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Outer Membrane of Gram-Negative Bacteria

XVIII. Electron Microscopic Studies on Porin Insertion Sites and Growth of Cell Surface of *Salmonella typhimurium*

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Salmonella typhimurium contains three "major proteins" or "porins" (34K, 35K, and 36K) in the outer membrane. A mutant strain producing only the 35K porin was first grown in media containing high concentrations of NaCl to "repress" the porin synthesis and then was shifted into a medium without NaCl. The newly made porin molecules were then labeled with the ferritin-coupled antibody at various times after the shift, and the samples were examined by whole-mount, freeze-etching, and thin-section electron microscopy. These experiments showed that newly inserted porins appeared as discrete patches uniformly distributed over the surface of the cell and, furthermore, that the sites of adhesion between the inner and outer membrane were most probably the pathway by which the newly made porin molecules appeared on cell surface. The 34K and 36K porins were also inserted in the same manner, since the appearance of new porins at discrete sites all over the cell surface was also observed when cells with wild-type porin phenotype were treated with unlabeled antibody to block existing antigenic sites, subsequently regrown, and labeled with the ferritin-coupled antibody. Since porins comprise a major portion of the densely packed, relatively immobile, "protein framework" of the outer membrane, these results lead us to conclude that the outer membrane grows predominantly by diffuse intercalation rather than by the zonal growth mechanism.

The gram-negative bacteria are covered by the outer membrane, which in many species constitutes the outermost structure of the cell (35). Protein content of the outer membrane is quite high, freeze-fracture electron microscopy shows a densely packed arrangement of particles in the concave fracture face (43), and treatment of intact cells with bifunctional reagents tends to cross-link most of the outer membrane proteins into a cell-sized giant complex (22). These observations suggest that proteins form a tightly packed, presumably rather immobile matrix or framework. The predominant species among these proteins are the so-called "major proteins," which often make up more than 50% of the mass of all outer membrane proteins. *Salmonella typhimurium* contains four species of these proteins, called 36K, 35K, 34K, and 33K on the basis of their apparent molecular weights (3), and the first three are also called porins, because they form transmembrane channels for the passive diffusion of nutrients and waste products across the outer membrane (32, 33). A part of each porin molecule is exposed on cell surface (25).

In this work, we studied the mode of growth of *S. typhimurium* cell surface by examining the insertion of the major proteins, or porins, into the outer membrane. The results indicate that newly made porins are inserted at "membrane adhesion areas" located all over the cell surface, and are thus incompatible with hypotheses postulating the zonal mode of growth for the outer membrane.

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MATERIALS AND METHODS

Bacterial strains and chemicals. The strains used are presented in Table 1. The mutant HN407, lacking both 34K and 36K porins, was isolated from strain HN202 (34K$^+$, 35K$^+$, 36K$^+$) as a rare spontaneous mutant by selecting for simultaneous resistance to two bacteriophages, PH51 and PH105 (gift of P. H. Mäkelä), which use the 34K and 36K porins as receptors (37). The absence of 34K and 36K porins was established by sodium dodecyl sulfate (SDS)-slab acrylamide gel electrophoresis on cell envelopes (3).

Preparation of porin. Cell envelopes were prepared from strain TA1014 (grown in Vogel-Bonner

TABLE 1. *Properties of the strains used*

Strain	Porin phenotype			LPS produced[a]	Other mutations	Reference
	34K	35K	36K			
LT2	++	++	++	S	*S. typhimurium* wild type	
TA1014	++	++	++	S	LT2 *dhuA1*	(2)
HN202	++	++	++	Rc	LT2 *galE503*	Strain M1 (18)
HN407	−	++	−	Rc	LT2 *galE503*	This paper
SH5551	++	±	−	Rb	LT2 *metA22 trpB2 fla-66 str*^{r} *metE511 rfa xyl-404*	(37)

[a] For the structure of various types of LPS, see reference 35.

medium [46] with glucose as carbon source) as described previously (3). Porins were purified from the cell envelope, also as described previously (Fig. 1 of reference 32).

Preparation of antiporin antibody. Phospholipids were isolated from HN202 as described by Ames (1). Porin-phospholipid vesicles were made as described by Nakae (33), except that lipopolysaccharide (LPS) was omitted, and water was used as the suspending medium. Rabbits were injected subcutaneously with vesicles containing 400 μg of protein. Eighteen days later, they were boosted in the same manner with vesicles containing 1 mg of porin. The rabbits were bled at 21 and 28 days after the boost. An equal amount of saturated $(NH_4)_2SO_4$ was added to the serum, and the slurry was kept at 4°C until used.

Portions of the slurry were centrifuged, and the pellet was resuspended in a small volume of 0.01 M phosphate buffer (pH 7.3) containing 0.15 M NaCl (PBS), dialyzed against this buffer, concentrated by dialysis against dry Ficoll, and then dialyzed overnight in PBS containing 3 mM NaN_3 at 4°C. For fluorescent antibody experiments, this was used without further purification.

For all ferritin antibody experiments, the immunoglobulin G (IgG) fraction was isolated by using ion-exchange chromatography (13). The eluted IgG fraction was dialyzed against dry Ficoll, and then against PBS containing NaN_3, and was kept refrigerated.

Fluorescent labeling of bacteria. Cells were grown in L broth (glucose omitted; 11) at 37°C with aeration to midexponential phase, centrifuged, washed once with PBS by centrifugation, and suspended to a concentration of 10^9 cells per ml. This suspension (1 ml) was centrifuged in a microfuge and suspended in the antiserum. After 10 min at 37°C, the cells were centrifuged down, washed twice with PBS, and then suspended in rhodamine-labeled goat anti-rabbit antibody (a gift of J. Kimura). After 10 min, the cells were centrifuged, washed twice, and suspended in PBS, and wet mounts were examined by using an Epiplan fluorescent microscope (Leitz/Opto-Metric Div. of E. Leitz Inc.).

