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High-Resolution Visualization by Field Emission Scanning Electron Microscopy of *Enterococcus faecalis* Surface Proteins Encoded by the Pheromone-Inducible Conjugative Plasmid pCF10

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Enterococcus faecalis **can acquire antibiotic resistance and virulence genes by transfer of pheromone-inducible conjugative plasmids such as pCF10, which encodes tetracycline resistance. Two pCF10-encoded cell surface proteins, Sec10 and Asc10, have been previously shown to play an important role in the transfer of this plasmid. We used high-resolution, field emission scanning electron microscopy to visualize these proteins on the surfaces of a series of isogenic strains of *E. faecalis*. Immunogold labeling, using both 6- and 12-nm colloidal gold, unambiguously demonstrated the expression and distribution of Sec10 and Asc10 on the surface of the *E. faecalis* cells. On unlabeled *E. faecalis* cells which expressed either Sec10 or Asc10, the former appeared to be more readily detected. Immunogold labeling of *E. faecalis* cells expressing both Asc10 and Sec10 clearly demonstrated the abundance and intermixing of both proteins on the cell surface except at septal regions. Sec10 was observed to be distributed over the cell surface. At regions of cell-cell contact, fine strands representing Asc10 were observed directly attaching adjacent cells to one another.**

Gene transfer in *Enterococcus faecalis* can occur by a mechanism involving pheromone-inducible conjugative plasmids, which to date has been found only in this organism (23). Pheromone-induced conjugation is characterized by the formation in liquid culture of aggregates of donor and recipient cells (10, 12) under conditions in which highly efficient plasmid transfer occurs (10). Most of our understanding of this type of gene transfer comes from work on the hemolysin plasmid, pAD1 (7), and a tetracycline resistance plasmid, pCF10 (9, 11). *E. faecalis* has become increasingly important in several diseases, including endocarditis (1, 2, 20) and urinary tract infections (20), particularly with respect to nosocomial infections. Conjugative plasmids are of clinical significance because of their role in the dissemination of antibiotic resistance genes and because of the potential virulence factors they encode, such as hemolysin (16) and bacteriocin (16, 36) production.

In the case of pCF10 (13), the induced expression of a plasmid-encoded cell surface protein called aggregation substance, Asc10, which mediates the formation of the mating aggregates (27) is important for efficient plasmid transfer. In addition, a surface exclusion protein, Sec10, that is important in preventing plasmid transfer between aggregated donor cells has been identified (15). There is also evidence suggesting that one or more additional pheromone-inducible surface proteins may be involved in the transfer of pCF10 (24).

As early as 1981, Handley and Jacob (22) examined *E. faecalis* by transmission electron microscopy of negatively stained cells and identified what were described as flexible, peritrichous fimbriae. However, it is not clear how these fimbriae correlate with the surface proteins associated with the pheromone-induced conjugative plasmids. Wanner et al. (32) used scanning electron microscopy to examine the aggregation substance, Asa1, encoded by pAD1. They were able to demonstrate the presence and distribution of surface proteins labeled with colloidal gold; however, these studies were hampered by an inability to clearly resolve the colloidal gold used in the study. In addition, it is possible that the surface exclusion protein encoded by pAD1 (33), unidentified at the time of the study by Wanner et al. (32), may have complicated the interpretation of their data.

In the present report, we describe the use of high-resolution, field emission scanning electron microscopy at low accelerating voltages (LVSEM) to study the expression and distribution of Asc10 and Sec10 on the *E. faecalis* cell surface. Using specific monoclonal antibodies (MAb) visualized with both 6- and 12-nm colloidal gold, we have labeled Asc10 and Sec10 on a series of isogenic *E. faecalis* strains constitutively expressing various combinations of the two proteins, and we have examined pheromone-induced expression of the proteins in wild-type donor cells. In conjunction with LVSEM at accelerating voltages of less than 4 keV, we have used a secondary electron (SE) detector for conventional topographical imaging and a specially designed high-resolution YAG backscatter electron (BSE) detector (17, 31) which by atomic number contrast permits unambiguous identification of the colloidal gold label. Utilizing this novel technology, we have visualized the pCF10-encoded proteins, Sec10 and Asc10, individually and together in a manner which clarifies their expression, distribution, and interaction on the cell surface and with other *E. faecalis* cells.

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TABLE 1. *E. faecalis* OG1RF and inserted plasmids

Strain or plasmid	Description[a]	Reference
OG1RF	Chromosomal Rif^r Fus^r Con$^+$	10
pCF10	Wild-type pheromone-inducible plasmid; Tet^r	13
pWM401	*E. coli-E. faecalis* shuttle vector, Cat^r Tet^r (*E. coli*); Cat^r (*E. faecalis*)	35
pINY1801	*Eco*RI c and e fragments (11.958 kb) of pCF10 cloned into pWM402	6
pINY4515	pINY1801 containing a Tn*5* insertion within the *prgA* structural gene of the *Eco*RI c fragment	24
pINY4561	pINY1801 containing a Tn*5* insertion within the *prgB* structural gene of the *Eco*RI e fragment	24

[a] Abbreviations: Rif, rifampicin; Fus, fusidic acid; Tet, tetracycline; Cat, chloramphenicol; Con, conjugation.

