

**Vibrio cholerae** O1 Strain TSI-4 Produces the Exopolysaccharide Materials That Determine Colony Morphology, Stress Resistance, and Biofilm Formation

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Cholera is a serious epidemic disease that has killed millions of people and continues to be a major health problem worldwide. *Vibrio cholerae*, the bacterium that causes cholera, is a motile, gram-negative, curved rod with a single polar flagellum. The hypothesis that *V. cholerae* occupies an ecological niche in the estuarine environment requires that this organism be able to survive the dynamics of various physiochemical changes, including variations in nutrient concentrations. As a response to nutrient depletion, copiotrophic (31, 42), heterotrophic bacteria may undergo considerable morphological, physiological, and chemical changes (13, 22, 23, 26–28). In fact, to survive energy- and nutrient-deprived conditions, non-spore-forming, heterotrophic bacteria are known to undergo an active adaptation program (28). Brown and Williams have provided detailed experimental evidence that the molecular composition of the bacterial cell walls is essentially plastic and is remarkably responsive to the cell's growth environment (5). Rice et al. (33) discovered that *V. cholerae* O1 from the Peru epidemic was able to shift to a phenotype having a wrinkled or rugose colony morphology. They also suggested that the *V. cholerae* rugose phenotype represents a fully virulent survival form of the organism that can persist in the presence of free chlorine. Morris et al. (29) reported that *V. cholerae* can shift to a rugose colony morphology associated with the expression of an amorphous exopolysaccharide (EPS) that promotes cell aggregation, and they also confirmed that rugose strains displayed resistance to killing by chlorophyll and complement-mediated serum bactericidal activity. They also indicated that these rugose strains cause human disease. However, the phenotypic characteristics associated with rugose morphology, relationships between these characteristics, and their relative importance in pathogenicity still remained to be identified.

A large variety of EPSs are synthesized by gram-negative bacteria. While some have been implicated in the pathogenicity of plant and mammalian hosts, others have not been assigned a function, but many serve a structural role, benefiting the bacterium by enabling attachment to surfaces, improving nutrient acquisition, or providing protection from environmental stresses and host defenses (36). The EPSs cover the surfaces of many gram-negative and gram-positive bacteria. They may form a capsule composed of a high-molecular-weight polysaccharide attached to the cell surface, or they may produce slime either loosely attached to the cell surface or released to the culture fluid. Bacterial cells initiate the process of irreversible adhesion by binding to the surface by using EPS glycolcalyx polymers and the development of microcolonies. The eventual production of a continuous biofilm on the colonized surface is a function of cell division within microcolonies and recruitment of bacteria from the planktonic phase. The biofilm concept has drawn attention to the bacterium's ecological and biotechnological importance (8–11). We must now accept the unequivocal evidence that bacteria respond to changes in their environment by profound phenotypic variations in enzymatic activity, cell wall composition (34), and surface structure (2).

In this study, we have isolated the rugose variants of *V. cholerae* O1 strain TSI-4 from starvation medium and determined EPS expression on the cell surface of the rugose strain by polycationic ferritin-labeled thin-section electron microscopy. While examining the morphological characteristics of these rugose strains, we found that they produced a continuous biofilm on the colonized surface and culture tube walls. Directly sampled, intact biofilms were subjected to electron microscopic analysis. We have also studied the role of the slime polysaccharide of *V. cholerae* TSI-4 in the bacterium's resistance to osmotic and oxidative stress.

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MATERIALS AND METHODS

Organism and microcosm conditions. *V. cholerae* O1 strain TSI-4 (El Tor Ogawa) was used in this study. Frozen stocks were maintained at −80°C in L broth (23) containing 50% glycerol. The original isolate of strain TSI-4 had a transmissible colony morphology. Cells of TSI-4 were routinely grown at 37°C on a rotary shaker in L broth. The culture was incubated to mid-log phase, which corresponded to an *A*_600 of 0.4. The cells were then harvested by centrifugation (13,000 × *g* for 10 min), washed three times with cold M9 salts (37), resuspended in standard minimal medium (M9 salts) to give a final concentration of approximately 5 × 10⁷ cells/ml, and incubated at 16°C without shaking. Strain TSI-4 exhibits a shift of colony morphology to the rugose form under starvation conditions at 2 months after inoculation. The rate of phase variation from the rugose form to the translucent form was assessed by inoculating an isolated rugose colony into L broth and incubating it overnight with shaking at 37°C and then plating serial dilutions of the bacteria onto L agar incubated overnight at 37°C.