Conjugation of antiporin antibody to ferritin (4). A typical conjugation mixture contained 50 mg of ferritin (Polysciences), 7 mg of IgG, and 0.1 M phosphate buffer (pH 6.8) in a total volume of 1.5 ml. Then 150 μl of a freshly prepared solution of 0.5% glutaraldehyde (Grade I, Sigma Chemical Co.) was slowly added, and the mixture was stirred for 75 min in a 5-ml Reactivial (Pierce Chemical Co.) at room temperature. This solution was dialyzed against 0.1 M ammonium carbonate for 2 to 3 h, and then overnight against 0.05 M phosphate buffer (pH 7.5) at 4°C. This mixture was then centrifuged and the supernatant was fractionated on a Sepharose 6B (Pharmacia Fine Chemicals, Inc.) column (1.27 by 43 cm) equilibrated with 0.05 M phosphate buffer (pH 7.5). Fractions between the exclusion volume and the unconjugated ferritin peak were pooled, concentrated by dialysis against dry Ficoll, and dialyzed overnight in PBS containing NaN_3. (It should be stressed that the pooling was done carefully so as to exclude most of the polymeric aggregates). Ferritin content was quantitated spectrophotometrically at 440 nm, assuming an extinction coefficient of 1.54 cm^{-1} ml mg^{-1}, and the solution was adjusted to a concentration of 5 to 6 mg/ml. The conjugate was kept at 4°C until use.

Several manipulations were performed on the conjugate before all labeling experiments. First, bovine serum albumin was added to a final concentration of 1 mg/ml, to reduce nonspecific "sticking" of the conjugate. The mixture was also lightly disrupted by sonic treatment to minimize aggregation. Second, for all experiments in which the conjugate was not absorbed with whole cells, it was absorbed with the appropriate type of LPS, prepared as described by Galanos (19). An amount of sonically treated LPS, equal to 10 or 20 times the amount of LPS present in the cells to be labeled, was added to the conjugate. After 1 h at room temperature, the mixture was centrifuged at 11,000 × *g* for 2 min to remove LPS. Third, tetracycline (20 μg/ml) was added. Together with NaN_3, the antibiotic prevented any growth of cells and, presumably, porin production during the labeling.

When it was necessary to have antibody directed against specific porins, the LPS treatment was omitted, and the conjugate was absorbed with whole cells of the appropriate porin-deficient phenotype. Cells equal to five times the number of cells to be later labeled were resuspended in the conjugate solution. After 1 h at room temperature, the suspension was centrifuged for 2 min in an Eppendorf model 3200 microfuge operated at 85 V. (Similar centrifugation procedures will hereafter be called "centrifugation in a voltage-regulated microfuge.") The supernatant

fraction was recentrifuged at high speed for another 2 min, and tetracycline was added. The effectiveness of this treatment was confirmed by the failure of the absorbed antibody to label the strain used for absorption.

Quantitation of the number of ferritin particles in the ferritin antibody "unit." The cross-linking of ferritin to IgG via glutaraldehyde will produce antibody species associated with "oligomers" of ferritin. Hence, it was necessary to find out what proportion of the labeling "units" contained more than one ferritin molecule. The conjugate was diluted to 20 to 50 μg of ferritin per ml with PBS, and was applied to Formvar-coated, carbon-stabilized copper grids with a spray-droplet method by using a glass nebulizer (Vapofine Co.). Ferritin treated with bovine serum albumin was used as a control. The electron-microscopic analysis indicated a Gaussian-like distribution in which 89% of the units contained three ferritin molecules or less, and 99.4% contained six or less. This technique probably exaggerates the proportion of larger units. In an untreated ferritin control, 25% of the units seen in the spray-droplet assay contained more than one ferritin molecule, whereas gel filtration yielded a single symmetrical peak with no indication of significant populations of dimers and trimers. Hence, we assume that perhaps 95% of the labeling units contained less than four ferritin molecules.

Vesicle preparation and labeling with ferritin-conjugated antibody. "Complete reconstitution" vesicles were prepared from phospholipids, LPS, and porin protein (33). Vesicles containing only phospholipid and LPS were prepared similarly, omitting the addition of protein. Vesicles containing only phospholipid and porin were prepared in the same manner as those used to immunize rabbits.

Vesicles were centrifuged in a microfuge at high speed for 4 to 6 min, and the pellet was suspended in ferritin-conjugate. After 1 h at room temperature, the suspension was centrifuged and washed 2 to 3 times with deionized water and observed in the electron microscope.

Time course of ferritin antibody labeling of HN407 after shifting from high NaCl medium to no NaCl medium. Cells were grown in 25 ml of a complex medium containing 1% yeast extract (Difco), 1% Neopeptone (Difco), and 0.5 M NaCl (YEN-NaCl) to a density of 6×10^8 to 8×10^8 cells per ml. Under these conditions, almost no 35K porin is produced (see Results). The cells were then harvested by centrifugation (2,000 × *g*, 10 min), and were washed and suspended in 0.01 M phosphate buffer (pH 7.2) containing 0.5 M NaCl and 5 mM $MgSO_4$. This suspension (3 ml) was then slowly diluted 10-fold with stirring over a period of 15 to 20 min with dropwise addition of the same buffer that did not contain NaCl. All of these manipulations were performed at room temperature. The cell suspension was then centrifuged and suspended in 25 ml of prewarmed YEN lacking NaCl and incubated at 37°C. Samples containing 1.5×10^8 cells were removed at various times, placed in microfuge tubes containing tetracycline, centrifuged at 85 V for 2 min in a voltage-regulated microfuge, and suspended in 150 μl of prepared conjugate. This mixture was incubated for 1 h at room temperature, diluted with 1 ml of PBS, and centrifuged. The pellet was washed (by suspension and centrifugation) first with water-PBS (1:1), then twice with water, and finally was suspended in 50 μl of water. Grids were then prepared for whole-mount electron microscopy.

When preparations for thin-section or freeze-etching electron microscopy were made, twice as many cells and ferritin-conjugate were used. For thin-section preparations, the cells were washed with PBS rather than with water.

Time course of ferritin-antibody labeling of HN202 after blocking with unlabeled antibody. Cells were grown in M-63 minimal medium (14) containing 0.5% glucose as carbon source to a density of 5×10^8 cells per ml. The culture was harvested by centrifugation (2000 × *g*; 10 min; all operations were carried out at room temperature unless noted otherwise), washed once, and suspended in PBS containing tetracycline (20 μg/ml) to a density of 8×10^8 cells per ml. One milliliter of cells was centrifuged at 95 V for 2 min in a voltage-regulated microfuge. The pellet was suspended in 1 ml of a solution containing antiporin IgG (5.5 mg of protein) and 20 μg of tetracycline. After 1 h of incubation at 37°C, the cells were washed twice by centrifugation with PBS, suspended in 1 ml of prewarmed M-63 glucose, and incubated at 37°C. Preliminary experiments showed that the cells began to grow with the same doubling time as the control (untreated) cells after a lag period of 4 to 5 min. Samples (250 μl each) were removed at 0, 5, 10, and 30 min and chilled, and tetracycline was immediately added. The preparations were centrifuged and suspended in 100 μl of prepared conjugate. After 10 to 15 min of incubation on ice, the mixtures were centrifuged, washed with PBS and then with water, and finally suspended in 75 μl of water. The cells were then viewed as whole mounts.

