was significantly inhibited (14% and 57% adhesion, respectively), whereas M9 minimal medium with or without amino acids had no effect on adhesion (Fig. 1). The involvement of hydrophobic interactions was tested by addition of surfactants. The presence of polysorbate 80 or polysorbate 60 did not inhibit adhesion (Fig. 1). The effects of ions on adhesion were also tested; a basic adhesion assay performed in the presence of 2 mM EDTA revealed no effect. Addition of 0.5 M NaCl to the phosphate buffer reduced the level of adhesion to 79%; however, when 0.2 M NaCl or 0.8 M NaCl was added, no effect on adhesion was observed (Fig. 1).

The role of bacterial proteins in adhesion was studied by using proteolytic and chemical treatments aimed at reducing adhesion. Pretreatment of *V. cholerae* with pronase E had only a minor effect on its adhesion to starch granules; however, pretreatment with proteinase K resulted in a significant reduction in the level of adhesion, to 60% (Fig. 1). Specific binding of glycosidic moieties was tested by using the modified sugar M6-ANDS (maltohexaose modified at the reducing end with 3-amino-naphthalene-2,7-disulfonic acid), which blocks the maltoporin channel when it is added from outside the membrane (3). The presence of 0.1 mg · ml⁻¹ M6-ANDS in the buffer reduced the level of adhesion to 75% (Fig. 1), but when

FIG. 1. Possible factors affecting adhesion of *V. cholerae* to starch granules: levels of adherence of *V. cholerae* under different conditions and with different treatments that may affect adhesion (see Table 1). The light gray bars indicate results obtained with various medium compositions or bacterial pretreatments. The dark gray bars indicate results obtained with starch granule pretreatments. Each experiment was repeated at least three times. The error bars indicate standard errors. Different letters above the bars indicate that the levels of adhesion are significantly different ($\alpha = 0.05$).

M6-ANDS was preincubated with the bacteria and then diluted 10-fold prior to the adhesion assay, the level of adherence was restored ($>80\%$; data not shown). When the unmodified sugar maltohexaose was present in the modified ORS (at a concentration of 0.5%), it reduced the level of adhesion to 30% (data not shown).

The relevance of *V. cholerae* polysaccharides to the adhesion process was evaluated by pretreating the cells with 0.01 mM sodium *m*-periodate, which oxidizes polysaccharides. This caused some reduction in the level of adhesion, to 81% (Fig. 1). Pretreatment of the starch granules with either protease or periodate did not affect adhesion (Fig. 1).

Adhesion under simulated upper GIT conditions. According to our hypothesis, adhesion to starch is a plausible explanation for the effect of starch in ORT. We therefore tested this possibility by conducting some of the adhesion experiments *in vitro* under conditions simulating the physiological conditions in the upper GIT. Figure 2 shows that at a pH higher than 4.0 there was no effect on adhesion, while the level of adhesion at pH 4.0 was slightly lower than that at a higher pH (74.5% and 88% to 93%, respectively). Lower pH values, ranging from pH 2.0 to 3.0, completely eliminated cell viability (data not shown).

The starch added to the ORS passes through the stomach before it encounters the bacteria in the duodenum and lower intestine; we therefore tested the effect of stomach pH and pepsin on the starch granules. As Fig. 2 shows, when starch

FIG. 2. Adhesion under conditions simulating the upper gastrointestinal tract. The open stippled bars indicate results obtained with different adhesion conditions, and the gray stippled bars indicate results obtained with starch granule pretreatments (see Table 2). Each experiment was repeated at least three times. The error bars indicate standard errors. Different letters above the bars indicate that the levels of adhesion are significantly different ($\alpha = 0.05$).

granules were preincubated under the conditions present in the stomach, there was no reduction in their adhesion capacity compared to that of untreated granules used as a control.

The GIT pH is not the only factor that may affect adhesion; surface-active agents, such as bile acids, may also have an influence. The presence of bile salts at a concentration of 3 g · liter⁻¹ reduced the level of adherence significantly (to 60%). However, 2-fold dilution of the bile (to 1.5 g · liter⁻¹) was sufficient to restore the level of adhesion to 83% (Fig. 2). Adhesion of *V. cholerae* to starch granules was also examined after pancreatin digestion. The adherence of *V. cholerae* to starch granules decreased dramatically (to a level of 11%) when the granules were pretreated with pancreatin for 1 h (Fig. 2). Longer incubation with pancreatin had a similar effect on adhesion (data not shown).