MATERIALS AND METHODS

Bacterial strain, media, and reagents. The *E. faecalis* strain (OG1RF) and plasmids used in this study are described in Table 1. *E. faecalis* OG1RF (10) was grown in M9-YE medium (30) and was transformed with chimeric plasmids by electroporation (14). Antimicrobial agents (Sigma Chemical Co., St. Louis, Mo.) were added to agar plates and broth in the following concentrations: chloramphenicol, 15 μg/ml; rifampin, 50 μg/ml; and fusidic acid, 10 μg/ml. A synthetic pheromone, cCF10, was used for pheromone induction as described previously (25).

Immunogold labeling of *E. faecalis*. Overnight or exponential-phase bacterial cultures were washed twice and resuspended to a concentration of 10^8 cells per ml in 10 mM phosphate-buffered saline (pH 7.4). Glass chips (4 by 8 mm) were cleaned with 95% ethanol and coated with 0.1% poly-L-lysine for 10 min. Excess poly-L-lysine was rinsed off, and 30 μl of each bacterial suspension was placed on individual chips for 10 min. Excess bacteria were gently washed from the chips with Hanks' balanced salt solution (pH 7.4) (HBSS), and unlabeled samples were placed in fixative (2.5% glutaraldehyde and 0.5% paraformaldehyde in a 0.1 M sodium cacodylate buffer containing 7.5% sucrose) for 1 h. Bacteria to be labeled with colloidal gold were washed with HBSS containing 0.5% bovine serum albumin, and 20 μl of hybridoma supernatants containing either a mouse anti-Asc10 MAb (29) or a rat anti-Sec10 MAb (15) was applied for 1 h at 37°C. Bacteria were then gently washed with HBSS containing 0.5% bovine serum albumin, and 20 μl of a 1:5 dilution of goat anti-mouse or anti-rat immunoglobulin G (IgG) conjugated to 12-nm colloidal gold particles (Jackson ImmunoResearch Laboratories, Inc., West Grove, Pa.) was applied for 10 min at room temperature. Finally, all samples were washed gently with HBSS and placed in the fixative described above. Double immunogold labeling was performed by labeling Sec10 and Asc10 successively with 6- and 12-nm gold conjugates to distinguish the two proteins. Experiments were performed in which either Asc10 or Sec10 was initially labeled in combination with the 6- or 12-nm gold conjugates to optimize the labeling of the proteins. As a control for cross-reaction of the goat colloidal gold conjugates, a series of samples were labeled with either the mouse anti-Asc10 MAb or rat anti-Sec10 MAb followed by an appropriate goat anti-mouse or anti-rat fluorescein isothiocyanate conjugate, respectively. The 12-nm gold–anti-IgG conjugate with the alternate species was then applied for 10 min at room temperature. With this approach, any observed labeling would be due to insufficient blocking or saturation of the primary MAb by its appropriate colloidal gold conjugate.

High-resolution LVSEM. Bacteria were prepared for viewing by LVSEM as previously described (34). Briefly, the fixative was washed from the samples twice for 10 min in 0.1 M sodium cacodylate with 7.5% sucrose buffer and postfixed for 30 min in 0.1 M sodium cacodylate containing 1% osmium tetroxide and 7.5% sucrose. The samples were then washed twice with 0.1 M sodium cacodylate, dehydrated with ethanol, critical-point dried by the CO_2 method of Anderson (3) with a Samdri-780A (Tousimis, Rockville, Md.), and coated with a 1- to 2-nm discontinuous layer of platinum by using an saddle field ion beam gun (VCR Group, South San Francisco, Calif.). *E. faecalis* cells were viewed with a Hitachi S-900 field emission scanning electron microscope operated at low accelerating voltages (1.3 to 4 keV), using an SE detector for conventional topographical imaging and a high-resolution YAG BSE detector (17, 31) for the visualization of colloidal gold by atomic number contrast.

RESULTS

Previous studies demonstrated the constitutive expression of both Sec10 and Asc10 on the surface of *E. faecalis* cells carrying pINY1801 (6, 24). Insertional mutagenesis of this plasmid with Tn*5* generated pINY4515 and pINY4561 with transposon inserts in the structural genes encoding Sec10 and Asc10, respectively, resulting in expression of either Asc10 or Sec10 (24). All broth cultures were examined for their characteristic phenotypic growth; strains expressing Asc10, strains OG1RF(pINY1801) and OG1RF(pINY4515), aggregate in broth, while cells carrying plasmid pWM401 or pINY4561 remain evenly distributed. Examination by LVSEM also reflected these phenotypes, as large aggregates of cells were observed with the former two strains, while an even monolayer of cells was distributed over the glass chips with the last two. Effective LVSEM examination of the clumpy cells required the viewing of smaller, isolated clumps because of the minimal depth of field present with the in-lens field emission scanning electron microscope.

When viewed by SE emission, all strains exhibited the classical diplococcus form. Differences between the four strains were also apparent. The *E. faecalis* OG1RF cells containing the vector pWM401 as a control were devoid of any apparent material on their cell surface (Fig. 1A), while those containing the cloned pCF10 DNA had pleomorphic surface protrusions on the cell surface. Interestingly, the expression of this material varied greatly from cell to cell. Strain OG1RF(pINY4561) (Fig. 1B) had a flocculent material evenly distributed over the cell surface, which was not observed attaching to other cells.