Whole-mount electron microscopy. Cells were deposited, unfixed and unstained, on Formvar-coated, carbon-stabilized grids which had been subjected to a glow discharge in a partial vacuum to render the surface somewhat more hydrophilic. Occasional lysed cells with quantities of ferritin trapped inside were ignored in all analyses.

Freeze-etching electron microscopy. Ferritin-labeled cells in deionized water were frozen on cardboard disks in Freon 22 kept partially frozen with liquid nitrogen. Care was taken to maintain the cells at room temperature or above before freezing, including warming of the forceps used to handle the disks. The frozen droplets were fractured at −100°C in a Balzers 360M apparatus and etched for 1 to 1.5 min. The surface was then shadowed with evaporated platinum and replicated with evaporated carbon. To clean the replicas, we placed them in methanol for 15 min and then transferred them to 6% sodium hypochlorite where they remained overnight.

Thin-section microscopy of plasmolyzed cells. Three methods of plasmolysis and primary fixation were used. (i) Labeled cells (3×10^8) in PBS were centrifuged and resuspended in 0.5 ml of PBS containing 20% sucrose at room temperature. After 5 min, 0.5 ml of 8% glutaraldehyde in PBS-sucrose was added.

(ii) The labeled cell pellet was suspended directly in 4% glutaraldehyde in PBS-sucrose. (iii) The labeled cell pellet was suspended in 0.5 ml of 0.1% glutaraldehyde in PBS-sucrose. After 5 min, 0.5 ml of 8% glutaraldehyde in PBS-sucrose was added.

All samples were then fixed in 4% glutaraldehyde for 2 h, and the cells were centrifuged and the supernatant fluid was completely removed. The pellet was then warmed to 45°C and quickly suspended in 5 μl of 1.5% Ionagar (Colab) in PBS-sucrose at 45°C. The mixture was chilled, removed from the microfuge tube, cut into pieces, and washed with PBS-sucrose. The cells were then postfixed for 2 h at room temperature in 1% OsO_4 in PBS-sucrose. The specimens were washed successively with PBS-sucrose, PBS, and water. They were directly dehydrated in acidified 2,2-dimethoxypropane (31). The bacteria were then infiltrated with Spurr resin (44), which was then polymerized at 70°C. Sections were cut with glass knives, mounted on uncoated 300-mesh copper grids, and viewed unstained, or were stained with 3% uranyl acetate in 50% ethanol followed by Reynold lead citrate (40).

All preparations were examined with a Siemens-Elmiskop 1A electron microscope. Micrographs were recorded on Electron Image Plates (Eastman Kodak Co.) developed for medium speed and contrast.

RESULTS

Production and purification of antibodies directed against porins. A cell envelope preparation of TA1014, containing 36K, 35K, and 34K porins in the ratio of 1:1.7:2.1 (based on the scanning of the Coomassie blue-stained gels), was used as the source for the immunogen. The porins were purified by taking advantage of the fact that only they and Braun lipoprotein remain tightly associated with the peptidoglycan when cell envelopes are treated with SDS at temperatures below 60°C (41). Under these conditions, porins are not significantly denatured (34). The SDS-insoluble complex was further treated with trypsin, which hydrolyzes Braun lipoprotein (12) as well as other contaminating proteins but does not cleave the porin (20, 23). The cleavage of Braun lipoprotein also results in the dissociation of porin oligomers from the peptidoglycan layer, that is, the porin oligomers become "solubilized" in SDS, as first shown by Nakae (32). Subsequent gel filtration in 0.1% SDS produced a preparation containing the three species of *Salmonella* porins, but little else, as judged by SDS-acrylamide gel electrophoresis (cf. reference 33).

After removal of SDS by dialysis, this porin preparation was highly aggregated, and the antigenic determinants normally present on cell surface could have been exposed rather inefficiently. Consequently, we incorporated the porins into liposome membranes containing bacterial phospholipids and used these vesicles for immunization (see Methods).

As a preliminary analysis of the antiserum, an immunofluorescent microscopy technique was used (see Methods). We found that each cell was uniformly labeled, and all cells of the population seemed labeled to the same degree. With preimmune serum, no fluorescence was observed.

The antibody caused extensive clumping of the strain with a wild-type LPS (LT2), but only a slight clumping of the strain with a defective, Rc-type LPS (HN202). This finding suggested that a significant fraction of the antibody population was directed to wild-type LPS. When the antiserum was first absorbed with the wild-type LPS, the clumping disappeared completely.

Since mutants with Rc-type LPS were used in the labeling experiments, and since ferritin was conjugated to purified IgG, whereas most of the anti-LPS activity resides in IgM (5), the interference from anti-LPS antibody was expected to be minimal. Nevertheless, we absorbed all ferritin antibody preparations with the LPS of the appropriate type (see Methods).

Effect of the ionic strength of the growth media on porin production. To label only the newly made porin molecules with antibodies, it was preferable to have a means of repressing the porin synthesis by manipulation of environmental conditions. We found accidentally that the level of 35K porin was very sensitive to the concentration of salts present in complex media. If HN202 cells were grown in YEN containing 1% NaCl or similar molarities of KCl, the 35K porin was nearly gone (not shown), and there appeared to be a compensating increase of the 34K porin, but no other visible changes. It should be emphasized that the reduction in 35K porin was not due to "solubilization" and release of this protein from the cell surface; the analysis of culture supernatants did not reveal any increase in protein bands of 35,000 daltons or less (not shown).

At this point, we realized that similar observations had been made earlier by Nakamura and Mizushima in *Escherichia coli* K-12, and that they used growth in salt-containing media to specifically repress the production of one of the porins, O-9 (34). Thus in its repression by salts, the 35K porin is quite similar to the O-9 (or 1a) porin in *E. coli* K-12.