Cell surface hydrophobicity. The hydrophobicity of the bacterial cell surface was evaluated to determine whether there is a correlation between hydrophobicity and adhesion to starch. A number of bacterial strains were tested, and two systems were used; the results varied, depending on the hydrocarbon used as the hydrophobic phase. When hexadecane was used, all of the bacterial species were characterized as weakly hydrophobic or nonhydrophobic (range, 14% adhesion for *P. aeruginosa* to 0% adhesion for *V. cholerae* O139) (Fig. 3). On the other hand, when xylene was used, all *V. cholerae* strains tested were highly hydrophobic (between 68 and 80% adhesion), while the non-*V. cholerae* strains were much less hydrophobic (range, 48% adhesion for *P. aeruginosa* to 36% adhesion for *S. enterica* serovar Typhimurium) (Fig. 3). A significant linear fit between cell surface hydrophobicity and adhesion to starch was found only for xylene ($R^2 = 0.77$, $P = 0.0217$) (Fig. 3).

FIG. 3. Correlation between adhesion to starch granules and cell surface hydrophobicity. The cell surface hydrophobicity of the strains was measured by using the test for bacterial adhesion to hydrocarbon. The hydrocarbons used were xylene (filled symbols) and hexadecane (open symbols). A linear fit was found only with xylene ($R^2 = 0.769$, $P = 0.0217$). Each experiment was repeated at least three times. The error bars indicate standard errors.

Effect of growth phase and carbon source in the medium.

The effects of both growth phase and the carbon source present in the growth medium were studied. We found that a number of carbon sources added to M9 minimal medium (and removed before the adhesion test) had no effect on the growth curve (Fig. 4B) or on the adhesion to starch granules (Fig. 4A). The adhesion to starch was tested at three points along the growth curve, two points in the logarithmic growth phase and one point in the early stationary phase. There was no difference in adhesion at any time point between the different carbon sources. For the different carbon sources used, the level of adhesion to starch granules was slightly lower at the first time point tested (78% on average) than at the other two time points (86% and 88%) (Fig. 4A).

Removal of adhered bacteria. To examine possible conditions for detachment of bacteria that have adhered to starch

FIG. 5. Removal of adhered bacteria. Adhered bacteria were resuspended and incubated in several solutions containing agents that might facilitate their removal from the starch granules. The percentage of bacteria released from the granules was estimated after 2 min and 15 min of incubation and compared to the values for two controls composed of oral rehydration solution (ORS) or phosphate buffer (PB) with no added reagent. Each experiment was repeated at least three times. The error bars indicate standard errors. Different letters above the bars indicate that the levels are significantly different ($\alpha = 0.05$).

granules, granules with adhered bacteria were resuspended and incubated with several solutions containing potential adhesion inhibitors (such as glucose and bile) and then compared to control solutions (modified ORS, phosphate buffer, and modified ORS containing 111 mM NaCl). As shown in Fig. 5, whenever the control solutions were used, the level of adherence was greater than 87% after both 2 and 15 min of reincubation. Significantly reduced adhesion was observed when glucose or bile was present in the solution used for resuspension

FIG. 4. Effect of growth phase and carbon source on adhesion. (A) Levels of adhesion of *V. cholerae* to cornstarch granules determined with three carbon sources, glucose, maltose, and DE19. The adhesion was examined at three points along the growth curve, two points in the log phase (90 min and 150 min) and one point in the early stationary phase (270 min). The error bars indicate standard errors. (B) Growth curves for *V. cholerae* in the presence of three carbon sources. Each experiment was repeated at least three times, and the results shown are averages.

TABLE 3. Candidate starch-binding proteins released from the granules by glucose and bile acid solutions

Protein (accession no.)	Elution with:			Predicted function	Reference(s)
	PBS	PBS + glucose	PBS + bile		
Outer membrane protein OmpA (NP_231844) ^a	—	+	+	May contribute to colonization of the intestine	43, 44
Outer membrane channel protein (NP_232066) ^a or outer membrane protein TolC (ABC41333) ^b	—	+	+	Role in bile resistance and colonization	5
Putative porin (NP_231488) ^a or outer membrane porin-like protein OmpT (AAC28105) ^b	—	+	—	Functions primarily as a channel for entry and exit of hydrophilic low-molecular-mass (<600-Da) molecules	28
Flagellin (NP_231774) ^a	—	+	—	Structural flagellar protein	22
MSHA pilin protein MshA (NP_230063) ^a	—	—	+	Plays a role in attachment to abiotic surfaces (zooplankton exoskeleton) and to nonnutritive substrates; involved in adherence to living cells	51
Hypothetical protein VC0414 (NP_230068) ^a or MshQ (AAC72349) ^b	—	+	—	Likely to be an outer membrane protein	27
Lipoprotein YaeC (ZP_01976792) ^c	—	—	+	Putative lipoprotein gene, function not determined yet	21
Hypothetical protein VC1055 (NP_230700) ^a	—	+	+		
Hypothetical protein VCA0689 (NP_233077) ^a	—	—	+		

^a Based on *V. cholerae* O1 biovar El-tor strain N16961.^b Based on references submitted directly to GenBank.^c Based on *V. cholerae* B33.

and reincubation (Fig. 5). The decrease was time dependent (2 min compared with 15 min) for both bile and glucose (Fig. 5) and was faster for glucose than for the bile acids.