The two clumpy strains OG1RF(pINY4515) and OG1RF (pINY1801) sharply contrasted with the previous strains in appearance by exhibiting fine filaments which appeared to mediate contact between adjacent cells. These structures are visible in Fig. 1C, in which two OG1RF(pINY4515) cells are shown. In the areas of direct contact, these fine filaments stretched between the two cells. The remaining surface of OG1RF(pINY4515) cells appeared to be similar to that of control cells containing pWM401. OG1RF(pINY1801) is

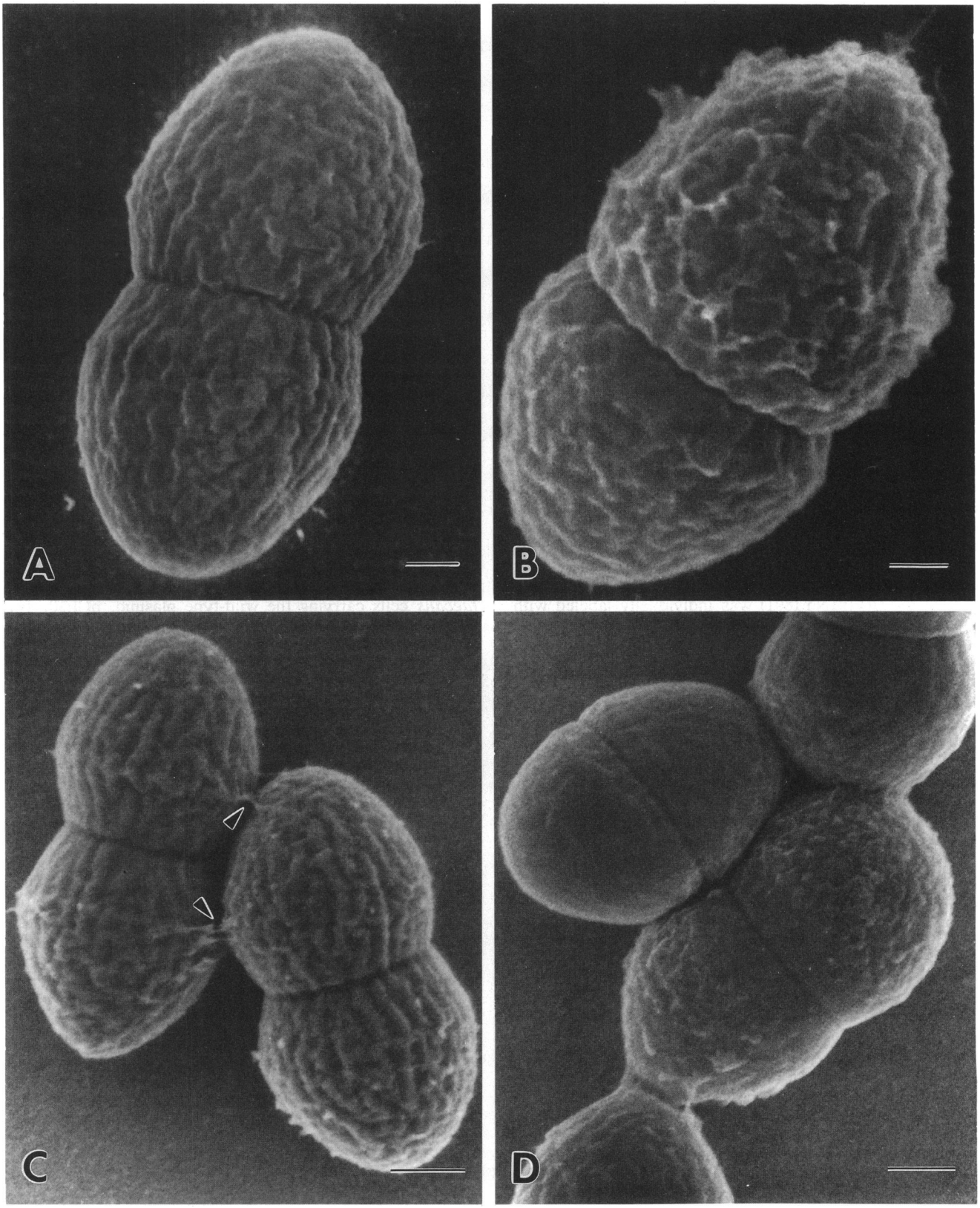

FIG. 1. Stationary-phase cultures of isogenic *E. faecalis* strains viewed by SE emission. (A) OG1RF(pWM401), used as a negative control because cells contain vector without cloned pCF10 fragments; cells exhibit a smooth surface presumably representing lipoteichoic acid and possibly other protein constituents associated with the cell wall. (B) OG1RF(pINY4561); this strain expresses only Sec10, which appears as a flocculent material extending out from the cell surface. (C) OG1RF(pINY4515); this strain expresses only Asc10, which appears more compact and tightly adherent to the cell surface. Fine filaments (arrowheads) at points of cell contact appear to hold the two cells in close apposition. (D) OG1RF(pINY1801), which expresses both Sec10 and Asc10; the presence of both Asc10 and Sec10 in the cell surface produces the appearance of clusters or aggregates protruding above the surface. Fine strands (also seen in panel C) can be observed at points of cell contact; these may be indicative of Asc10 expression. Variation of protein expression can be observed, with the adjacent cell seemingly devoid of surface protein. Bars: A and B, 100 nm; C and D, 200 nm.

shown in Fig. 1D; again, in areas of direct contact with the adjacent cells, fine strands could be seen stretching across to the other cell, holding them in tight apposition. However, in contrast to the case with OG1RF(pINY4515), there was a great amount of additional material apparent on the surface of OG1RF(pINY1801). Differences between individual cells were also readily apparent, as can be seen in Fig. 1D, in which the amount of material on the surface of each cell varies.