We found, furthermore, that the reduction of 35K porin level occurs also in HN407, which is a mutant incapable of producing both 36K and 34K porins. HN407 cells were grown in YEN containing 0.5 M NaCl and were shifted to YEN without NaCl as described in Methods (Fig. 1). Right after the shift, there was no visible 35K porin band. However, the porin began to be produced immediately and reached about 30% of normal level after 40 min (track d, Fig. 1).

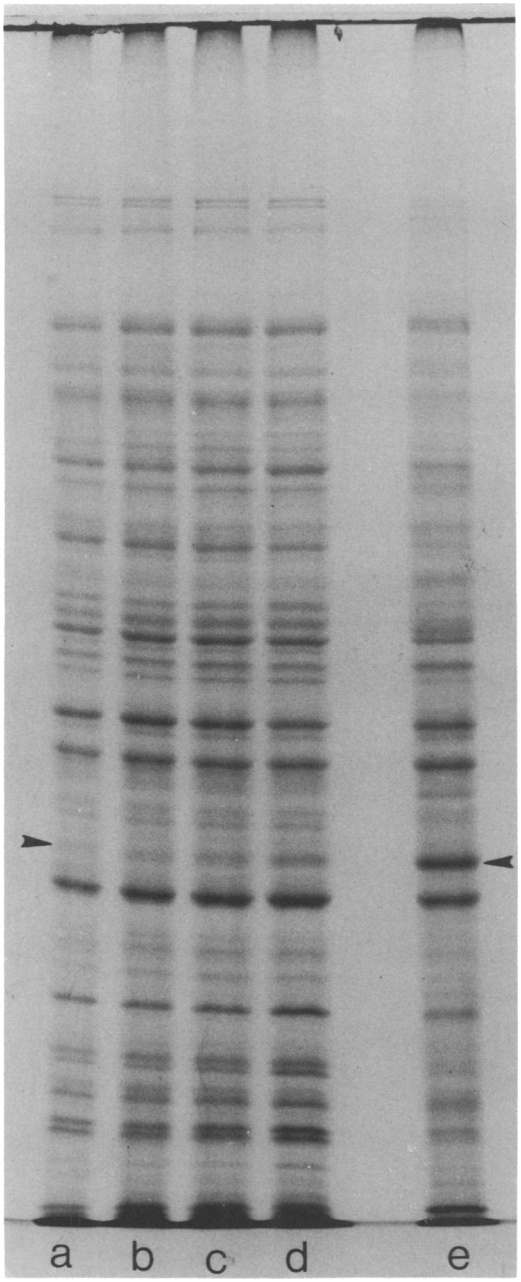

FIG. 1. *Sodium dodecyl sulfate-slab polyacrylamide gel electrophoresis (9% acrylamide) of cell envelopes of HN407 grown in YEN-NaCl and shifted into YEN. (a, b, c, d) Samples from cells 0, 10, 20, and 40 min, respectively, after the shift. (e) Control, corresponding to envelopes of cells grown in YEN for many generations. Each well received a sample containing 30 μg of protein.*

Ferritin labeling of newly synthesized porin molecules in HN407. Cells were grown in YEN containing 0.5 M NaCl and shifted into YEN without NaCl (Methods). Samples were removed at 0, 5, 10, 15, 25, and 35 min, placed in microfuge tubes containing tetracycline, and centrifuged immediately. The cells were then labeled with the ferritin-antibody conjugate and examined by the whole-mount technique.

Examination of the sample taken at zero time showed that the great majority of cells (>90%) were essentially unlabeled, containing 20 or less ferritin molecules per cell (Fig. 2b) in striking contrast to unrepressed control cells (Fig. 2a). In some cells of the zero time sample, small patches of ferritin were seen all over the surface of the cells (Fig. 2c). Since it takes several minutes to suspend the YEN-NaCl-grown cells in YEN, it seems likely that these patches correspond to the appearance of porin molecules, newly made in response to the shift to YEN.

In a control experiment, cells were grown in YEN-NaCl and shifted to YEN, except that tetracycline was present in YEN and buffers. Samples were taken before as well as 30 min after the shift. In both cases, no cells could be found with any significant number (>20 per cell) of ferritin molecules adhering to them.

Figures 2d–f chronicle the subsequent appearance of more ferritin dots on cells selected from subsequent time points and show the two most significant points. First, the newly made porins appeared on the cell surface as clusters or patches located at discrete sites. Second, these sites were distributed uniformly over the entire cell surface, and no preferential labeling of either the cell pole or septal region was seen. In addition, these patches did not seem to grow in size, but their number increased rapidly.

The emergence of newly made porins did not occur synchronously in all cells. This lack of synchrony is best understood by examining the pattern of growth after the shift from high-salt into low-salt YEN. There was a reproducible 10- to 15-min lag period, and then growth resumed at a rate comparable to cells grown in YEN (data not shown). Hence, during the lag phase, individual cells apparently began growing at various times and the time at which porin synthesis was begun also showed significant scattering.

An early time point (10 min) after media shift was also examined by freeze-etching electron microscopy (Fig. 3b). Pictures such as this confirm the whole-mount observation of discrete clusters of ferritin appearing over the entire surface of the cell. Figure 3a depicts a positive control preparation in which the HN407 cells grown in YEN were labeled with ferritin-antibody conjugate. Here where we view only one surface of the cell, one can see that the label is not distributed in a completely homogeneous manner.