Isolation of candidate starch-binding proteins. A suspension of *V. cholerae* cells subjected to complete lysis was incubated with starch granules. After a series of nonspecific washes, proteins adhering to the starch granules were removed by resuspension with either PBS (control), phosphate buffer containing 111 mM glucose, or phosphate buffer containing 3 g · liter⁻¹ bile extract. Nine candidate starch-binding proteins were identified by LC—MS—MS analysis (Table 3); three of these proteins were identified as outer membrane proteins, four proteins were identified as outer membrane-associated proteins, and the two other proteins were identified as hypothetical proteins. As Table 3 shows, three of the proteins were found when elution was performed with either glucose or bile (OmpA, TolC, and VC1055), three proteins were found only after elution with glucose (OmpT, flagellin, and MshQ), and the other three proteins were identified only after elution with bile (MshA, YaeC, and VCA0689). None of the proteins were detected when elution was performed with the control solution (PBS).

DISCUSSION

The survival of microorganisms in some niches depends on their ability to adhere to surfaces and substrates. Bacteria often prefer to grow on available surfaces rather than in the surrounding aqueous phase (1, 33). *V. cholerae* is known to adhere to different surfaces, such as the human lumen (20), chironomid egg masses (6), chitin (46), zooplankton (9, 18), and cornstarch granules (15). Its ability to adhere is related to the survival of the bacteria in the environment (9), as it facilitates both adherence to available nutrition sources and protection against environmental stresses (52). It is also associated

with colonization in the human intestine, which is a prerequisite for establishment of a productive infection (20, 41).

The mechanism underlying the adherence of *V. cholerae* to starch granules is still unknown. Our findings suggest that there is involvement of both nonspecific hydrophobic and electrostatic interactions, as well as specific binding through a cell-associated ligand(s).

Adhesion mechanisms. Hydrophobic interactions play an important role in bacterial interactions with surfaces, either as a primary mechanism of adhesion or by facilitating a closer approach to the surface (14). Although addition of surfactants, such as polysorbate 80 and polysorbate 60, did not inhibit adhesion (Fig. 1), addition of a bile solution resulted in a dramatic decrease in the level of adhesion, to 60% (Fig. 2). Bile acids are strong detergents with an amphipathic structure and have both hydrophobic and hydrophilic sides (45), and this suggests that hydrophobic interactions may play a role in adhesion of *V. cholerae* to starch granules.

Long-range electrostatic forces may also affect the initial phase of bacterial adhesion to solid surfaces (30). Such interactions can affect the adhesion process in a bidirectional manner (i.e., as attractive or repulsive forces) (14, 30). As shown in Fig. 1, addition of 0.5 M NaCl reduced the level of adhesion to 79%. This concentration of salt has previously been shown to provide enough counterions to block the electrostatic interactions between starch and bacteria (10), suggesting that some electrostatic forces may also be involved in adhesion of *V. cholerae* to cornstarch granules. It should be noted that this is not always the case; for example, adhesion of bifidobacteria to Hylon VII starch was not affected by NaCl at this concentration (10). Interestingly, in contrast, higher (0.8 M) or lower (0.2 M) NaCl concentrations did not affect the adhesion of *Vibrio cholerae*; in the case of *Vibrio alginolyticus* attachment to chitin, the level of attachment increased as the NaCl concentration increased from 0.5 to 3% (0.5 M), while at higher salt concen-

trations the level of attachment decreased (36). Many studies have shown that divalent cations are required to mediate adhesion (33, 50), but this is not likely to be the case for adherence of *V. cholerae* to starch; adhesion assays performed in the presence of 2 mM EDTA showed no change in adhesion (Fig. 1). Previous studies have also shown that the presence of divalent cations has no effect on attachment of *V. alginolyticus* to chitin (36).

The bacterial surface is a heterogeneous structure with a complex chemical composition. Numerous macromolecules, including outer membrane proteins, lipopolysaccharides, fimbriae, etc., interface with the surrounding medium (14). Therefore, it has been suggested that these polymeric surface structures mediate adhesion and facilitate firmer adhesion of the bacteria to a surface (1).

