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Immunoelectron Microscopic Analysis of Elongation of Type 1 Fimbriae in *Escherichia coli*

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Using 10- and 20-nm-diameter gold particles conjugated to an antifimbrial monoclonal antibody, we analyzed the location of assembly of newly formed subunits on growing type 1 fimbriae of *Escherichia coli*. Fimbriae were removed from an *E. coli* K-12-derived strain, CSH50, by blending. Blended cells were allowed to regenerate their fimbriae in growth medium for approximately 25 min, after which they were labeled with a 20-nm-gold–monoclonal antibody probe. Continued outgrowth of these labeled fimbriae was allowed for additional time intervals, after which they were labeled with a 10-nm-gold–monoclonal antibody probe. The resulting fimbriae, double labeled with 10- and 20-nm-diameter gold particles, were examined in an electron microscope. The pattern of labeling on individual fimbrial organelles indicated morphologically that newly synthesized subunits are added to a growing organelle at its base.

Virtually every species of gram-negative bacteria that has been examined makes nonflagellar, filamentous appendages, or fimbriae (or pili) (3, 7). These organelles are responsible for the adherence of the bacteria to a variety of eucaryotic cell surfaces (3, 7) and facilitate bacterial colonization of host tissues. In *Escherichia coli*, the most prevalent class of fimbriae are the type 1 fimbriae, which are adhesins (3, 7) that mediate the binding of *E. coli* to specific receptors containing mannose residues (7, 24). There are 100 to 200 of these peritrichously arranged organelles located on the surface of a single *E. coli* cell. Each organelle consists of approximately 1,000 identical subunits, each with a molecular mass of approximately 17,000 daltons (2).

Essentially nothing is known regarding the mechanism by which the type 1 fimbrial subunits are assembled into an elongating organelle. For example, it is not known whether fimbriae grow from the tip out or from the base out; that is, are newly synthesized subunits incorporated at the tip or at the base of the growing organelle? Flagella, on the other hand, have been found to be assembled by the addition of newly synthesized flagellin monomers onto growing flagella at the tips of the organelles (9, 12, 13).

In this report, we describe morphologically the pattern of assembly of type 1 fimbrial subunits by labeling *E. coli* CSH50 cells (whose fimbriae were initially removed by blending and then allowed to regrow) with an antifimbria monoclonal antibody conjugated to gold particles with two different diameters. The results of this double gold labeling indicate that the type 1 fimbria assembles at the base, close to the surface of the outer membrane.

MATERIALS AND METHODS

Strains and culture conditions. *E. coli* K-12 strain CSH50 has the genotype F^- *ara* Δ(*lac-pro*) *rpsL thi* (4–6, 17). Cells from plate cultures were initially inoculated and grown statically overnight in 4 ml of minimal medium A (17) supplemented with 5 g of glucose per liter, 10 mM $MgSO_4$, 1 μg of thiamine per ml, and 20 μg of proline per ml. These cells were then inoculated into fresh minimal medium A to an optical density of 0.05 at 550 nm. The culture was then grown on a reciprocating shaker at 37°C to an optical density of 0.3 or to approximately 5×10^8 cells per ml.

Monoclonal antibody preparation. Monoclonal antibodies against purified *E. coli* CSH50 type 1 fimbriae were generated as previously described (8), with the following modification. BALB/c female mice were immunized intraperitoneally with 10 μg of pure fimbriae in complete Freund adjuvant on day 1. On day 28, mice were reimmunized with an additional 10 μg of fimbriae in incomplete Freund adjuvant. On day 42, 3 days before cell fusion, mice were given a final intravenous immunization of 10 μg of fimbriae in phosphate-buffered saline (PBS) or physiological buffer saline.