Ferritin antibody labeling of strain

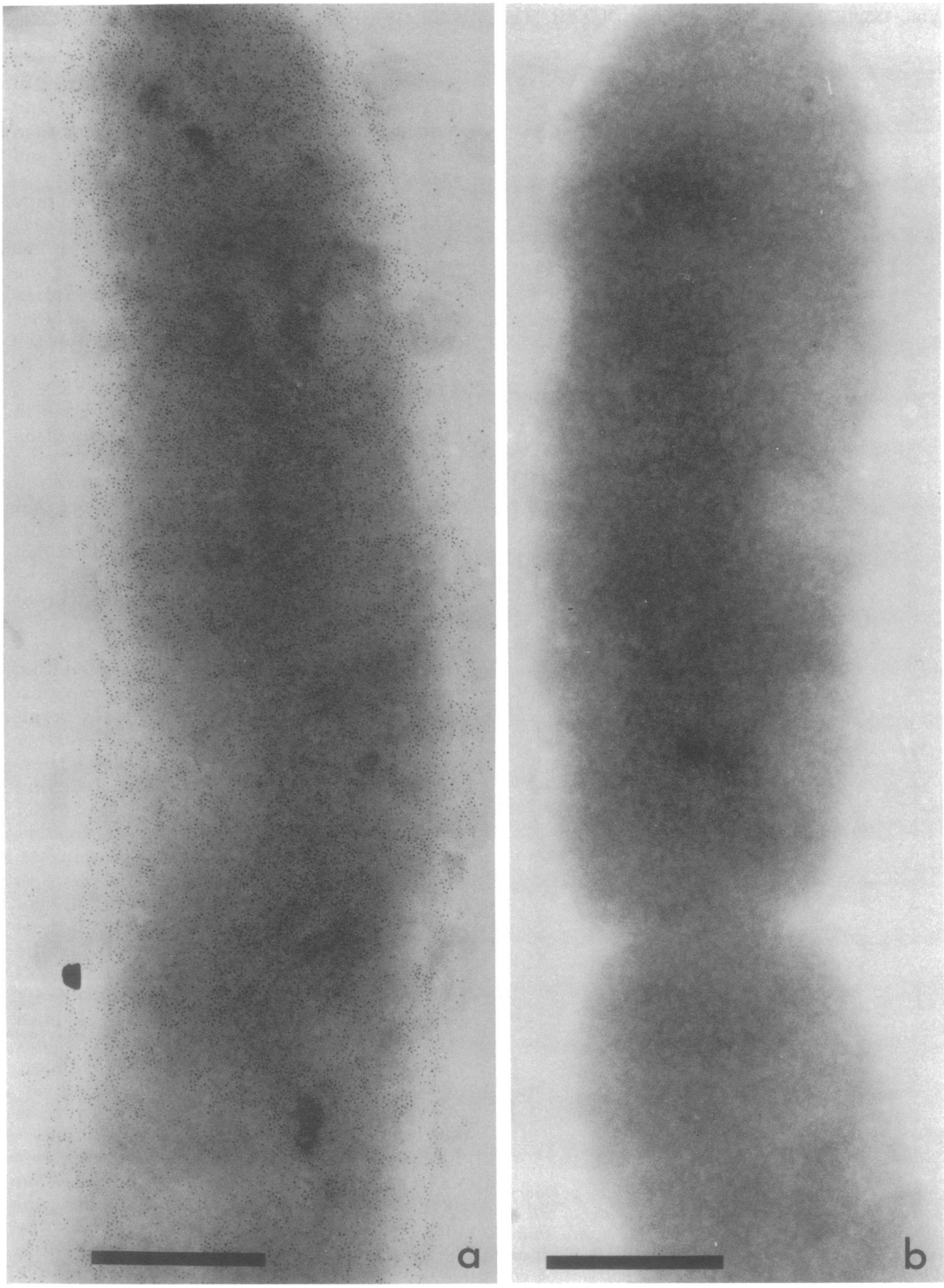

FIG. 2. *Whole mounts of HN407 cells labeled with the ferritin-antiporin conjugate. Scale markers correspond to 0.5 μm. (a) A member of the culture grown in YEN; this served as a positive control. (b, c, d, e, f) Cells grown in YEN-NaCl and then shifted into YEN; the samples were taken from the same culture 0, 0, 0, 15, and 15 min after the shift, respectively. Note that the degree of labeling is not completely homogeneous among the population at any time point. For example, at zero time, most of the cells look like (b), but there are also some cells that look like (c), and a few cells that look like (d). For details, see text.*