Proteins may function as adhesins in specific attachment mechanisms, but they can also be involved in nonspecific attachment (14). Specific starch-binding proteins have been observed in several intestinal bacteria, such as *Bacteroides thetaiotamicron*, some *Bifidobacterium* strains (10), and *Lactobacillus amylovorus* (19). In the case of *V. cholerae* (and other vibrios), adherence to chitin (one of the most abundant polysaccharides, composed of β -1,4-linked *N*-acetylglucosamine residues) and to chitin-containing plankton has been studied extensively in the last 2 decades (36, 46, 47, 52). Several specific ligands involved in the adhesion to chitin have been found (37) and are localized in the outer membrane of the bacteria (32, 36, 46, 52). Therefore, it is possible that a starch-binding protein(s) is present in *V. cholerae* and functions like the known chitin-binding proteins. Pretreatment of *V. cholerae* with pronase E had only a minor effect on its adhesion to starch granules. However, pretreatment with proteinase K resulted in a significant reduction in the level of adhesion, to 60% (Fig. 1). This observation suggests that proteins, among other factors, are indeed involved in the adhesion process.

The lipopolysaccharide layer dominates the outer leaflet of the outer membrane of Gram-negative bacteria, and as such it may affect the adhesive interaction (14). This layer contains a lipid A region anchored to the outer leaflet of the outer membrane, core polysaccharides, and O-antigen polysaccharide regions that project outward (8). Pretreatment of the bacteria with 0.01 mM sodium *m*-periodate, which oxidizes polysaccharides, reduced the level of adherence to 81%. Periodate oxidation of polysaccharides results in cleavage of adjacent hydroxyl-containing carbon-carbon bonds and the formation of highly reactive aldehyde groups (17). Although the aldehyde groups are more hydrophobic than the sugar's original hydroxyl groups, the level of adhesion decreased. This suggests that polysaccharide oxidation influenced the adhesion by some mechanism other than alteration of cell surface hydrophobicity, such as, for example, disruption of some sugar-binding moieties of the bacteria. In other studies, however, polysaccharide oxidation had no influence on attachment of *V. alginolyticus* to chitin particles (36) or on other *Vibrio* spp. tested to determine their abilities to attach to chitin particles and copepods (47).

The ability to interfere with the specific binding of glycosidic moieties was tested at the single-channel level using the modified sugar M6-ANDS, which causes blockage of the maltoporin channel (purified from *E. coli*) when it is added from the

outer side of the membrane (3). Maltoporin of *V. cholerae* forms trimetric pores that function in the uptake of maltose and maltodextrins (23, 24). This channel has a specific binding site for maltosaccharides, which is also accessible to polysaccharides, such as starch (39). The blocking effect of M6-ANDS on the maltoporin channel is irreversible (3, 11). In this study, preincubation of a bacterial suspension with M6-ANDS followed by 10-fold dilution did not affect the adhesion of the bacteria to starch. Therefore, from these results, it appears that the inhibitory effect of M6-ANDS is due to reversible blocking of the starch-binding sites by the maltohexaose sugar residue rather than irreversible blocking of the maltoporin channel. This hypothesis is supported by the low level of inhibition (~15%), which seemed to be due to the low M6-ANDS concentration used (0.01%) compared to the higher sugar concentrations used previously (0.5% to 2%) (15).

After examining several binding mechanisms, we explored the effect of growth medium components on bacterial adhesion to starch. As we showed previously, low-molecular-weight carbohydrates present in the ORS during adhesion prevent adhesion to starch. Of the sugars tested, those that significantly influenced adhesion of *V. cholerae* to cornstarch were the sugars that can be utilized by *V. cholerae* (15). Both yeast extract and tryptone contain some carbohydrates (according to the BD Bionutrients technical manual), explaining the significant reduction in the level of adhesion when the adhesion assay was performed in the presence of LB or spent LB medium (14% and 57%, respectively) (Fig. 1). When spent LB medium was used, some of the carbohydrates had already been utilized during bacterial growth, and the inhibition was therefore significantly lower than that observed with fresh LB medium. The amino acids present in both yeast extract and tryptone apparently do not influence adhesion, as reflected by the results of the experiments performed with M9 minimal medium with and without Casamino Acids.

Adhesion under simulated upper GIT conditions. To examine the significance of the adhesion of *V. cholerae* to starch in the context of ORT, this adhesion was tested under conditions mimicking the human intestine, assuming that adhesion can indeed occur *in vivo* during diarrhea. The first effector tested was medium pH, which might influence bacterial adhesion by changing the surface characteristics of both the bacteria and the surface being tested (30). The results suggested that neither *V. cholerae* nor cornstarch granules are affected by pH (Fig. 2). This is in agreement with previous studies in which a pH higher than 4.0 was found to have no effect on the adhesion of bifidobacteria to Hylon VII starch (10) or on adherence of *V. alginolyticus* to chitin (36). Overall, it appears that the pH gradient in the upper GIT is not a barrier to adhesion of the bacteria to starch granules.