Purification of monoclonal antibodies. Antifimbria monoclonal antibodies were purified over a protein A column based on the method of Mishell and Shiigi (18). Briefly, 1 liter of hybridoma supernatant was centrifuged at 10,000 × *g* for 5 min at 4°C, adjusted to pH 8.6, and applied to a protein A column. The antibody was eluted from the washed column (0.05 M Tris–0.15 M NaCl, pH 8.6), with either 3.5 M $MgCl_2$ or 0.05 M citrate–0.02 M NaCl, pH 3.5. Citrate-eluted fractions were immediately neutralized with 400 μl of 1 M Tris (pH 7.8), and fractions containing high concentrations of antibody (optical density at 280 nm) were pooled. Salt-eluted antibodies were dialyzed for 12 to 20 h at 4°C against PBS, concentrated, and stored at −20°C until used.

ELISA. The enzyme-linked immunosorbent assay (ELISA) technique employed previously by us was used to detect and to determine the titers of antifimbria monoclonal antibodies (5, 8).

SDS-PAGE. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of both type 1 fimbriae and monomeric subunits was performed in a 1.5-mm slab gel apparatus (Hoefer Scientific Instruments, San Francisco, Calif.) based on the method of Laemmli (15), as previously described (5). Since native type 1 fimbrial organelles are typically resistant to disaggregation by SDS (2, 5, 15), these polymerized structures will not enter the separation gel of an SDS-PAGE (5, 16), whereas fimbrial subunits that are released after treatment of native organelles with hot acid or

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guanidine hydrochloride will enter the separating gel (2, 10). Therefore, before SDS-PAGE, pure fimbriae were routinely acidified with HCl (pH <2), heated to 100°C for 5 min, cooled to room temperature, and neutralized. Samples were mixed 1:1 with sample buffer (0.125 M Tris, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.02% bromophenol blue, pH 6.8), heated to 100°C for 3 min, applied to gel, and electrophoresed for approximately 4 h.

Immunoblot analysis. The specifications of the antifimbria monoclonal antibodies were determined by Western blots of intact native fimbriae (in the stacking gel) and monomeric subunits (in the separating gel) with a TransBlot apparatus (Bio-Rad Laboratories, Richmond, Calif.) by the method of Towbin et al. (26) as previously applied in our laboratory (8).

Preparation of gold-protein A conjugates. Protein A was conjugated to 10- and 20-nm-diameter gold particles by the method of Robinson et al. (23). All gold-protein A conjugates were suspended in 10 ml of stabilizing buffer and stored at 4°C until used.

Preparation of gold-protein A-monoclonal antibody probe. Antifimbria monoclonal antibody (100 μl; diluted in stabilizing buffer plus 0.1% Tween 20, pH 8.0) was conjugated to gold-protein A by the method of Robinson et al. (23). This gold-protein A-monoclonal antibody was mixed by mild agitation on a shaking platform (Bellco Biotechnology, Vineland, N.J.) for 30 min at room temperature and stored at 4°C. An anti-*E. coli* lipopolysaccharide monoclonal antibody was used in gold labeling experiments as a negative control for fimbrial labeling.

Growth and removal of fimbriae. To study the location of assembly and elongation of fimbriae on the *E. coli* CSH50 cell surface, we first removed fimbriae by shearing the cells in a blender. Chloramphenicol was also used to deplete the small pool of fimbrial subunits that exist unassembled in *E. coli* cells. Importantly, chloramphenicol, which reversibly inhibits protein synthesis, does not affect assembly of these subunits into organelles (6); consequently, at the concentration used here, chloramphenicol inhibits new subunit synthesis and depletes the small pool of subunits present in the cell. Electron microscopic examination of the chloramphenicol-treated *E. coli* CSH50 cultures showed a more uniform speed of extension of fimbrial length on individual cells as compared with that of untreated cells. Bacterial cells were grown to an optical density of 0.3, at which point 150 μg of chloramphenicol per ml was added. Incubation was continued in the presence of chloramphenicol for 30 min at 37°C, after which the cells were centrifuged at 3,000 × *g* for 6 min at 4°C and suspended in 27 ml sterile, prechilled PBS. The fimbriae were sheared from the cells by blending for 2 min at the highest speed in a Waring commercial blender (Waring Products Division Dynamics Corp. of America, New Hartford, Conn.). This blending procedure was carried out twice with 1 min of cooling in ice between blending periods. The blended cells were collected by centrifugation at 3,000 × *g* for 6 min at 4°C, washed in 7 ml of PBS, recentrifuged, and finally resuspended in 2 ml of PBS. All cells were examined for fimbriation by negative-staining electron microscopy.