Based upon genetic and biochemical analyses of these four isogenic strains (6, 24), it seems reasonable to speculate that the material observed on the *E. faecalis* cells containing pINY4561 is Sec10 and that observed on cells containing pINY4515 is Asc10. The material seen on cells carrying pINY1801 would be made up of a combination of both Asc10 and Sec10. From observations of the former two strains, it appears that Sec10 is visually most predominant on the surface of the cells containing pINY4561 or pINY1801. To verify these predictions, colloidal gold labeling of the cell surfaces was carried out to confirm the expression of Asc10 and Sec10 on the appropriate clones and to specifically identify the distributions of these proteins. The absence of cross-reactivity by the mouse anti-Asc10 MAb and the rat anti-Sec10 MAb was confirmed first by indirect immunofluorescence and colloidal gold labeling, using the isogenic strains expressing either Asc10 [OG1RF(pINY4515)] or Sec10 [OG1RF(pINY4561)] alone (data not shown).

Strain OG1RF(pINY1801) was individually labeled with either mouse anti-Asc10 or rat anti-Sec10 to investigate the distributions of the proteins on the *E. faecalis* cell surface. Cellular clumping of this strain was readily apparent at low magnification (Fig. 2A and B); on many cells, the majority of the 12-nm gold label was observed on only one half of the diplococcus. Individual OG1RF(pINY1801) cells could be seen at higher magnification (Fig. 2C and D, labeled with anti-Sec10 or anti-Asc10, respectively). Both proteins appeared evenly distributed over the surface of the *E. faecalis* cells except in the septal regions.

To further investigate the expression of Sec10 and Asc10 on the surface of cells expressing both proteins, OG1RF (pINY1801) was double labeled by using 6- and 12-nm colloidal gold. Figure 3 shows both Asc10 and Sec10 on OG1RF(pINY1801) labeled with 6- and 12 nm colloidal gold, respectively. The cell surface proteins are intimately associated, as is reflected by the intermixing of the two gold labels. By sequentially labeling each protein, cross-reactivity by the second colloidal gold conjugate was eliminated. This loss of cross-reactivity was confirmed by treating OG1RF(pINY4515) cells with the mouse anti-Asc10 MAb followed by an anti-mouse FITC conjugate and finally the anti-rat 12-nm colloidal gold conjugate (Fig. 4). These cells were entirely devoid of 12-nm gold label. In addition, neither the order in which Sec10 and Asc10 were labeled nor the size of the gold particle used had an apparent effect on the labeling of OG1RF(pINY1801) cells; Fig. 4B demonstrates Asc10 and Sec10 labeled with 12- and 6-nm colloidal gold, respectively. As observed earlier, some cells expressed only Asc10 or Sec10.

In a similar fashion, OG1RF cells carrying wild-type pCF10 were investigated to compare the protein expression of the constructed isogenic OG1RF strains with the wild-type expression of Asc10 and Sec10 (Fig. 4). A low level of Sec10 expression could be seen on uninduced OG1RF (pCF10) cells (Fig. 4C), as indicated by the 12-nm colloidal gold label, which is consistent with previous work demonstrating constitutive Sec10 expression by Western blot (immunoblot) (6, 24). When induced by the synthetic pheromone cCF10 (Fig. 4D), these cells expressed both Sec10 and Asc10, as reflected by the 6- and 12-nm colloidal gold, respectively, in a manner identical to that observed with OG1RF(pINY1801) (see above). The two surface proteins appeared to be intimately associated and evenly distributed over the surface of the induced OG1RF(pCF10) cells, again with the exception of the region adjacent to the septum.

DISCUSSION

In previous reports, two surface proteins associated with conjugal plasmid transfer in *E. faecalis* were identified (6, 30). These proteins, encoded by the pheromone-inducible conjugative plasmid pCF10, are now referred to as Sec10, responsible for surface exclusion (15), and Asc10, shown to be the aggregation substance (27). We recently reported the complete DNA sequence of the *Eco*RI c and e fragments encoding these proteins (24). Recent advances in electron microscopy have provided the means by which surface structures on biological samples can be visualized at extremely high resolution (18, 21). We have employed this technology to study Asc10 and Sec10 on four isogenic strains of *E. faecalis* with respect to their presence and distribution, their structure on the cell surface, and their interaction with each other and with other *E. faecalis* cells. We have also compared this protein expression with that of other *E. faecalis* cells carrying the wild-type plasmid, pCF10. The resulting electron micrographs show a fine fibrillar, peritrichous material distributed over the surface of the *E. faecalis* cells.