Although a significant reduction in adhesion was observed in the presence of bile salts (Fig. 2), it is reasonable to assume that in acute diarrhea bile salts are ineffective at reducing adhesion of *V. cholerae* to starch due to massive dilution. Bile salt concentrations in fasting individuals have been found to range from 570 to 5470 μ M in the duodenum and jejunum (35). Based on the molecular weight of taurocholic acid (515.7 g $\cdot$ mol⁻¹), which was present at the highest concentration, estimated concentrations of bile salts ranging from 0.29 to 2.82 g $\cdot$ liter⁻¹ in the intestine were calculated. While removal of

adhered bacteria from the starch granules was observed with $3 \text{ g} \cdot \text{liter}^{-1}$ bile salts (Fig. 2), it is very likely that in cases of acute diarrhea, the concentration of bile salts decreases due to dilution in the secreted fluids.

Pancreatic secretions contain amylolytic enzymes, which may affect the starch granule surface. Here, a dramatic reduction in the level of adhesion was observed only after pancreatic digestion (Fig. 2). This suggests that adhesion involves some specificity for glycosidic moieties on the surface of the granule rather than being mediated by proteins associated with the starch granule. Reduced adherence to starch granules following pancreatin pretreatment has also been found for several *Bifidobacterium* strains with Hylon VII starch granules (10). The pancreas normally produces 10-fold more amylase than is needed for complete starch digestion. However, nongelatinized starch granules are not completely digested in the small intestine. In the course of diarrhea, along with the dilution effect on amylase activity due to the massive secretion of fluids, the contact time for digestion decreases, and this is likely to affect the completeness of starch digestion (7). Together with the 74% loss in amylase activity that occurs while the GIT contents move from the duodenum to the ileum (49), it appears that amylolytic activity in the small intestine may only slightly decrease the adhesion *in vivo* during acute diarrhea.

Cell surface hydrophobicity. Cell surface hydrophobicity was estimated by the BATH test, using two different hydrocarbons as the hydrophobic phase. As found in previous studies (25), our results showed different degrees of adherence to the different hydrocarbons (Fig. 3). When cell surface hydrophobicity was tested with several *Vibrio* species, none of the strains exhibited hydrophobic properties when hexadecane was used as the hydrophobic solvent. On the other hand, when *n*-octane was used as the solvent, all strains exhibited strong or moderate hydrophobicity (25). In a previous study, it was found that cells might not adhere to hexadecane at neutral pH or in diluted buffers but do adhere at low pH or in high-ionic-strength media (13). Interestingly, the BATH assay revealed very high surface hydrophobicity of the cornstarch granules (more than 90%) in both solvents (data not shown). These findings suggest that hydrophobicity plays at least a partial role in adhesion, as it has been found that bacteria with hydrophobic properties favor materials with hydrophobic surfaces (30). However, while the starch granules are highly hydrophobic and *V. cholerae* hydrophobicity was confirmed with only one system (xylene), it is reasonable to conclude that hydrophobic interactions explain only part of the adhesion.

Effect of growth phase and carbon source in the medium on adhesion. The growth phase may influence the composition of the outer membrane protein, cell surface hydrophobicity, and other cell surface properties that are likely to be involved in the adhesion mechanism (14, 30). Based on our results, although some statistically significant differences were found in adhesion along the growth curve, the differences were minor and very likely not biologically relevant.

Outer membrane moieties that may be involved in adhesion may also be affected by the type of carbon source present in the minimal growth medium. O'Riordan et al. (34) showed that *Bifidobacterium* spp. grown on maltose or amylomaize starch as the carbon source exhibit improved binding to starch granules. In our study, a series of carbon sources added to M9 minimal

medium (and removed before the adhesion test) had no effect on the bacterial growth curve (Fig. 4B) or on the adhesion to starch granules (Fig. 4A). This suggests that in the case of *V. cholerae*, adhesion is not affected by the carbon source present in the growth medium prior to its adhesion to starch.

Removal of adhered bacteria. As a complementary procedure to verify the suggested adhesion mechanisms, we conducted a set of experiments to test the removal of adhered bacteria by putative competitors of the binding moieties. The results suggested that two key forces are involved in adhesion: carbohydrate-binding moieties and nonspecific hydrophobic interactions. Thus, the significance of these forces was tested by measuring the ability of competing agents (glucose and bile salts, respectively) to remove the adhered bacteria. Since the incubation time with the sugars was very short, it is likely that the inhibitory effect of these compounds was due to their competition for specific binding sites rather than due to gene regulation. Lectins, a diverse group of multivalent carbohydrate-binding proteins, are one possible binding moiety. Bacterial lectins have been found to mediate adhesion to host tissues, one of the key events in pathogenesis (33, 42). In many of the lectin-binding sites, hydrophobic amino acids were found to surround the carbohydrate-specific site, stabilizing it. This might explain the ability of bile acid detergents and glucose to inhibit adhesion and remove the adhered bacteria. Moreover, when the BATH assay was performed in the presence of 111 mM glucose, there was no effect on adhesion to xylene (data not shown), indicating that a specific adhesion mechanism is involved. An inhibitory effect of low-molecular-weight sugars has been demonstrated previously for attachment of *V. alginolyticus* (36) and *V. cholerae* (46) to chitin particles, as well as for adhesion of *Bifidobacterium* (10) and *V. cholerae* to starch granules (15). However, this is the first time that low-molecular-weight sugars have been used to release adhered *V. cholerae* cells.