Fimbrial regeneration and gold labeling. The location of subunit assembly in the growth fimbriae was studied by double gold labeling, with 10- and 20-nm-diameter gold particles. A 0.5-ml sample of blended cells was incubated in 2.5 ml of minimal medium A for 25 min at 37°C, which corresponded to regeneration of fimbriae to approximately 20% of their preblended lengths. A 0.7-ml sample of cells was centrifuged at 10,000 × *g* for 4 min at 4°C, and the pellet was washed in 100 μl of PBS, recentrifuged as before, resuspended in 140 μl of PBS, and gold labeled by incubation with 200 μl of 20-nm-diameter gold particles conjugated to an antifimbria monoclonal antibody for 20 min at 37°C with continuous rotation of the tube. Labeled cells were then centrifuged at 10,000 × *g* for 3 min at 4°C, washed with 100 μl of PBS, recentrifuged at 10,000 × *g* for 3 min, and suspended in 0.5 ml of minimal medium A. These 20-nm-gold-labeled fimbriated cells were then allowed to regenerate their fimbriae to full length by reincubation at 37°C with aeration. At various times during incubation, 130-μl samples were removed, immediately fixed with 1% (vol/vol) formaldehyde, and centrifuged at 10,000 × *g* for 3 min at 4°C. The pellet was suspended in 20 μl of PBS, and the cells were gold labeled with 30 μl of the 10-nm-diameter gold-antifimbria monoclonal antibody conjugate. These cells were incubated for 30 min at 4°C with continuous rotation of the tube. The resulting double-gold-labeled cells were centrifuged at 10,000 × *g* for 3 min at 4°C, washed with 50 μl of PBS, recentrifuged as before, suspended in 25 μl of distilled H_2O, and examined by electron microscopy.

Electron microscopy. All samples were applied to 200-mesh Formvar-coated copper specimen grids and negatively stained with 0.5% (wt/vol) NH_4MoO_4; pH 7.2). Briefly, approximately 3 μl of sample was placed on the coated grids followed by approximately 3 μl of NH_4MoO_4. These cells were stained for 30 s to 1 min, and the drops were removed from the surface of the grid by absorption with Kimwipes. The stained cells were examined in either a JEOL JEM-100CX electron microscope or a Philips 301 transmission electron microscopy, operating at 60 kV. Representative electron microscopic fields were photographed at both low and high magnification, and the extent of fimbrial outgrowth was determined by counting each cell. For each of the nine independent gold labeling experiments, several photographic fields were examined, and at least 100 separate fimbriae were characterized according to the distribution of gold label. For every field examined, all evaluable (i.e., radially projecting) fimbriae were counted.

RESULTS

Isolation and characterization of monoclonal antibodies. Culture supernatants from 630 tissue culture wells were initially screened for antifimbria antibodies by ELISA. Of these, 303 clones reacted positively with purified fimbriae coated onto 96-well microtiter plates. Fifty-nine clones giving strong positive ELISA reactions were frozen and stored in liquid nitrogen, and six hybridomas were single-cell cloned by limiting dilution and characterized further. Four of the monoclonal antibodies, FG2, FG3, FG4, and FG6, were of the immunoglobulin G1 (IgG1) isotype, while the remaining two monoclonal antibodies, FG1 and FG5, were of the IgG3 and IgG2b isotypes, respectively. Our laboratory has previously isolated and characterized an IgM monoclonal antibody that reacts only with the polymerized organelle and not with monomeric subunits (8). Three of the monoclonal antibodies, FG1, FG3, and FG6, recognized epitopes present on both the native fimbriae and on individual subunits. The remaining three monoclonal antibodies, FG2, FG4, and FG5, reacted only with epitopes present on intact fimbriae. Monoclonal antibody FG5 was used in the gold-antibody probe experiments because of its IgG2b isotype, which permits high-affinity binding to protein A (11).

