Examination of Enterotoxigenic *Escherichia coli* H10407 (Colonization Factor Antigen I+) by Scanning Electron Microscopy with Conductive Staining

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We have used the scanning electron microscope to examine enterotoxigenic *Escherichia coli* H10407, which expresses colonization factor antigen I pili. The use of low accelerating voltages and conductive staining procedures allowed us to obtain images of colonization factor antigen I pili and other structural details which were obscured by conventional gold-coating techniques.

Previously, it was necessary to apply conductive metal coatings to the surfaces of biological specimens before they could be examined in the scanning electron microscope (SEM). While this process alleviated the charge-up phenomenon and reduced specimen damage by the electron beam, it restricted the use of the SEM in microbiology because the metal coating obscured surface detail on microorganisms. In recent years, improvements in SEM technology have allowed specimens to be examined with reduced accelerating voltages. This has made it possible to utilize the conductive properties of heavy-metal stains such as osmium tetroxide to reduce sample charge-up in the SEM (6, 8). Conductivity and resolution can be enhanced by using cross-linking reagents (6) such as tannic acid (TA) or thiocarbohydrazide (TCH) to increase the amount of heavy metal taken up by the specimen (B. Giammara and J. Hanker, Proc. Electron Microsc. Soc. Am. Annu. Meet. 1988, p. 20). To demonstrate the suitability of the technique for microbiological imaging problems, we have used TA- and TCH-based conductive staining techniques to obtain SEM images of diarrheagenic, enterotoxigenic *Escherichia coli* H10407. These pathogens express colonization factor antigen I (CFA/I) pili (3), which are thought to bind to receptors on intestinal enterocytes and facilitate bacterial colonization of the luminal surface of the duodenum. Consequently, there is considerable interest in studying CFA/I pili with a view to obtaining a better understanding of their roles as virulence factors in disease caused by enterotoxigenic *E. coli*.

The enterotoxigenic *E. coli* H10407 (O78:H11, CFA/I+) and H10407P (O78:H11, CFA/I+) strains used in this study have been described previously (7, 9). Strain H10407P did not contain the 60-megadalton plasmid which is required for CFA/I pili biosynthesis (7). After 24 h of growth at 37°C on CFA/I agar (2), bacterial colonies were gently scraped from the plates and fixed (without agitation) in 1 ml of 3.5% glutaraldehyde in 0.1 M sodium phosphate buffer (SPB [pH 7.3]). The bacteria were incubated in the glutaraldehyde solution for 10 min, washed thoroughly in SPB, and stained with 2% osmium tetroxide in SPB. Anti-CFA/I monoclonal antibodies (9) were added to H10407 bacteria which had been suspended in SPB. The agglutinated bacteria were then fixed in glutaraldehyde and stained in osmium tetroxide as described above.

Conductive staining was performed by incubating the SPB-washed, fixed cells for 30 min in 1% TA in distilled water. The TA-treated cells were then gently washed with SPB and treated once more with 2% osmium tetroxide. Alternatively, the bacteria were treated with osmium-ruthenium red and TCH and then osmium-ruthenium red by the method of Giammara and Hanker (Proc. Electron Microsc. Soc. Am. Annu. Meet. 1988). The fixed bacteria were then dehydrated in ethanol, critical point dried, and examined in a Hitachi model S2500 SEM. After the stained specimens had been examined in the SEM, they were removed from the microscope and lightly coated with gold. The gold-coated specimens were reexamined in the SEM to determine the effect of the coating process on the morphology of surface structures identified on the conductive stained bacteria. When careful attention was paid to the secondary electron detector bias voltage and spot size, we found that it was possible to obtain SEM images of bacteria that had been fixed only with glutaraldehyde and osmium tetroxide (Fig. 1) by using accelerating voltages of less than 5.0 kV. Nonetheless, it was difficult to avoid specimen damage and sample charge-up. However, when we used the conductive staining techniques, specimen damage and charge-up were greatly reduced, and the surfaces of the cells could be observed even when we employed accelerating voltages of up to 15.0 kV (Fig. 2; see also Fig. 4).

The TA-treated H10407 (CFA/I+) bacteria appeared to be completely covered with fine strands, and in many cases, it was impossible to observe the outer membranes of the organisms through the amorphous mass of fibrils (Fig. 2). Many of the H10407 bacteria were entangled in the fibrillar surface coat that contained some strands spanning gaps which approached 2 μm in length. The fibrillar surface coating was not observed on the TA-treated H10407P (CFA/I+) organisms, although numerous flagella were apparent (Fig. 2). Since we did not observe any differences (other than charge-up) between the bacteria fixed with glutaraldehyde and osmium and those treated with TA (compare the H10407 bacteria shown in Fig. 2 with those shown in Fig. 1), we concluded that the TA treatment had not altered the fine structures on the H10407 bacterial surfaces.