Isolation of candidate starch-binding proteins. Amylolytic microorganisms that can adhere to starch-containing substrates have been found to have a competitive advantage over nonadherent amylolytic microorganisms (19). Thus, the expression of starch-binding proteins by the amylolytic organism *V. cholerae* could be advantageous in the rich starch environment of the human GIT. The significant reduction in the bacterium's adhesion to starch granules observed after treatment with proteases (Fig. 1) is an indication of the participation of surface proteins in the adhesion process. Therefore, we attempted to identify candidate starch-binding proteins in *V. cholerae* that could be removed from the starch granule using glucose or bile solutions. The isolated proteins were further identified by LC-MS-MS analysis (Table 3). All of the proteins identified were outer membrane-associated proteins, and some of these proteins are known to function as adhesins (OmpA [43, 44], TolC [5], and MshA [51]). Interestingly, some of the proteins were found only when a glucose solution was used, while some were found only after the use of bile. This might also indicate that several mechanisms are involved in the adhesion process. Further investigation is required to study the nature of these proteins and their roles in starch catabolism.

Bacterial adhesion is a complex process affected by many factors, including the characteristics of the bacteria, the surfaces involved, and the surrounding medium (30, 33). Bacterial

adhesion commonly depends on the position of the adhesin(s) on the bacterial surface. Most bacterial species can express more than one type of adhesin, which may consist of proteins, polysaccharides, and lipids (33). Adhesion of *V. cholerae* to starch seems to be mediated by both a specific cell surface protein(s) and nonspecific hydrophobic and electrostatic interactions. Our findings indicate that although several inhibitors of adhesion are present in the human GIT, adhesion of *V. cholerae* to starch is likely to occur during the course of diarrhea, and this phenomenon may be responsible for some of the beneficial effects when resistant starch is added to an ORS.