Regeneration of type 1 fimbriae after blending. Electron microscopic examination of sheared *E. coli* CSH50 cells revealed approximately 99% of the cells to be devoid of fimbriae after blending (Fig. 1A and B). Fimbrial regenera-

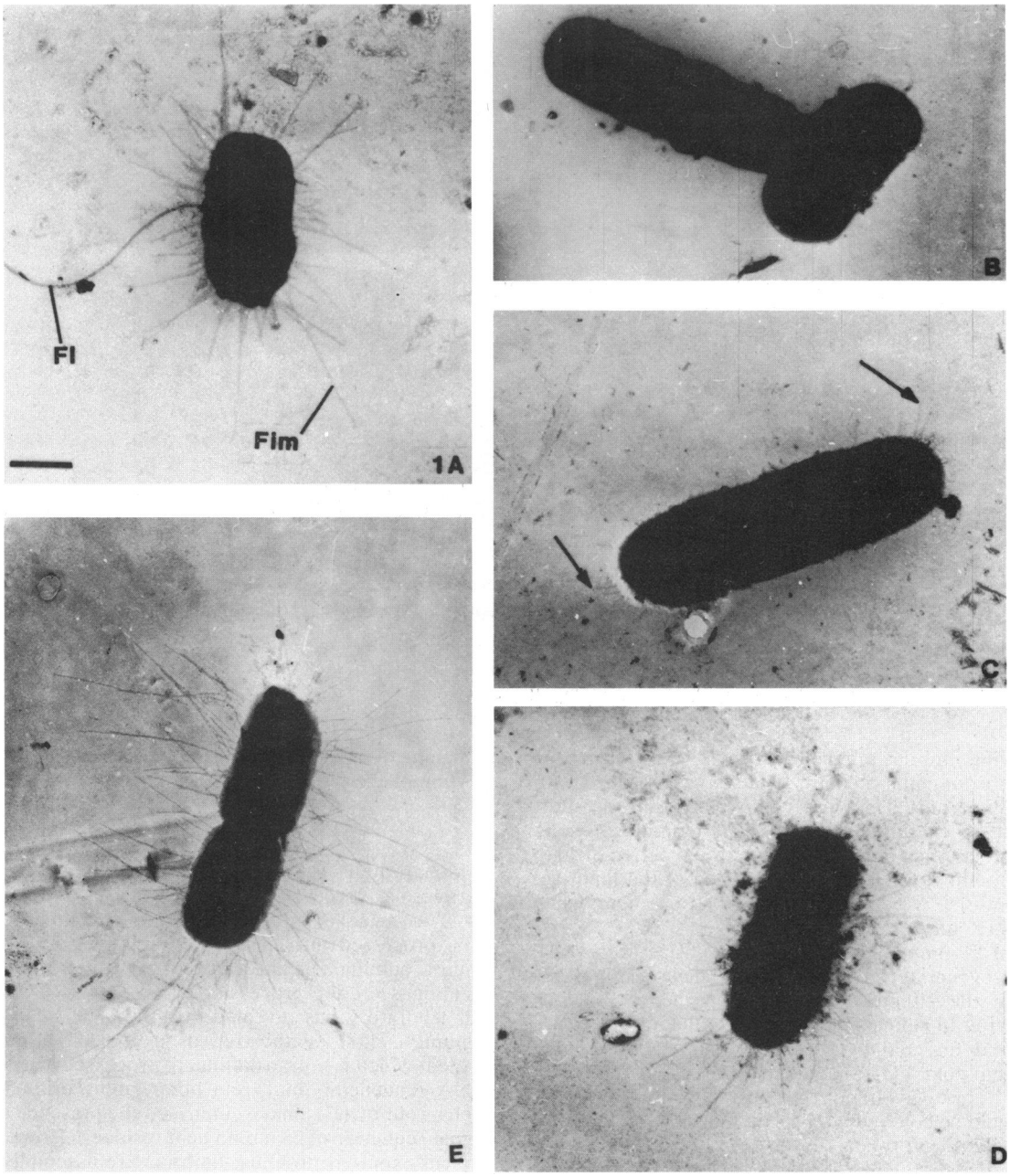


FIG. 1. Fimbrial regeneration of *E. coli* CSH50. A control cell before blending (A) contains a long flagellum (Fl) and numerous thin fimbriae (Fim) of various lengths. After blending (B) these cells contain no visible fimbriae. Panels C through E are representations of fimbrial regeneration in minimal medium at 37°C for 25 min (C), 40 min (D), and 1 h (E). Arrows in panel C indicate the beginnings of fimbrial regeneration. Cells were negatively stained with 0.5% ammonium molybdate, pH 7.2. Bar, 0.5 μm.