When both Sec10 and Asc10 are expressed on the same cell, as with OG1RF(pINY1801), and imaged by SE emission (Fig. 1D), it is difficult to distinguish the two proteins from each other, as they appear to intertwine. However, it is likely that both proteins are expressed, as reflected by the thick mat of material on the cell surface. In addition, using 6- and 12-nm colloidal gold to label Sec10 and Asc10, we can unambiguously demonstrate their expression, distribution, and intimate association on the cell surface. Only Asc10, whether expressed on OG1RF(pINY4515) or OG1RF (pINY1801), was observed interacting with adjacent *E. faecalis* cells (Fig. 1). By SE emission, the protein appeared as a fine filamentous material, at times stretched and taut, indicating a tenacious interaction with the other cells.

In this study, both Sec10 and Asc10 were closely associated with each other, and neither protein was partitioned to specific areas of the cell. Interestingly, however, the expression of the individual proteins varied greatly from cell to cell, as seen in Fig. 3D, perhaps indicating an association with cell cycle and surface protein expression. Three pieces of evidence support this supposition. First, while the structural genes *prgA* and *prgB*, encoding Sec10 and Asc10, respectively, are carried on an *Escherichia coli-E. faecalis* shuttle vector, instability in the form of deletions or alterations has not been observed in these constructs (6, 24). Second, a similar variability in the expression of the cell surface proteins in pheromone-induced *E. faecalis* cells carrying pCF10 was also observed. This variation is seen even at saturation levels of pheromone cCF10, reflecting an inability either to respond to pheromone or to express these proteins in individual cells. Last, this cell-to-cell variation has been observed in other genera with respect to other surface structures as pilus and flagellum expression (26).

The structure of each protein as seen on the surface of the *E. faecalis* cells seems to support the predicted structure.