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REFERENCES

- An, Y. H., R. B. Dickinson, and R. J. Doyle. 2000. Mechanisms of bacterial adhesion and pathogenesis of implant and tissue infections, p. 1–28. In Y. H. An and R. J. Friedman (ed.), *Handbook of bacterial adhesion: principles, methods and applications*. Humana Press Inc., Totowa, NJ.
- Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl. 1999. *Short protocols in molecular biology*. John Wiley & Sons, Inc., New York, NY.
- Berkane, E., F. Orlik, A. Charbit, C. Danelon, D. Fournier, R. Benz, and M. Winterhalter. 2005. Nanopores: maltoporin channel as a sensor for maltodextrin and lambda-phage. *J. Nanobiotechnol.* 3:3.
- Bhattacharya, S. K. 2000. Therapeutic methods for diarrhea in children. *World J. Gastroenterol.* 6:497–500.
- Bina, J. E., and J. J. Mekalanos. 2001. *Vibrio cholerae* *tolC* is required for bile resistance and colonization. *Infect. Immun.* 69:4681–4685.
- Broza, M., and M. Halpern. 2001. Pathogen reservoirs. Chironomid egg masses and *Vibrio cholerae*. *Nature* 412:40.
- Caspary, W. F. 1992. Physiology and pathophysiology of intestinal absorption. *Am. J. Clin. Nutr.* 55:299S–308S.
- Chatterjee, S. N., and K. Chaudhuri. 2003. Lipopolysaccharides of *Vibrio cholerae*. I. Physical and chemical characterization. *Biochim. Biophys. Acta* 1639:65–79.
- Chiavelli, D. A., J. W. Marsh, and R. K. Taylor. 2001. The mannose-sensitive hemagglutinin of *Vibrio cholerae* promotes adherence to zooplankton. *Appl. Environ. Microbiol.* 67:3220–3225.
- Crittenden, R., A. Laitila, P. Forsell, J. Matto, M. Saarela, T. Mattila-Sandholm, and P. Myllarinen. 2001. Adhesion of bifidobacteria to granular starch and its implications in probiotic technologies. *Appl. Environ. Microbiol.* 67:3469–3475.
- Danelon, C., T. Brando, and M. Winterhalter. 2003. Probing the orientation of reconstituted maltoporin channels at the single-protein level. *J. Biol. Chem.* 278:35542–35551.
- Desjeux, J. F., A. Briand, and J. D. Butzner. 1997. Oral rehydration solution in the year 2000: pathophysiology, efficacy and effectiveness. *Bailliere's Clin. Gastroenterol.* 11:509–527.
- Doyle, R. J. 2000. Contribution of the hydrophobic effect to microbial infection. *Microbes Infect.* 2:391–400.
- Fletcher, M. 1996. *Bacterial adhesion: molecular and ecological diversity*, p. 1–24. Wiley-Liss Inc., New York, NY.
- Gancz, H., O. Niderman-Meyer, M. Broza, Y. Kashi, and E. Shimoni. 2005. Adhesion of *Vibrio cholerae* to granular starches. *Appl. Environ. Microbiol.* 71:4850–4855.
- Guerrant, R. L., B. A. Carneiro-Filho, and R. A. Dillingham. 2003. Cholera, diarrhea, and oral rehydration therapy: triumph and indictment. *Clin. Infect. Dis.* 37:398–405.
- Hermanson, G. T. 2008. Bioconjugate techniques, p. 129–131. Academic Press, New York, NY.
- Huq, A., E. B. Small, P. A. West, M. I. Huq, R. Rahman, and R. R. Colwell. 1983. Ecological relationships between *Vibrio cholerae* and planktonic crustacean copepods. *Appl. Environ. Microbiol.* 45:275–283.
- Imam, S. H., and R. E. Harry-O'kuru. 1991. Adhesion of *Lactobacillus amylovorus* to insoluble and derivatized cornstarch granules. *Appl. Environ. Microbiol.* 57:1128–1133.
- Kaper, J. B., J. G. Morris, Jr., and M. M. Levine. 1995. Cholera. *Clin. Microbiol. Rev.* 8:48–86.
- Kim, Y. R., S. E. Lee, C. M. Kim, S. Y. Kim, E. K. Shin, D. H. Shin, S. S. Chung, H. E. Choy, A. Progulske-Fox, J. D. Hillman, M. Handfield, and J. H. Rhee. 2003. Characterization and pathogenic significance of *Vibrio vulnificus* antigens preferentially expressed in septicemic patients. *Infect. Immun.* 71:5461–5471.
- Klose, K. E., and J. J. Mekalanos. 1998. Differential regulation of multiple flagellins in *Vibrio cholerae*. *J. Bacteriol.* 180:303–316.
- Lang, H., M. Maki, A. Rantakari, and T. K. Korhonen. 2000. Use of the OmpS-display system to localize the receptor-binding region in the PapG adhesin of uropathogenic *Escherichia coli*. *Adv. Exp. Med. Biol.* 485:133–136.
- Lang, H., and E. T. Palva. 1993. The ompS gene of *Vibrio cholerae* encodes a growth-phase-dependent maltoporin. *Mol. Microbiol.* 10:891–901.
- Lee, K. K., and K. C. Yui. 1996. A comparison of three methods for assaying hydrophobicity of pathogenic vibrios. *Lett. Appl. Microbiol.* 23:343–346.
- Li, J., and L. A. McLandsborough. 1999. The effects of the surface charge and hydrophobicity of *Escherichia coli* on its adhesion to beef muscle. *Int. J. Food Microbiol.* 53:185–193.
- Marsh, J. W., and R. K. Taylor. 1999. Genetic and transcriptional analyses of the *Vibrio cholerae* mannose-sensitive hemagglutinin type 4 pilus gene locus. *J. Bacteriol.* 181:1110–1117.