tion occurred with a progressive lengthening of individual organelles as a function of time (Fig. 1C to E). By 40 min (Fig. 1D), fimbriae regenerated to approximately 70% of their preblended lengths (i.e., 0.52 ± 0.13 μm), while regeneration of fimbriae to more than 100% of their preblended lengths occurred by 1 h (i.e., 1.04 ± 0.36 μm; Fig. 1E). These results are consistent with previous immunochemical (6) and ultrastructural (19) experiments on blending and regeneration of fimbriae.

Specificity of FG5-gold probes. Monoclonal antibody FG5 conjugated to 20-nm-diameter gold particles reacted specifically with the fimbriae on the *E. coli* CSH50 cells (Fig. 2). A similar binding pattern was also observed when FG5 was conjugated to 10-nm-diameter gold particles (data not shown), while an anti-*E. coli* lipopolysaccharide monoclonal antibody conjugated to 20-nm-diameter gold particles labeled the bacterial surface but not the fimbriae (data not shown).

Immunogold probing of fimbrial regeneration. Blended *E. coli* CSH50 cells were gold labeled with gold particles with two different diameters during the course of fimbrial regeneration to determine the location of assembly of the subunits

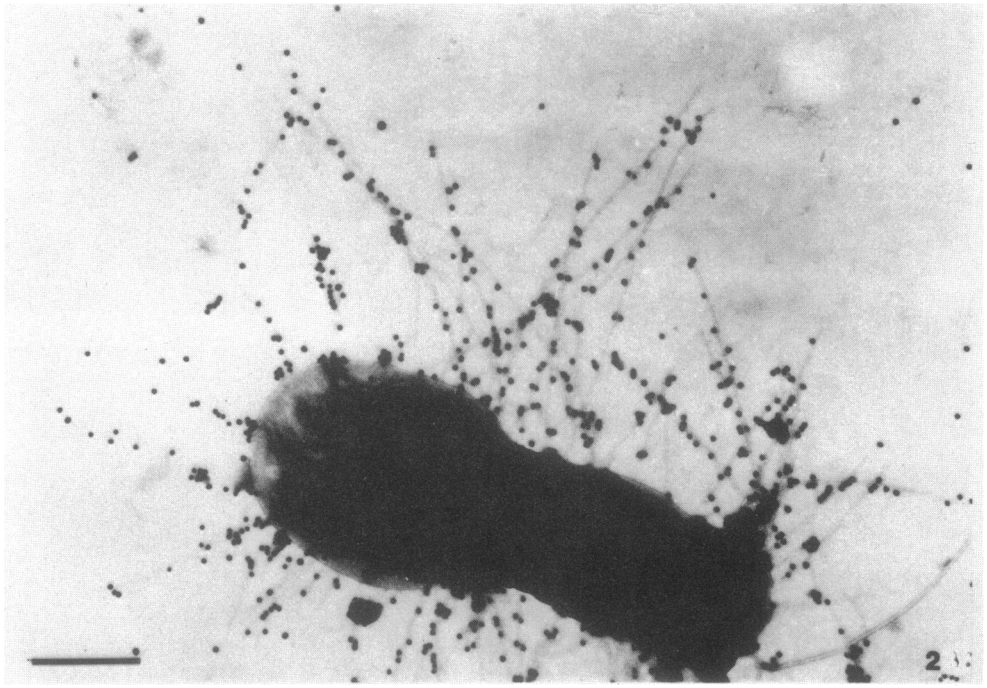


FIG. 2. Specificity of antifimbria monoclonal antibody shown by immunoelectron microscopy. Bacterial cells were labeled with monoclonal antibody FG5 conjugated to 20-nm-gold–protein A particles. Bar, 0.5 μm.

on the growing fimbrial organelle. After 25 min of fimbrial regeneration in minimal medium A, cells were probed with 20-nm-gold–FG5 particles (Fig. 3A). Virtually all the short segments of regenerated fimbriae were completely gold labeled. We found that a fimbrial regeneration period of 20 to 25 min was optimal for initial gold labeling of the fimbriae. Thus, the fimbriae were approximately 0.16 μm long when labeled, or 21% of their final length.

