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Isolation and Characterization of Rugose Form of *Vibrio cholerae* O139 Strain MO10

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An extracellular exopolysaccharide (slime) is produced by *Vibrio cholerae* O139 MO10 in response to nutrient starvation. The presence of this slime layer on the cell surface and its subsequent release have been shown to be associated with biofilm formation and the change from a normal smooth colony morphology to a rugose one. An immunoelectron microscopic examination demonstrated that there is an epitope common to the exopolysaccharide antigen of *V. cholerae* O1 and that of O139 MO10.

Vibrio cholerae is the causative agent of cholera, which in its most severe form is characterized by profuse diarrhea, vomiting, and muscle cramps. *V. cholerae* strains have been divided into two groups, O1 and non-O1, based on their ability to cause cholera epidemics. To date, there have been seven recorded pandemics of this severe dehydrating diarrheal disease caused by *V. cholerae* strains of serotype O1, and it was therefore assumed that only this serotype has epidemic potential. The new serogroup, designated O139 synonym Bengal, is the first recorded serogroup other than O1 to cause epidemic cholera. *V. cholerae* O139 closely resembles *V. cholerae* O1 biotype El Tor strains of the seventh pandemic (5, 12, 21, 40). The major differences between *V. cholerae* O139 and O1 are the composition and lengths of the O side chains of the cell wall lipopolysaccharide (LPS) and the presence of a capsular polysaccharide (CPS) in O139 strains that is not found in *V. cholerae* O1 strains (7, 15, 20). Serological and genetic studies suggested that CPS of O139 *V. cholerae* has the same repeating unit as the O antigen (8, 39).

V. cholerae strains are natural inhabitants of brackish water and estuarine systems (6). As a response to nutrient depletion, copiotrophic (32) heterotrophic bacteria may undergo considerable morphological, physiological, and chemical changes (11, 22, 23, 26, 27, 29). In fact, to survive energy- and nutrient-deprived conditions, non-spore-forming, heterotrophic bacteria are known to undergo an active adaptation program (29). Wai et al. (38) reported that *V. cholerae* O1 TSI-4 can shift to a rugose colony morphology associated with the expression of an amorphous exopolysaccharide (EPS) that promotes biofilm formation, and they also indicated that rugose strains displayed resistance to osmotic and oxidative stress.

Many microorganisms produce EPSs which are located outside the cell wall, either attached to it in the form of capsules or secreted into the extracellular environment in the form of slime. Extracellular polysaccharide excretion (slime or capsule) is a common phenomenon for many bacteria following the exhaustion of the nitrogen supply under otherwise nutrient-sufficient conditions (28). Bacterial cells initiate the process of irreversible adhesion by binding to the surface by using EPSs, glycocalyx polymers, and the development of biofilms. A biofilm is a functional consortium of microorganisms organized within an extensive exopolymer matrix comprised mainly of hydrated polysaccharides (43). Biofilms are produced by a wide variety of environmentally and medically important microorganisms, including *Staphylococcus*, *Pseudomonas*, *Desulfovibrio*, *Thermococcus*, and *Methanosarcina* (4, 9, 10, 18, 35, 36). The production of biofilm may enhance the survival of cells in dynamic environments by allowing the formation of colonies containing thousands of cells.

This study demonstrates that *V. cholerae* O139 MO10 is able to shift to a phenotype having a rugose colony morphology associated with the excretion of slime in response to starvation. This form promotes biofilm formation. Interestingly, the antiserum against *V. cholerae* O1 TSI-4 EPS (38) is reactive with the slime produced by *V. cholerae* O139 rugose strains. It may support the hypothesis that *V. cholerae* O139 arose from an O1 El Tor strain.

Isolation of the rugose strain of *V. cholerae* O139 MO10. *V. cholerae* O139 opaque encapsulated MO10 (40) was used in this study. The original isolate of strain MO10 had a smooth