Polyacrylamide gel electrophoresis (PAGE). Separation of proteins was carried out in 12% polyacrylamide slab gels at 30 mA for 2 h in a buffersystem containing 0.1 M Tris (pH 8.8) and 0.19 M glycine. The gels were stained with 0.1% (wt/vol) Coomassie brilliant blue R-250 in 50% methanol and 10% acetic acid followed by destaining with the same solution without Coomassie blue. The gels were photographed with a digital camera. The molecular masses of the proteins were estimated using a molecular mass marker kit (Sigma). The gels were stained for 1 h with 0.25% (vol/vol) Coomassie brilliant blue R-250 in 50% methanol and 10% acetic acid for 30 s and destained with 10% acetic acid in 50% methanol for 30 min. The gels were scanned with a BioRad Gel Doc Gel Documentation System (BioRad, Hercules, Calif.) and analyzed with Quantity One software (BioRad). The gel images were printed after normalization of the images.

**RESULTS**

Isolation of the rugose strain and phase variation. We have found that *V. cholerae* O1 strain TSI-4 is able to shift to a phenotype having a rugose colony morphology under starvation conditions. To isolate spontaneous rugose variants, bacteria from smooth colonies were starved in M9 salts for 2 months at 16°C and then plated on L agar at 37°C. TSI-4 underwent phase variation, converting from translucent to rugose in M9 salts and back again at a low rate in L broth. Rugose TSI-4/R colonies inoculated into L broth and subcultured on L agar produced translucent TSI-4/T colonies at a frequency of 1.5 × 10⁻⁵. The two distinct colony morphologies are shown in Fig. 1.

Thin-section electron microscopy. To determine the nature of the colony morphology differences, bacterial pellets were...
stained with polycationic ferritin and thin sections were observed by electron microscopy. Representative profiles are shown in Fig. 2. EPS materials of TSI-4/R were recognized as a heavy, fibrous, electron-dense, ferritin-stained layer completely surrounding the cell (Fig. 2A), but TSI-4/T did not appear to have this external layer surrounding its cells (Fig. 2B). The staining of the extracellular fibrous layer by polycationic ferritin strongly suggests the presence of acidic polysaccharide, and it shows the electrostatic binding of a derivative of ferritin to anionic sites on the cell surface (12, 20).

**Immunoelectron microscopy.** The reactivity of the anti-*V. cholerae* O1 monoclonal antibody with the cell surfaces was tested with both strains TSI-4/R and TSI-4/T. The reaction of anti-O1 serum with both strains is shown in Fig. 3. The gold particles were specifically found on the outer membrane surfaces and were believed to be the antibody reacting with the surface O antigens of both strains. The anti-EPS serum prepared by specific adsorption of anti-TSI-4/R serum with strain TSI-4/T was reactive only with TSI-4/R and not with TSI-4/T (Fig. 4A and B). The gold particles were specifically bound to

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**FIG. 2.** Thin sections of *V. cholerae* stained with polycationic ferritin showing a thick, electron-dense EPS layer completely surrounding TSI-4/R cells (A) and the absence of this layer surrounding a cell of TSI-4/T (B). Bars, 0.5 μm.
Biofilm growth of *V. cholerae* TSI-4/R and scanning electron microscopy. The biofilms of *V. cholerae* TSI-4/R were clearly visible on the upper surface of L broth and culture tube walls after 5 days of static incubation at 37°C, whereas TSI-4/T did not have the biofilm-forming property and produced a smooth suspension of bacteria. Figure 5 shows a biofilm under scanning electron microscopy; the surface of the film was completely covered with a layer of contiguous bacterial cells embedded within a polymeric matrix. Throughout the biofilm, cells were interconnected by a finger-like glycocalyx matrix that extended from the substratum to the outer boundaries of the biofilm.