The initial 20-nm-gold–FG5-labeled fimbriae were then allowed to regenerate so as to expose new epitopes for binding with the 10-nm-gold–antibody probe. At various time points after blending and initial gold labeling, cells were labeled with 10-nm-gold–FG5 particles (Fig. 3B, C, D, and E). The 10-nm-gold–FG5 probe should bind only to newly synthesized epitopes since the previously synthesized epitopes should be covered after the initial gold labeling with the 20-nm-gold–FG5 probe.

We found that the double-labeling pattern of the 10- and 20-nm-diameter gold particles (Fig. 3B, C, D, and E) was reproducible in repeated fimbrial regeneration experiments, with the 10-nm-gold–FG5 particles consistently found on the proximal portions of the fimbrial organelles. The 20-nm-diameter gold particles, however, were consistently observed to be on the tip (10% distal portion away from the cell surface) of the fimbrial organelles. Although there was occasional binding of the 10-nm-diameter gold particles to the tip portions of the double-labeled fimbriae (Fig. 3B and C), this result occurred infrequently in replicate experiments (Table 1) and might be due to incomplete labeling with the 20-nm-gold–antibody probe.

To ensure that the size of the gold particle had not biased the results, we repeated the experiment but reversed the order of the two sizes of gold particles. Results were unchanged; the first label consistently was seen preferentially (75%; Table 1) at the tip, with the second label attaching to newly synthesized epitopes along the basal 10% portion of the fimbrial organelle surface.

DISCUSSION

Most proteins of *E. coli* that are ultimately localized in the periplasmic space or outer membrane are initially synthesized as signal sequence-containing precursors which are then processed into mature proteins (20, 22). The type 1 fimbrial subunit acts like a typical outer membrane protein in its initial secretion-processing pathway (4). Previous work in our laboratory has revealed that the 17,000-dalton fimbrial subunit is first synthesized as a 19,000-dalton precursor protein which is cotranslationally processed (4). Subsequent DNA sequencing data from other laboratories (14, 21), as well as our own (1), have confirmed the presence of a typical signal sequence of 23 amino acid residues. However, unlike typical exported proteins, fimbriae are assembled after the secretion step outside of the inner membrane, possibly in the periplasm, or within or on the surface of the outer mem-

TABLE 1. Frequency of location along fimbriae of 10-nm gold particles conjugated to antifimbria monoclonal antibody FG5

Sequence of double labeling	% at base[a]	% at tip[a]	% throughout fimbriae[a]
20-nm-gold–FG5, then 10-nm-gold–FG5	65 ± 9[b]	12 ± 9	23 ± 15
10-nm-gold–FG5, then 20-nm-gold–FG5	14[c]	75	11

[a] Base defined as portion of fimbriae seen within 10% of length closest to cell surface; tip, outermost 10% of visible length; throughout, labeling not confined to inner 10% or outer 10%.

[b] Mean percentage ± standard deviation (n = 8 independent labeling experiments, in which ≥100 fimbriae were counted per experiment).

[c] Only a single determination was performed to rule out labeling artifact. Several hundred fimbriae were counted.