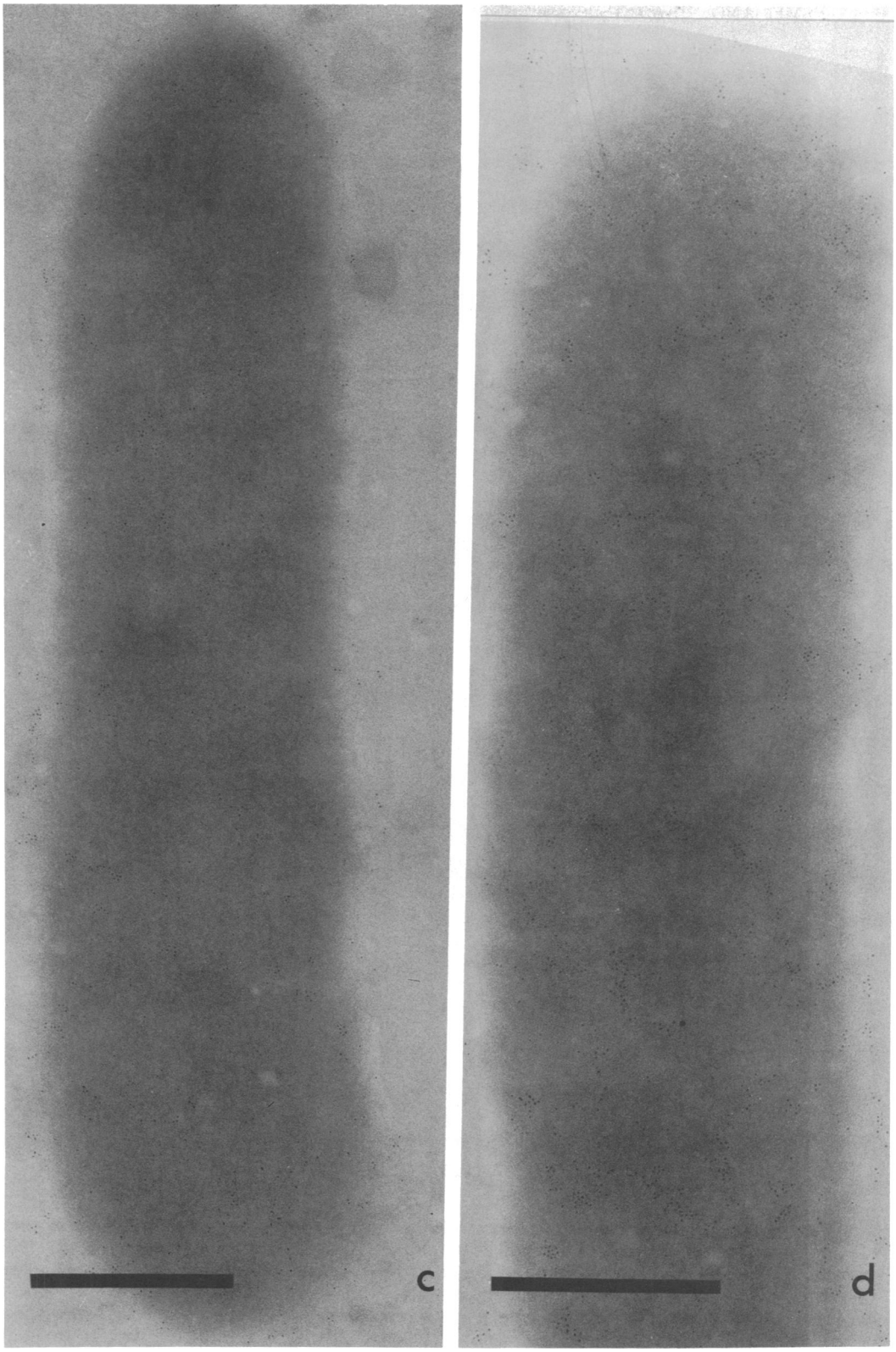

FIG. 2 c–d

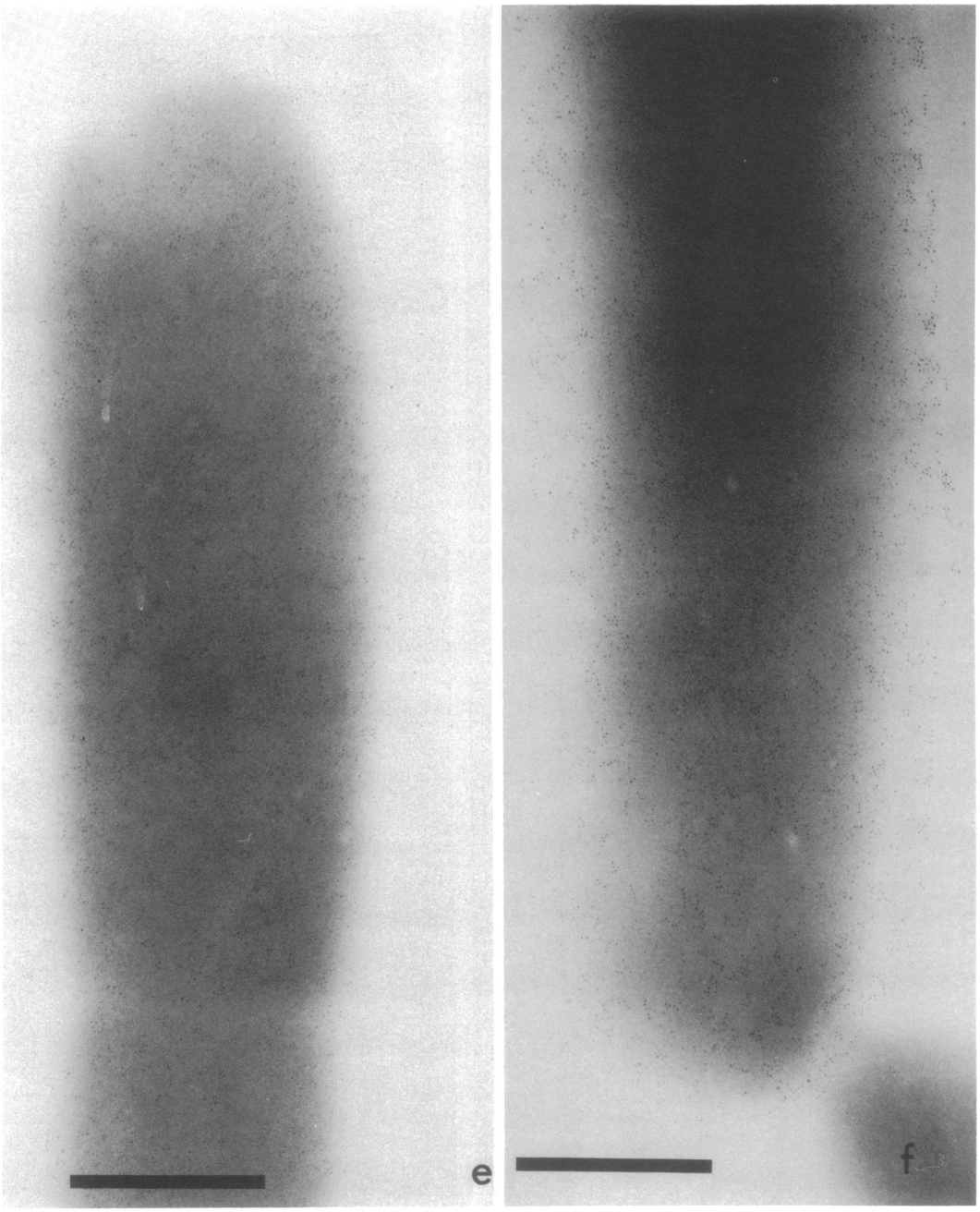

FIG. 2 e–f

HN202. To make certain that the pattern of new porin insertion observed in strain HN407 was not due to the unusual porin composition of this mutant or to the NaCl derepression procedure per se, a strain with a wild-type porin composition, HN202, was labeled after covering the preexisting porins with unlabeled antibody molecules. Exponential phase cells of HN202 were treated with unlabeled antiserum, and were then allowed to grow again (see Methods). Portions were withdrawn at various times and labeled with ferritin antibody. At zero time after