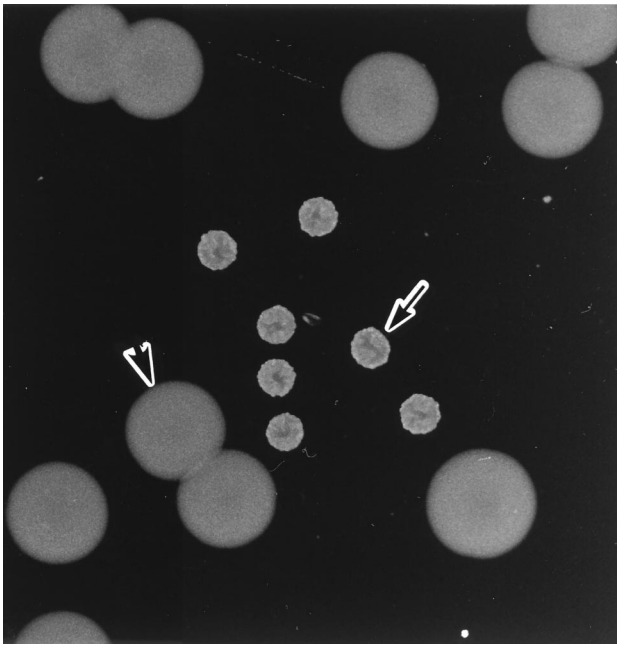

FIG. 1. Photomicrograph of *V. cholerae* O139 MO10/SPR (arrow) and MO10/NSPS (arrow head) colonies. Bacteria were incubated on an L agar plate at 37°C for 18 h.

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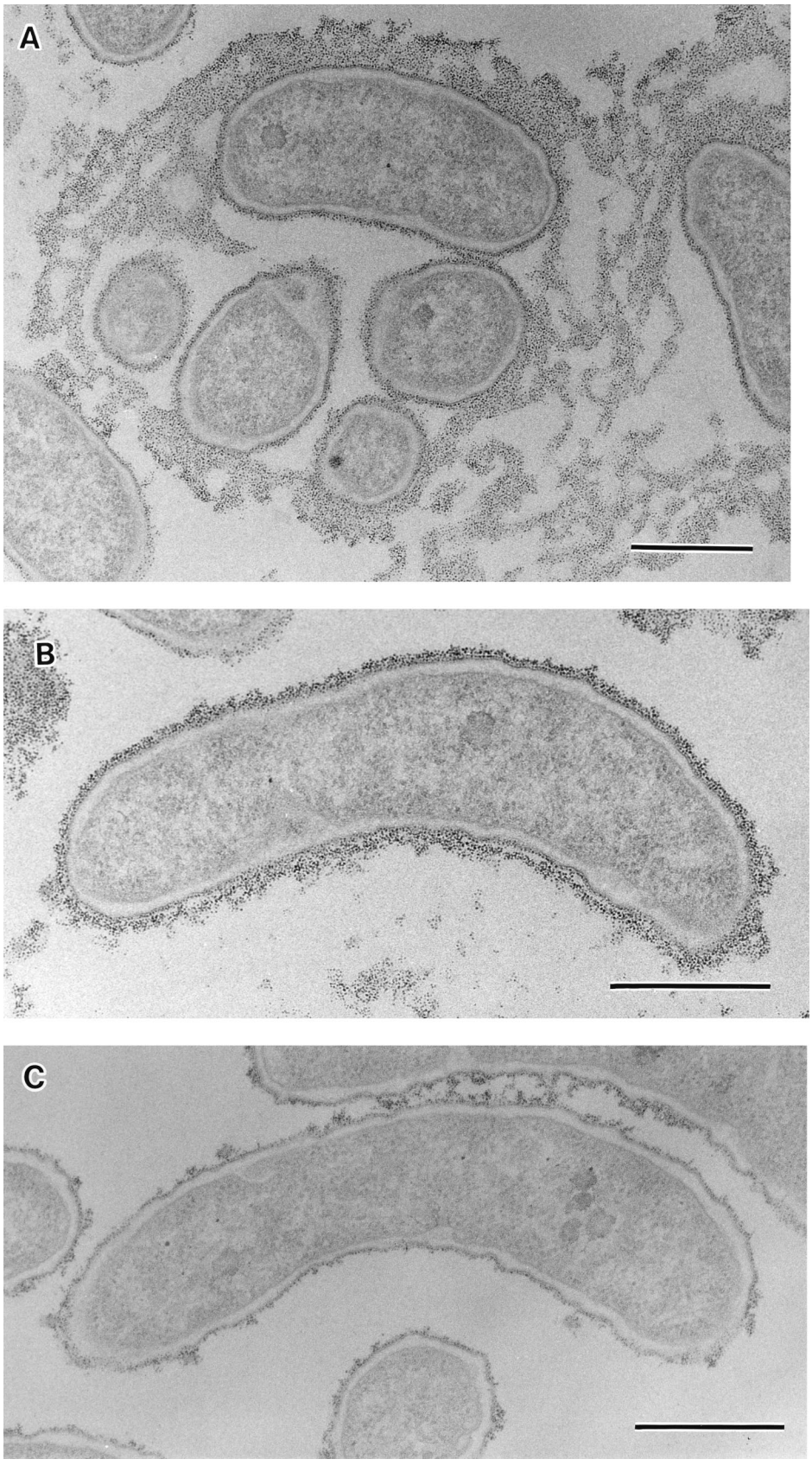