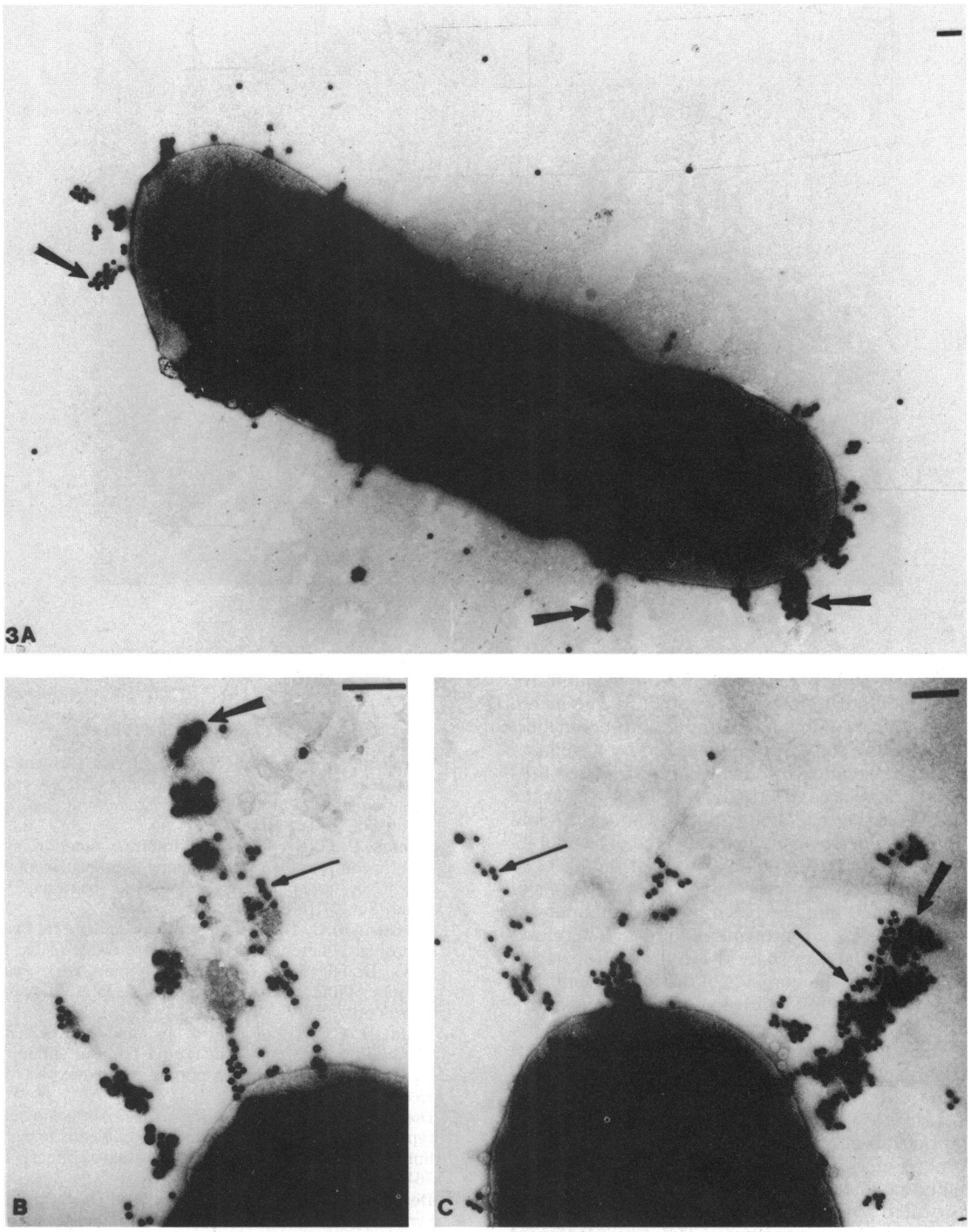
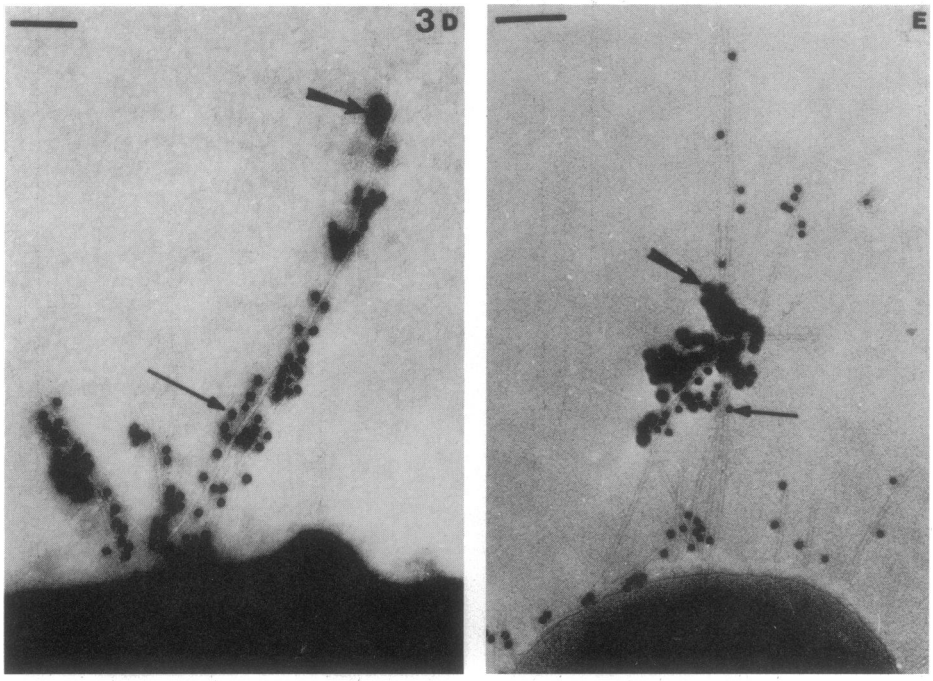