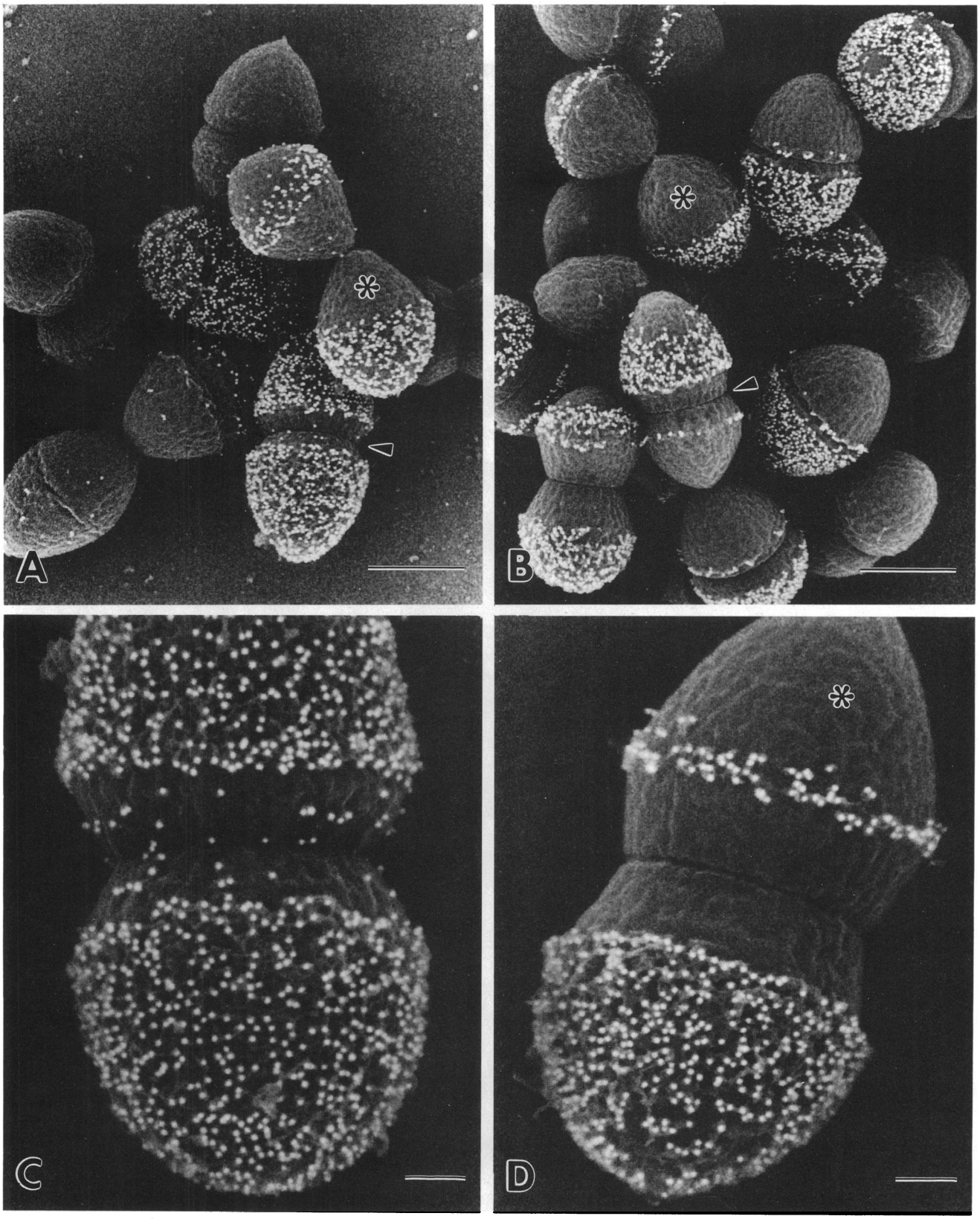

FIG. 2. Exponential-phase *E. faecalis* OG1RF(pINY1801) labeled with 12-nm colloidal gold as described in Materials and Methods and visualized by BSE. Heterogeneity of the staining for either Sec10 or Asc10 on cell surfaces was apparent; both proteins were seen evenly distributed in polar caps, while the septal regions were devoid of immunogold staining (arrowheads). As cellular division progressed, this region of new cell wall synthesis increased, and upon completion of division, cells appeared with one half devoid of surface material (asterisks) or with a narrow band of protein. (A and B) Low magnification of aggregated cells, using anti-Sec10 MAb and anti-Asc10 MAb, respectively; (C and D) higher magnification of individual cells labeled with anti-Sec10 MAb and anti-Asc10 MAb, respectively. Bars: A and B, 500 nm; C and D, 100 nm.

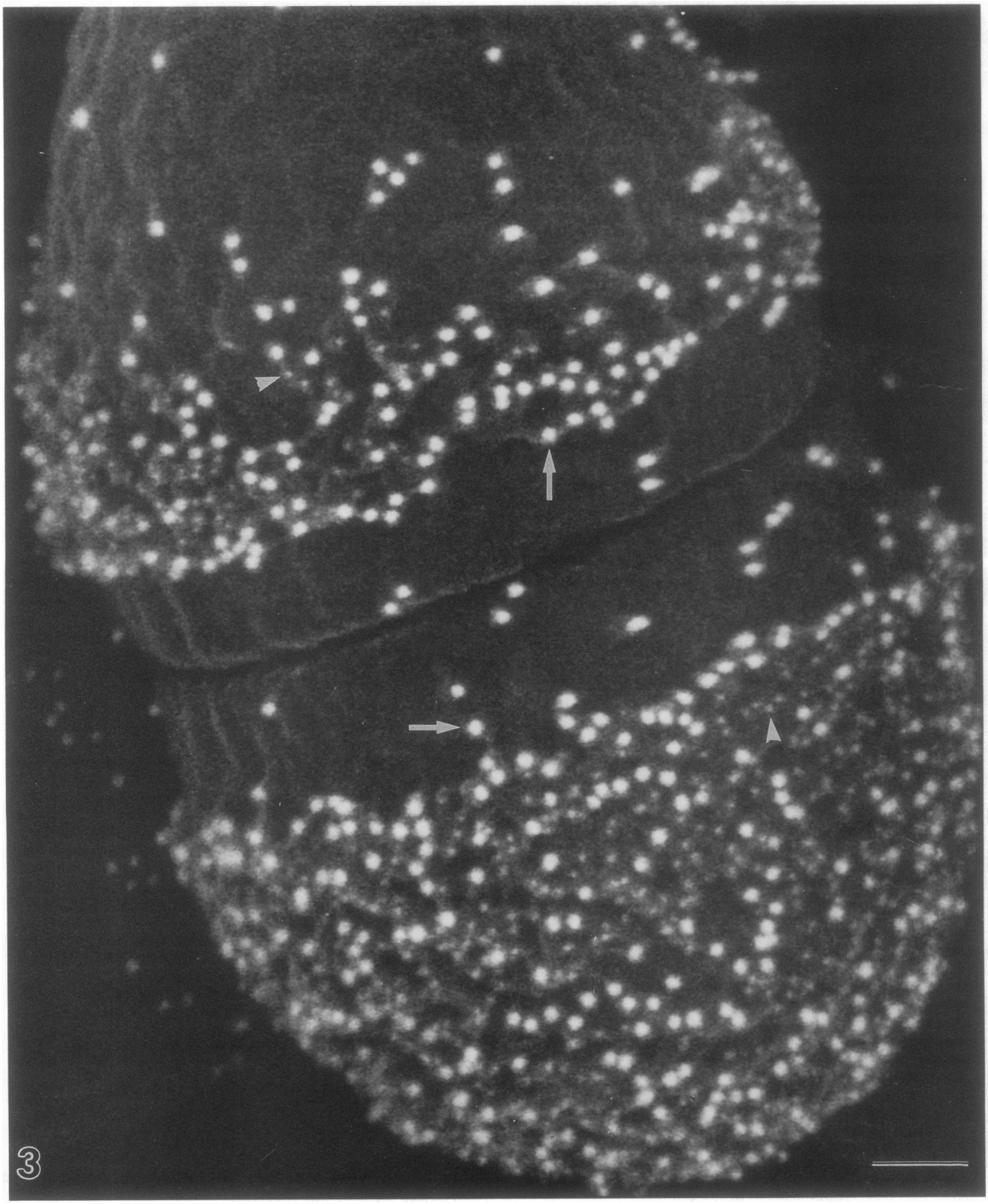

FIG. 3. Double immunogold labeling of OG1RF(pINY1801) with 6- and 12-nm colloidal gold visualized by BSE. Asc10 was first labeled with 6-nm colloidal gold (arrowheads), and then Sec10 was labeled with 12-nm gold (arrows), as described in Materials and Methods; the two colloidal gold labels were interspersed in regions along what appeared to be the same linear arrays. Occasionally, some Sec10 was detected in the region of the septum. Bar, 50 nm.