**Rugose TSI-4 exhibits elevated resistance to osmotic and oxidative stress.** Cells of both TSI-4/T and TSI-4/R were collected at mid-exponential phase and tested as described in Materials and Methods. *V. cholerae* O1 strain TSI-4/R was much more resistant to oxidative and osmotic stress than was strain TSI-4/T, showing viability more than 10 times greater than that of strain TSI-4/T (Fig. 6).

**DISCUSSION**

When bacteria are transported from one environment to another, the environmental changes with which they are confronted include changes in temperature, nutrient concentration, salinity, osmotic pressure, pH, and many other factors. However, bacterial cells dynamically adapt to shifts in environmental parameters by employing a variety of genotypic and phenotypic mechanisms (7). Starvation-induced changes in bacterial surfaces have been reported for several strains of marine bacteria by Kjelleberg and Hermansson (21). The cells of *V. cholerae* demonstrate a predilection for association with chitinaceous surfaces and the mucilaginous sheath of algae that can be interpreted as an ecological advantage (3).

In this paper, we report that the growth of *V. cholerae* O1 strain TSI-4 (El Tor, Ogawa) in starvation medium resulted in a change to a wrinkled or rugose colony morphology from the normal translucent colony morphology and, at the same time, significant production of EPSs, some of which are loosely attached to the cell surface and some of which are released into the intercellular spaces. Analogies to rugosity can be found in a number of other bacterial species, including the expression of alginate by mucoid strains of *Pseudomonas aeruginosa* and the expression of an adhesive EPS by members of the marine genus *Hyphomonas*. Wrangstadh et al. (41) demonstrated that energy and nutrient starvation of marine *Pseudomonas* sp. strain S9 induced the production and release of an EPS with resulting pronounced effects on the adhesion and aggregation of the bacterial cells. Our data indicate that cell surface EPS materials confer a rugose colony morphology, biofilm formation ability, and resistance to osmotic and oxidative stress. We suggest that the spontaneous and reversible variation in cell-associated and cell-free EPS production represents an optimal adaptive mechanism that facilitates survival in stressful environments.

The rugose form of *V. cholerae* was first described in 1938 by Bruce White, who recognized that it might serve as a survival form of the organism (39). Rice et al. (33) suggested that the *V. cholerae* rugose phenotype represents a fully virulent survival form of the organism that can persist in the presence of free chlorine and that this phenotype may limit the usefulness of chlorination in blocking the endemic and epidemic spread of cholera. Morris et al. (29) have supported and confirmed his finding that rugose strains appear to produce an EPS that promotes cell aggregation and causes human disease. This aggregation may shield individual cells from killing by disin-

![FIG. 3. Immunoelectron micrographs of the surface labeling of strains TSI-4/R (A) and TSI-4/S (B) with anti-*V. cholerae* O1 monoclonal antibody. Bars, 0.5 μm.](http://aem.asm.org/Downloaded from)
fectants, such as chlorine, or lysis by complement. He also suggested that the EPS produced by *V. cholerae* plays a role in marine biofilm formation; this, in turn, may contribute to attachment of bacteria to marine organisms, such as plankton. Our findings confirm and support their suggestions that EPS-producing rugose vibrios promote cell aggregation in a shaking culture at stationary phase and that this adhesive EPS and rugose vibrio can form a biofilm in a static culture. We iden-

FIG. 4. Strains TSI-4/R (A) and TSI-4/T (B) treated with anti-EPS serum and labeled with a gold probe. Bars, 0.5 μm.
FIG. 5. Scanning electron micrographs of biofilm formation by *V. cholerae* O1 strain TSI-4/R. (A) Most of the surface has been colonized with actively dividing rod cells, and finger-like projections of extracellular polymeric material are present. Bar, 5 μm. (B) High magnification indicates the presence of extracellular polymeric materials on the surfaces of bacterial cells. Bar, 1 μm.
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REFERENCES