FIG. 3. Regenerating fimbriae sequentially labeled with antifimbria monoclonal antibody FG5 coupled with 20- and 10-nm-diameter gold particles. (A) First labeling with 20-nm-gold–FG5 (large arrows) after 25 min of regeneration. (B through E) Second labeling with 10-nm-gold–FG5 (small arrows) after initial 20-nm gold labeling and after an additional 15 (B), 20 (C), and 35 (D and E) min of regrowth. Bars, 0.1 μm.

brane. Except for work by Novotny et al. (19), who examined the rapidity of regeneration of F pili, flagella, and type 1 fimbrial subunits, little had been published on fimbrial assembly. It was not known where on the growing organelle the newly processed subunits were added.

To date the best studied organelle has been, and still is, the flagellum, which is also a polymer of monomeric subunits (flagellin). Unlike fimbrial subunits, though, flagellins are not synthesized first as signal sequence-containing precursors (27). Moreover, flagellar assembly occurs by the addition of newly synthesized flagellin subunits at the tips of growing organelles (9, 12, 13), most likely by transportation of subunits from the cytoplasm through the large hollow core present in the flagellar filament (13, 25). Although the type 1



fimbria also contains an axial hole, its smaller diameter (2 to 2.5 nm) makes it unlikely to be used as a conduit for transport of the larger-size fimbrial subunits (2). The mechanism by which fimbrial subunits assemble into organelles, therefore, could not be the same as that used by flagella.

Depending on the location of newly synthesized subunits on the growing fimbrial organelle (either at the base or at the tip), different double-gold-labeling patterns with the 10- and 20-nm-gold–FG5 conjugates would be expected. The double-gold-labeling pattern of 10-nm gold particles at the bases of the fimbrial organelles and 20-nm gold particles at the tips of the organelles indicated that newly synthesized subunits are assembled at the bases of growing fimbriae. Therefore, although fimbriae and flagella share the atypical property of being assembled outside the confines of the inner membrane after the secretion step, they differ markedly in their mechanisms of assembly. The monomeric fimbrial subunits are assembled at their bases after the nascent subunits have first been cotranslationally processed with removal of their signal sequences. The exact mechanism of assembly of fimbrial subunits into the growing organelle is still undetermined. Possibly, fimbrial subunits may assemble in the periplasm as dimers or trimers, etc., after which they are translocated to the outer membrane for addition to the organelle, or subunits may be translocated to the outer membrane and added on to the growing organelle as monomers. The two classes of antifimbria monoclonal antibodies, one recognizing both free subunits and assembled subunits (i.e., organelles), the other recognizing only assembled subunits, might be useful in determining the site within the membrane of subunit assembly.

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