When the TA-treated bacteria were coated with gold and reexamined in the SEM, the fine fibrillar structures on the surfaces of the H10407 organisms were not apparent, and it was difficult to differentiate between the H10407 and

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FIG. 1. SEM image of H10407 (CFA/I+) bacteria fixed with glutaraldehyde and postfixed with osmium tetroxide. The image was obtained with an accelerating voltage of 5.0 kV. Bar, 1 μm.

FIG. 2. SEM images of glutaraldehyde-osmium-fixed H10407 (CFA/I+) and H10407P (CFA/I-) bacteria treated first with TA and then again with osmium tetroxide. The H10407 and H10407P images were obtained with accelerating voltages of 5.0 and 2.0 kV, respectively. Bars, 1 μm. The arrows in the left panel indicate fibrillar surface coat strands which span gaps almost 2 μm long. The arrow in the right panel indicates numerous flagella.

H10407P strains (Fig. 3). Short, rodlike structures were observed on both strains, and the outer membranes of the organisms could be seen. The curved appearance of some of the rods suggests that they were probably gold-coated flagella.

The fibrillar surface coating on H10407 bacteria that had been agglutinated with anti-CFA/I monoclonal antibodies (Fig. 4) was not well defined. Individual strands appeared much thicker than those shown in Fig. 1 and 2, suggesting direct interaction between the monoclonal antibodies and the fibrils. Furthermore, in the absence of CFA/I monoclonal antibody, the fibrils were lost if the glutaraldehyde was left for more than 10 min or if the bacteria were handled too vigorously during preparation (data not shown). Taken together, these observations suggest that cross-linking with the divalent monoclonal antibodies stabilized the surface coating and that the fibrils seen on the H10407 organisms in Fig. 1 and 2 were CFA/I pili.

In previous studies, Knutton et al. (4) examined thin sections of H10407 bacteria bound to erythrocytes and intestinal epithelial cells. These studies revealed an electron-translucent zone approximately 1 μm wide between the bacteria and the host cells. The gap appeared to be maintained by rigid pili protruding from the surfaces of the organisms. It is apparent that it would be difficult for the surfaces of the H10407 bacteria to come into close contact with the surfaces of host tissues (Fig. 2). Therefore, the SEM images presented in this article support the findings of Knutton et al. (4) and provide additional images of the surface coating of enterotoxigenic E. coli H10407.

Some bacteria possess an extracellular glycocalyx (1) composed of acidic polysaccharides. The glycocalyx can be
visualized in the electron microscope by using ruthenium red staining (1, 5). During their investigations, Knutton et al. detected ruthenium red staining of H10407 surface components (4). Therefore, we used osmium-ruthenium red in combination with TCH to determine if the surface coats of H10407 bacteria contained any carbohydrate. In the osmium-ruthenium red procedure, TCH was used instead of TA to increase the conductivity of the specimens. The

FIG. 3. Gold-coated H10407 and H10407P viewed in the SEM. Both images were obtained with an accelerating voltage of 5.0 kV. Bar, 1 μm. The arrows indicate the curved appearance of some of the rods.

FIG. 4. SEM images of glutaraldehyde-osmium-fixed H10407 bacteria agglutinated with anti-CFA/I monoclonal antibodies and treated first with TA and then with osmium tetroxide or, in the absence of antibodies, treated with osmium-ruthenium red, TCH, and again with osmium-ruthenium red. The images were obtained with accelerating voltages of 10.0 and 15.0 kV, respectively. Bar, 1 μm. The arrow indicates the clearly visible flagella.
appearance of ruthenium red-stained bacteria was similar to that of the organisms which had been treated with monoclonal antibody (Fig. 4). Although flagella were clearly visible, the fibrils appeared thicker and the coating was condensed, similar to that of the organisms treated with monoclonal antibody. This observation suggests direct interaction between ruthenium red and extracellular material (possibly glycocalyx) associated with the CFA/I pili.

Previously, images of the surface of enterotoxigenic *E. coli* H10407 were obtained by examining thin sections of organisms (4) or negative-stained preparations in the transmission electron microscope. However, transmission electron microscopes can produce only two-dimensional images, whereas the SEM can produce three-dimensional images. The electron micrographs in this article demonstrate that, unlike gold coating (Fig. 3), the conductive staining procedures make it possible to utilize the SEM to study microbiological specimens bearing fragile surface structures such as pili (Fig. 2).

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