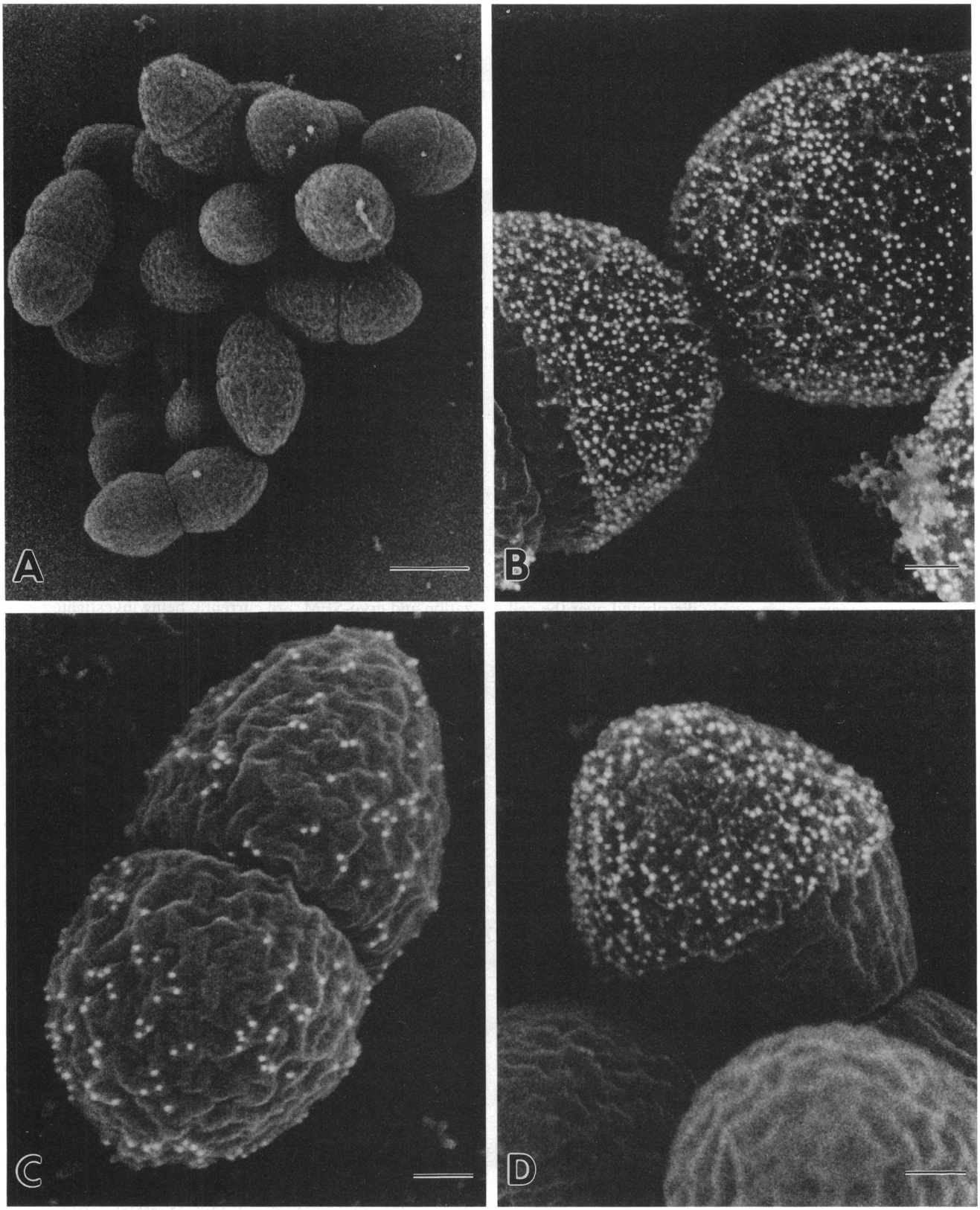

FIG. 4. BSE imaging of colloidal gold labeling of surface proteins on isogenic strains of *E. faecalis* OG1RF(pINY1801 [Asc10 and Sec10] and pINY4515 [Asc10]) compared with *E. faecalis* carrying wild-type pCF10. (A) Labeling control. OG1RF(pINY4515) cells were labeled first with mouse anti-Asc10 MAb, then with a goat anti-mouse fluorescein isothiocyanate conjugate, and then with the goat anti-rat 12-nm colloidal gold conjugate, as described in Materials and Methods. Cells are completely devoid of gold label, indicating complete saturation and blockage of the primary MAb. (B) OG1RF(pINY1801). Asc10 was first indirectly labeled with 12-nm colloidal gold, and then Sec10 was indirectly labeled with 6-nm gold. (C) Uninduced OG1RF(pCF10). Sec10 was indirectly labeled with 12-nm gold; a low-level constitutive expression of this protein was evident in these cells. (D) Induced OG1RF(pCF10) double labeled, using first 12-nm colloidal gold to detect Asc10 and then 6-nm gold to detect Sec10. The pattern of labeling is identical to that of OG1RF(pINY1801) (Fig. 3A and 4B). Bars: A, 500 nm; B, C, and D, 100 nm.