FIG. 2. Thin sections of *V. cholerae* O139 stained with polycationic ferritin showing a thick, electron-dense slime layer in addition to a thin electron-dense layer of capsule surrounding MO10/SPR cells (A and B) and no slime layer surrounding MO10/NSPS (C). Bars, 0.5 μm.

colony morphology. Cells of MO10 were routinely grown at 37°C with shaking or in a static condition in Luria (L) broth (25). MO10 cells were 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 starvation medium (M9 salts) to give a final concentration of approximately 5×10^7 cells per ml, and incubated at 4°C without shaking. Strain MO10 exhibits a shift of colony morphology to the rugose form under starvation conditions at 2 weeks after inoculation. To establish the criteria for slime production and rugose colony morphology, *V. cholerae* O139 MO10 strains have been classified as either slime-producing rugose type (MO10/SPR) or non-slime-producing smooth type (MO10/NSPS). MO10/SPR grown overnight with shaking in L broth produced MO10/NSPS colonies at a frequency of 1.5×10^{-5}. Colony counts for rugose strains represent the number of particles not the number of cells (34). This makes the determination of a frequency of phase variation difficult. Two distinct colony morphologies are shown in Fig. 1. The larger size of the smooth colonies is due to the difference of the growth rates of smooth and rugose colonies. Both colony types were tested for agglutination with anti-O139 Bengal sera (Denka, Seiken, Co. Ltd., Tokyo, Japan) and showed positive reactions. The antiserum against *V. cholerae* O1 TSI-4 EPS (38) agglutinated MO10/SPR, whereas it did not agglutinate MO10/NSPS.

Like the O1 TSI-4 rugose strain (38), *V. cholerae* MO10/SPR was much more resistant to osmotic, oxidative, and acidic stress than MO10/NSPS (data not shown).

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 as described previously (38). Both strains were surrounded by relatively thin electron-dense capsule (Fig. 2). Slime materials released by MO10/SPR were recognized as a heavy, electron-dense ferritin-stained layer surrounding the cell in addition to a thin electron-dense layer of capsule (Fig. 2A and B), but MO10/NSPS did not appear to have this slime layer surrounding its cells (Fig. 2C).

Immunoelectron microscopy. Immunoelectron microscopy was performed with anti-EPS serum of the rugose form of *V. cholerae* O1 TSI-4 as described previously (38). The antiserum against *V. cholerae* O1 TSI-4 EPS (38) was reactive only with *V. cholerae* O139 MO10/SPR and not with MO10/NSPS (Fig. 3A and B). The gold particles were specifically bound to the slime layer surrounding MO10/SPR cells and at the intercellular spaces (Fig. 3A).

Outer membrane and LPS profiles. The outer membrane was prepared from a broth culture of *V. cholerae* O139 MO10/NSPS or MO10/SPR according to the method of Filip et al. (13). LPS was prepared from 1 ml of an overnight culture (11). LPS and outer membrane samples were electrophoresed and detected by silver staining as previously described (16). No outer membrane protein or LPS differences between cell types were detected (data not shown).

Biofilm growth of *V. cholerae* O139 MO10/SPR and scanning electron microscopy. *V. cholerae* O139 MO10/SPR was cultured overnight in L broth at 37°C without shaking. The biofilms growing on the upper surface of the L broth and on the wall of a culture tube were sampled and prepared for scanning electron microscopy as described previously (38). The specimens were examined with a scanning image-observing device (ASID) equipped with a JEOL JEM 2000EX electron microscope. Figure 4 shows a biofilm examined by scanning electron microscopy; the surface of the film was completely covered with a layer of rod cells, rounded cells, and filamentous cells