- Mathur, J., and M. K. Waldor. 2004. The *Vibrio cholerae* ToxR-regulated porin OmpU confers resistance to antimicrobial peptides. *Infect. Immun.* 72:3577–3583.
- Maulen-Radovan, I., P. Gutierrez-Castrellon, M. Hashem, M. Neylan, G. Baggs, R. Zaldo, L. I. Ndife, P. F. Pollack, and M. Santosham. 2004. Safety and efficacy of a premixed, rice-based oral rehydration solution. *J. Pediatr. Gastroenterol. Nutr.* 38:159–163.
- Merritt, K., and Y. H. An. 2000. Factors influencing bacterial adhesion, p. 53–72. In Y. H. An and R. J. Friedman (ed.), *Handbook of bacterial adhesion: principles, methods and applications*. Humana Press Inc., Totowa, NJ.
- Molla, A. M., A. Bari, W. B. Greenough III, A. M. Molla, P. Budhiraja, and P. N. Sharma. 2000. Bangladeshi rural mothers prepare safer rice oral rehydration solution. *Acta Paediatr.* 89:791–794.
- Montgomery, M. T., and D. L. Kirchman. 1993. Role of chitin-binding proteins in the specific attachment of the marine bacterium *Vibrio harveyi* to chitin. *Appl. Environ. Microbiol.* 59:373–379.
- Ofek, I., and R. J. Doyle. 1994. Bacterial adhesion to cells and tissues, p. 1–92. Chapman & Hall, New York, NY.
- O'Riordan, K., N. Muljadi, and P. Conway. 2001. Characterization of factors affecting attachment of *Bifidobacterium* species to amylo maize starch granules. *J. Appl. Microbiol.* 90:749–754.
- Perez de la Cruz Moreno, M., M. Oth, S. Deferme, F. Lammert, J. Tack, J. Dressman, and P. Augustijns. 2006. Characterization of fasted-state human intestinal fluids collected from duodenum and jejunum. *J. Pharm. Pharmacol.* 58:1079–1089.
- Pruzzo, C., A. Crippa, S. Bertone, L. Pane, and A. Carli. 1996. Attachment of *Vibrio alginolyticus* to chitin mediated by chitin-binding proteins. *Microbiology* 142:2181–2186.
- Pruzzo, C., L. Vezzulli, and R. R. Colwell. 2008. Global impact of *Vibrio cholerae* interactions with chitin. *Environ. Microbiol.* 10:1400–1410.
- Ramakrishna, B. S., S. Venkataraman, P. Srinivasan, P. Dash, G. P. Young, and H. J. Binder. 2000. Amylase-resistant starch plus oral rehydration solution for cholera. *N. Engl. J. Med.* 342:308–313.
- Ranquin, A., and P. Van Gelder. 2004. Maltoporin: sugar for physics and biology. *Res. Microbiol.* 155:611–616.
- Reidl, J., and K. E. Klose. 2002. *Vibrio cholerae* and cholera: out of the water and into the host. *FEMS Microbiol. Rev.* 26:125–139.
- Sack, D. A., R. B. Sack, G. B. Nair, and A. K. Siddique. 2004. Cholera. *Lancet* 363:223–233.
- Saha, N., and K. K. Banerjee. 1995. Carbohydrate-dependent binding of the cell-free hemagglutinin of *Vibrio cholerae* to glycoprotein and glycolipid. *J. Bacteriol.* 177:758–764.
- Smith, S. G., V. Mahon, M. A. Lambert, and R. P. Fagan. 2007. A molecular Swiss army knife: OmpA structure, function and expression. *FEMS Microbiol. Lett.* 273:1–11.
- Song, T., F. Mika, B. Lindmark, Z. Liu, S. Schild, A. Bishop, J. Zhu, A. Camilli, J. Johansson, J. Vogel, and S. N. Wai. 2008. A new *Vibrio cholerae* sRNA modulates colonization and affects release of outer membrane vesicles. *Mol. Microbiol.* 70:100–111.
- Stamp, D., and G. Jenkins. 2008. An overview of bile-acid synthesis, chemistry and function, p. 1–13. In J. Jenjind and L. J. Hardie (ed.), *Bile acids toxicology and bioactivity*. Royal Society of Chemistry, London, United Kingdom.
- Tarsi, R., and C. Pruzzo. 1999. Role of surface proteins in *Vibrio cholerae* attachment to chitin. *Appl. Environ. Microbiol.* 65:1348–1351.
- Vezzulli, L., E. Pezzati, B. Repetto, M. Stauder, G. Giusto, and C. Pruzzo. 2008. A general role for surface membrane proteins in attachment to chitin particles and copepods of environmental and clinical vibrios. *Lett. Appl. Microbiol.* 46:119–125.
- Wapnir, R. A. 2000. Recent progress and future aims in the formulation and use of oral rehydration solutions. *Int. Pediatr.* 15:205–214.

49. Whitcomb, D. C., and M. E. Lowe. 2007. Human pancreatic digestive enzymes. *Dig. Dis. Sci.* **52**:1–17.
50. Yu, C., A. M. Lee, B. L. Bassler, and S. Roseman. 1991. Chitin utilization by marine bacteria. A physiological function for bacterial adhesion to immobilized carbohydrates. *J. Biol. Chem.* **266**:24260–24267.
51. Zampini, M., L. Canesi, M. Betti, C. Ciacci, R. Tarsi, G. Gallo, and C. Pruzzo. 2003. Role for mannose-sensitive hemagglutinin in promoting interactions between *Vibrio cholerae* El Tor and mussel hemolymph. *Appl. Environ. Microbiol.* **69**:5711–5715.
52. Zampini, M., C. Pruzzo, V. P. Bondre, R. Tarsi, M. Cosmo, A. Bacciaglia, A. Chhabra, R. Srivastava, and B. S. Srivastava. 2005. *Vibrio cholerae* persistence in aquatic environments and colonization of intestinal cells: involvement of a common adhesion mechanism. *FEMS Microbiol. Lett.* **244**:267–273.