From DNA sequence analysis, the predicted protein structure of Sec10 closely resembles that of the type 6 M protein of *Streptococcus pyogenes*, with extended regions of α-helical structure (24). This prediction seems consistent with the current observation of this protein appearing to assume a more defined rigid structure outside the cell, as seen in Fig. 1B. In contrast, Asc10 is predicted to be more globular (24) and in this study appeared closely associated with the cell surface (Fig. 1C).

In stationary cultures, the expression of Sec10 and Asc10 was evenly distributed over the cell surface. In actively dividing cells, however, their expression was restricted to regions presumably of old cell wall. A model for cell wall synthesis in streptococci was presented in 1962 by Cole and Hahn (8), who used fluorescein-conjugated anti-group A *Streptococcus* antibody to label cells at different time points during *S. pyogenes* growth and cell division; their study demonstrated new cell wall formation at the septal regions and is consistent with many of our observations. In 1970 (22a), Higgins and Shockman began an extensive analysis of cell wall synthesis in enterococci and proposed a similar model for these organisms. In the present study, little if any colloidal gold label was observed in the septal regions, and bands of proteins appeared on some cells (Fig. 2). These patterns of protein expression are associated with cell wall synthesis and cell division (8, 22a). Similar observations were also made by Wanner et al. (32). It appears that as cells reach late log phase growth and cell division slows, the proteins again become more evenly distributed over the cell surface.

As early as 1981, Handley and Jacob (22) identified structures, which they termed fimbriae, by transmission electron microscopy on negatively stained *E. faecalis*. How these structures relate to the pCF10-encoded surface proteins, Sec10 and Asc10, is not known, but "self-transferable" plasmids were carried by one of the strains observed. Wanner et al. (32) used field emission SEM to study the pAD1-encoded aggregation substance, Asa1 (same as Asc10 in this study), on the surface of *E. faecalis* cells. This study was important in showing the presence and distribution of surface material on pheromone-induced cells, but it was limited in two respects. First, the colloidal gold label used to identify Asa1 was not clearly resolved, partially because of the higher accelerating voltages and the lower resolution obtained with below-the-lens field emission SEM (28). Second, only Asa1 had been identified at the time of the study (32). The pAD1-encoded surface exclusion protein, Sea1, has been identified and reported only recently (33). As a consequence, all the surface material observed in the study by Wanner et al. (32) was interpreted to be Asa1. In addition, this interpretation is based upon the formation of large clusters or aggregates of gold particles giving an artifactual appearance to the surface topography. Our observations indicate that the bulk of material seen on the surface of these cells is actually the surface exclusion protein. This conclusion has been made possible by the use of isogenic strains which express the two surface proteins both separately and together, allowing for a clear analysis of their presence on the *E. faecalis* cell surface.

When the *E. faecalis* were examined cells by SE emission, charging artifacts (unwanted excessive emission of SEs) were a constant problem in resolving the surface morphology of the samples. Detection of surface proteins requires topographical resolution on the order of 10 to 30 nm. To achieve this resolution we found it necessary to use a very thin coat of platinum. In conventional thermionic source SEM, samples are coated with a relatively thick layer of gold (10 to 20 nm) to minimize these effects with higher beam voltages (20 keV) and to generate an adequate SE signal; however, in doing so, much of the fine surface detail (10 nm or less) is obscured. To help achieve the high-resolution detail of the samples presented in this study, a very thin (1-nm), discontinuous coat of platinum was used (28). Unfortunately, at times this thin coat is inadequate to disperse the energy generated by the primary electron beam which accumulates in these topographically complex biological samples. This could be minimized by depositing a thicker coat of platinum on the surface of the samples, but doing so would again cover up some of the fine detail. An alternative is the use of the BSE detector, which images by atomic number contrast (best for visualizing the colloidal gold) and which gives topographical *z*-contrast as well (28). The BSE method proved superior for detailed images of the cell surfaces because it is relatively independent of imaging artifacts.

As the technology in electron microscopy advances, so must the associated procedures for specimen preparation. The gyriform appearance of the cell wall suggests that shrinkage has occurred, and collapse of the cell surface structures is possible. These observations may be a result of current fixation procedures (4, 5) and become apparent with high-resolution LVSEM. Although present, these fixation artifacts are less obvious with other SEM techniques. Alternatives to these methods of fixation, such as cryo-immobilization and cryo-SEM, are currently being investigated in an effort to preserve surface proteins in a more native state. The results suggest that surface shrinkage can be eliminated (5). In addition, new methods utilizing stereoscopic imaging have been developed which allow for a more accurate means of measuring size, surface area, and extension of proteins from the cellular surface (19). Together, these methodologies may permit quantitation of cell surface proteins and allow an accurate measurement of changes related to the cell cycle.

In conclusion, the use of the isogenic *E. faecalis* strains which express well-defined combinations of surface proteins has proven to be a valuable tool in conjunction with LVSEM technology. Utilizing LVSEM, we have been able to unambiguously demonstrate the presence of Asc10 and Sec10 on the *E. faecalis* cell surface and provide information regarding their distribution on the cell surface and their interaction with other *E. faecalis* cells. The methods described should be useful tools for further topographical studies of other biological samples.

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