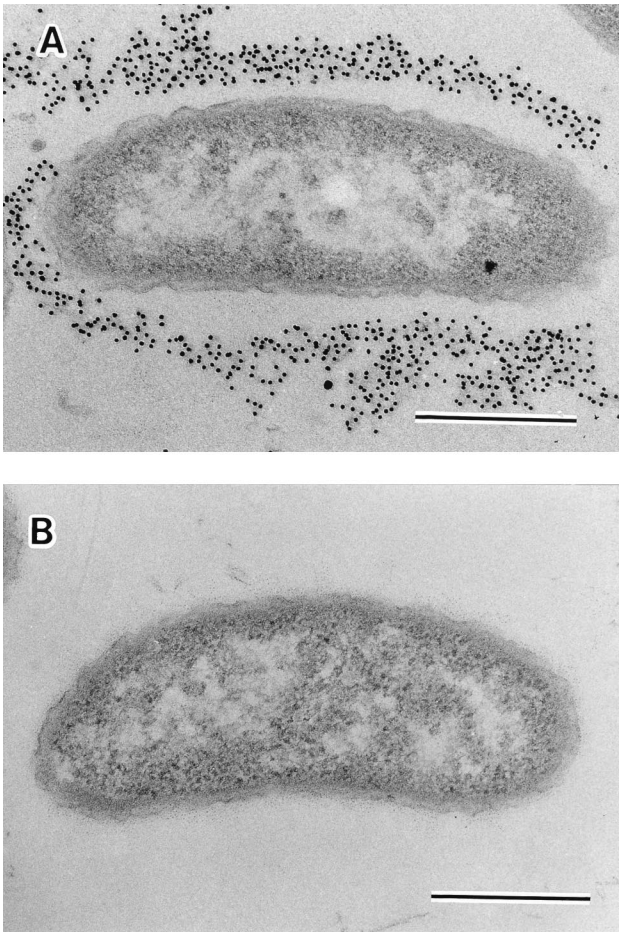

FIG. 3. Immunoelectron micrographs of the surface labeling of *V. cholerae* O139 MO10/SPR (A) and MO10/NSPS (B) with antiserum against EPS of rugose *V. cholerae* O1 TSI-4. Bars, 0.5 μm.

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. Interestingly, some of the surface of the biofilm was covered by a twisting long filamentous growth of bacteria.

The rugose form of *V. cholerae* was first described in 1938 by Bruce White, who recognized that it might be a survival form of the organism (42). Rice et al. (33, 34) 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. (30) have supported and confirmed that rugose strains appear to produce an EPS that promotes cell aggregation and causes human disease. Recently, Wai et al. (38) reported that *V. cholerae* O1 TSI-4 can shift to a rugose colony morphology from its normal translucent colony morphology in response to nutrient starvation. They also observed that EPS material on the surface of the *V. cholerae* O1 TSI-4 rugose strain promoted biofilm formation and resistance to the effects of osmotic and oxidative stress, as in the case of the O139 rugose strain. These observations suggest that the persistence of this type of setting may, in turn, contribute to the further spread of the infection in human populations. It is suggested that an improved understanding of starvation survival and nongrowth biology is an essential goal in microbiol-