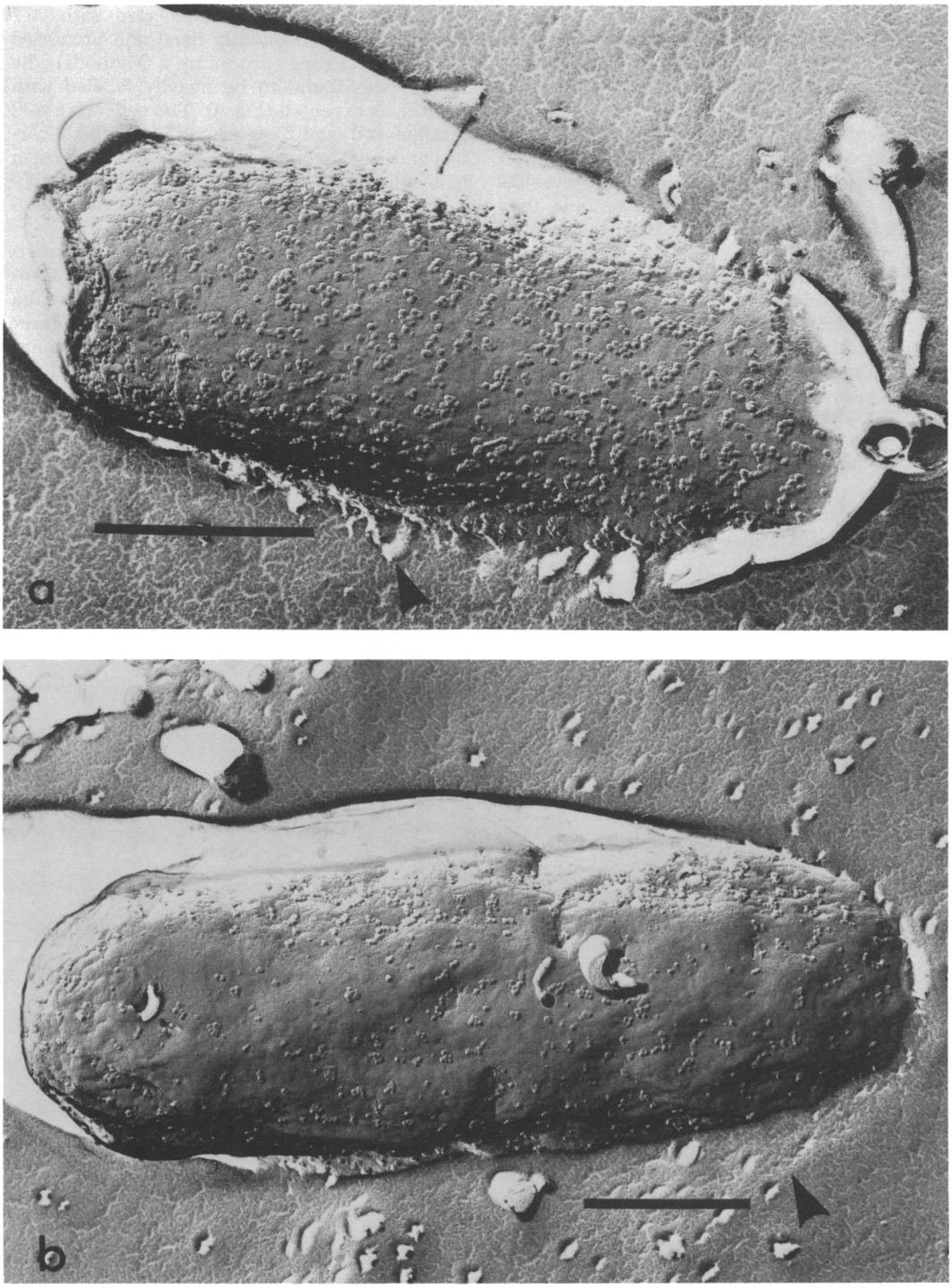

FIG. 3. *Freeze-etching micrographs of HN407 cells labeled with ferritin-antiporin conjugates. (a) A member of the culture grown in YEN. (b) A member of the culture grown in YEN-NaCl, shifted into YEN, and then grown for 10 min. Note the clustering of ferritin antibodies on cell surface in both specimens. Arrows indicate the direction of shadowing. Scale markers correspond to 0.5 μm.*

the treatment with unlabeled antibody, the degree of labeling was markedly reduced compared with the normal labeling (not shown). After 30 min of growth, many discrete clusters of ferritin could be clearly seen on the surface of some cells (not shown). These clusters were uniformly distributed over the surface of the cells. At earlier times, there was similar evidence of discrete sites in some cells, but these were fewer and they were much harder to discern from the level of background labeling. In cells "regrown" in the presence of tetracycline, no clusters could be found (not shown).

Thin-section analysis of ferritin antibody labeling of HN407 cells. The studies described above indicated that porin is inserted into the outer membrane at discrete sites. This suggests that the insertion might occur at those areas of fusion between the cytoplasmic membrane and the outer membrane, first reported by Bayer (6, 7). This possibility was examined by thin sectioning of plasmolyzed cells. When positive control cells (HN407 grown in YEN) were labeled with ferritin antibody, plasmolyzed with 20% sucrose, and subsequently fixed and processed for thin sectioning (procedure i, Methods), the surface was found to be heavily labeled with ferritin, as expected (Fig. 4). The cells were well plasmolyzed, and there seemed to be little correspondence between the location of ferritin molecules and that of the "strands," presumably membrane material, connecting the two membranes.

However, neither this procedure (procedure i) nor the simultaneous plasmolysis fixation (procedure ii, Methods) produced adequate results with media-shifted HN407 cells, the former causing the apparent "sucking-in" of ferritin antibody into periplasmic space (Fig. 5a), and the latter failing to produce any plasmolysis (Fig. 5b).

Adequate results with media-shifted HN407 cells were finally obtained by plasmolyzing them in dilute glutaraldehyde (procedure iii, Methods). Figures 5c and 5d show cells labeled 15 and 30 min, respectively, after shifting from the high-