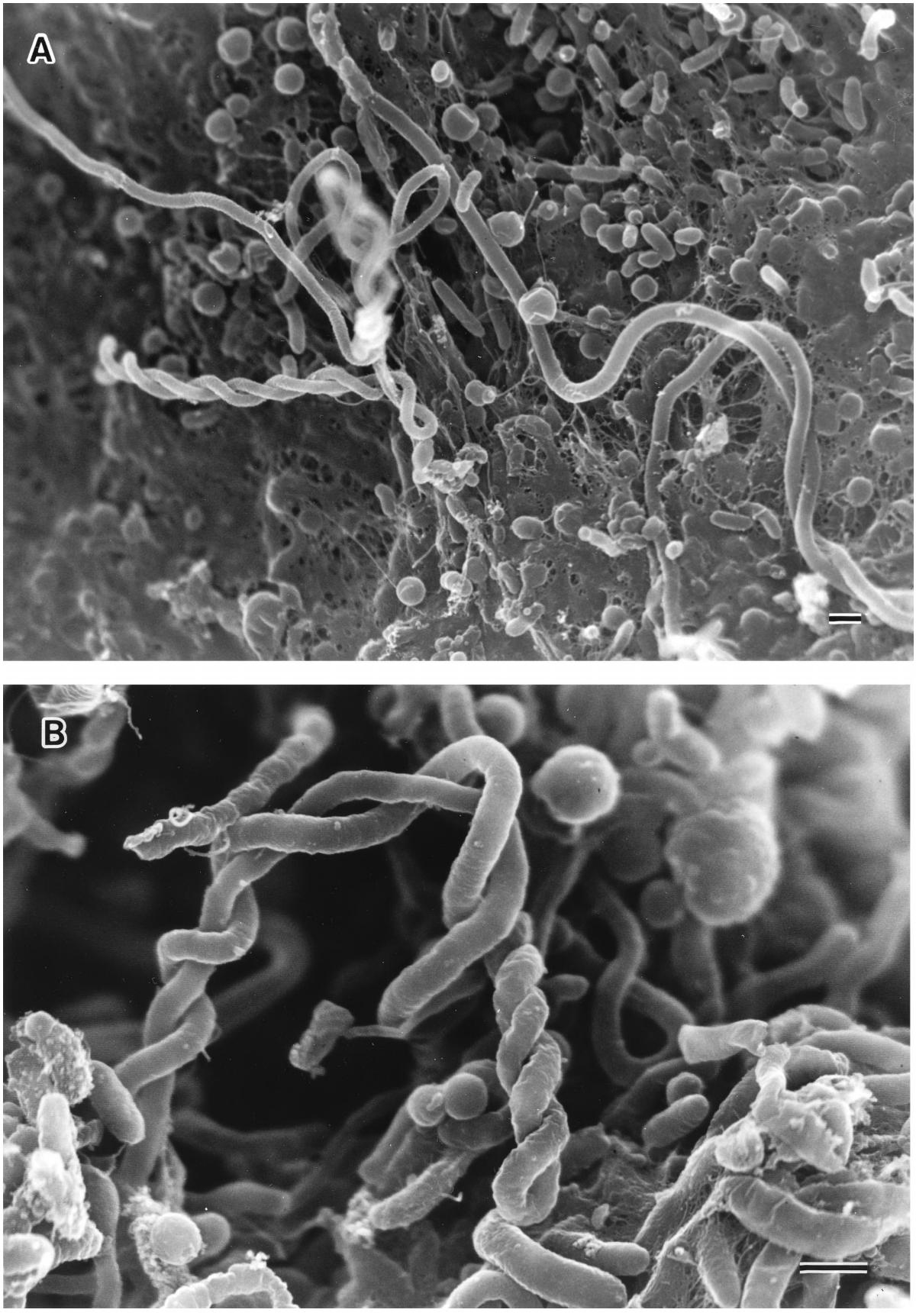

FIG. 4. Scanning electron micrographs of biofilm formation by *V. cholerae* O139 MO10/SPR. (A) Most of the surface has been colonized by rod cells, rounded cells, and twisting filamentous cells, and finger-like projections of extracellular polymeric material are present. Bar, 1 μm. (B) High magnification shows extracellular polymeric materials on the surface of bacterial cells and long twisting filamentous cells. Bar, 1 μm.

ogy, with far-reaching implications for bacterial physiology and ecology, as well as for applied bacteriology and biotechnology.

V. cholerae O139 is replacing O1 strains in some areas, and it has been suggested that the O139 strain may cause the eighth cholera pandemic (14, 19). *V. cholerae* O139 Bengal is the second most common etiologic agent of cholera, and the disease caused by this organism has now become endemic in the Indian subcontinent and neighboring countries (1). Prior infection with *V. cholerae* O1, the traditional causative agent of cholera, does not cross-protect against infection with *V. cholerae* O139 (2, 3), since the LPS antigens of the two vibrios are different (15). In addition, unlike *V. cholerae* O1, *V. cholerae* O139 possesses a CPS (20, 21, 39, 41), and it is likely that this CPS can potentially mask certain critical surface antigens, with a resulting decrease in the host immune response (31). Effective vaccines against O1 strains have been developed and are being tested in field trials (17, 24), and they do not cross-protect against *V. cholerae* O139 infection.

To facilitate the development of vaccines effective against both *V. cholerae* O1 and O139, many researchers have been studying the genes encoding O antigen and capsular synthesis in O1 and O139 strains. In our study, interestingly, antiserum against the EPS of *V. cholerae* O1 TSI-4 showed a cross-reaction with EPS materials on the surface of rugose *V. cholerae* O139 MO10. We suggest that the study of the genes encoding the EPS (slime) in *V. cholerae* O1 and O139 may facilitate the development of vaccines effective against both *V. cholerae* O1 and O139.

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