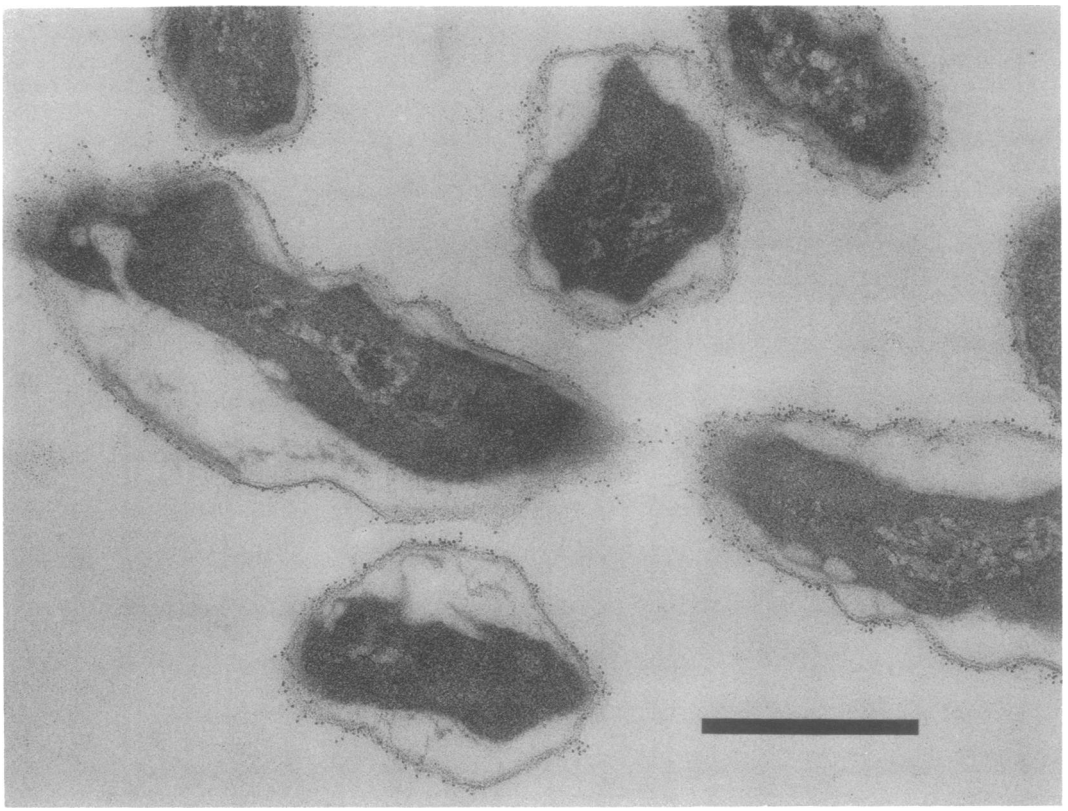

FIG. 4. *Thin-section micrograph of HN407 cells grown in YEN, labeled and prepared by method i (see text). One can see a moderate amount of ferritin on the membrane, exterior to the cell. There appears to be no correlation between the location of ferritin and the location of the adhesion or fusion sites of the inner and outer membranes. There is also no indication of ferritin in the periplasmic space created by the sucrose plasmolysis. Scale marker corresponds to 0.5 μm.*

salt to the low-salt medium. Ferritin molecules appear to be attached to the membrane in areas in which the outer and inner membranes are connected (arrows). Furthermore, although it was impossible to discern this relationship in some areas due to insufficient plasmolysis, there were very few instances in which ferritin was attached to the membrane at sites where fusion regions clearly did not exist. Our conclusion, then, is that porin was inserted into the membrane at these fusion regions and did not rapidly diffuse away laterally from these sites.

Labeling of 34K and 36K porins. Similar results obtained with strain HN407 and HN202 suggested that the 34K and 36K porins present in the latter strain were also inserted into the outer membrane in the same manner as the 35K porin of HN407. However, this conclusion is based on the assumption that the antibody preparation we used contained those specific for 34K and 36K porins as well. This was tested as follows. The 34K porin in HN202 was labeled with the ferritin conjugate absorbed with SH5551 ($36K^+$) and HN407 ($35K^+$; see Methods). To visualize the labeling of only the 36K porin, SH5551 was treated with the conjugate absorbed with HN407 cells. In both cases, ferritin was bound to cells in significant numbers. This indicated that not only was there activity to all three porins, but their antigenic determinants were sufficiently different so that a significant quantity of antibody specific to each porin remained after absorption by the other porins.

Ferritin antibody labeling of vesicles. Labeling of phospholipid-LPS vesicles with a ferritin-preimmune serum conjugate yielded almost no ferritin molecules on the vesicles (not shown). In contrast, phospholipid or phospholipid-LPS vesicles containing porin were heavily labeled with ferritin antibody (not shown).

Other labeling experiments. Attempts were made to label *Salmonella* cells with wild-type LPS structure (LT2). After absorption of serum with wild-type LPS, little labeling was observed; presumably, the long O chain polysaccharide prevented the access of antibody to the porins. Thus, for all labeling experiments described above, we used strains with a "rough" LPS phenotype.

Since *Salmonella* and *E. coli* are closely related, and several analogies have been made concerning their porins, an attempt was made to label *E. coli* cells with ferritin-conjugated antibody against *Salmonella* porins. Neither *E. coli* B nor K-12 could be labeled, although both contained rough LPS (38, 39).

DISCUSSION

Whole-mount and freeze-etching observations. The most significant observations obtained with HN407 were that (i) the porin molecules were inserted at discrete sites, and (ii) these sites were distributed uniformly over the cell surface. There was no preferential labeling of either the central zone or the pole of the cell. These observations are not artifacts due to the NaCl derepression procedure or the absence of 34K and 36K porins in HN407, since antibody-blocking experiments with the "wild-type" HN202 also gave very similar results.

The diffuse intercalation process observed is not likely to be a consequence of lateral diffusion after the localized insertion in the cell pole or the septal region. First, since porins span the membrane (25, 33, 41), a large portion of these molecules must be embedded in LPS, which constitutes a medium of low fluidity (36). Indeed, LPS in the outer membrane is known to have a lateral diffusion coefficient (D) of around 3×10^{-13} cm^2/s at 37°C, about 10^4 times lower than that of phospholipids in "typical" biological membranes (30). Even if the porin molecules had mobility similar to LPS, the average time (t) needed for them to travel the distance (x) of 1 μm, calculated from the relation $x^2/2t \cong D$ (27), would be of the order of 10 h, whereas our experiment lasted only for 30 min. Second, in the HN202 experiment the lateral diffusion of porins must have been slowed down further by performing the antibody labeling at 0°C and by the presence of the full complement of outer membrane proteins, which are known to be packed very densely (22, 43).

In the HN202 labeling experiment, it may be argued that the ferritin antibody might be merely labeling old porins from which the blocking antibody has dissociated, rather than newly inserted porins. This possibility can be ruled out by the result of an experiment showing that the number of ferritin antibody-labeled sites does not increase during incubation in the presence of tetracycline (see Results). It should also be recalled that experiments with similar "antibody-blocked" cells have been successfully performed with gram-positive bacteria without any difficulty arising from the dissociation of blocking antibodies (16, 24).

Freeze-etching electron microscopy of cells labeled shortly after the osmotic shift confirmed the results of the whole-mount technique (Fig. 3b). This technique further permits visualization of ferritin attached to only one side of the bacterial surface. When a positive control (cells with unrepressed porin) was examined, many clusters, instead of ferritin molecules spaced at approximately equal distances apart, were seen (Fig. 3a). Thus, possibly even under normal growing conditions, the porin molecules simply

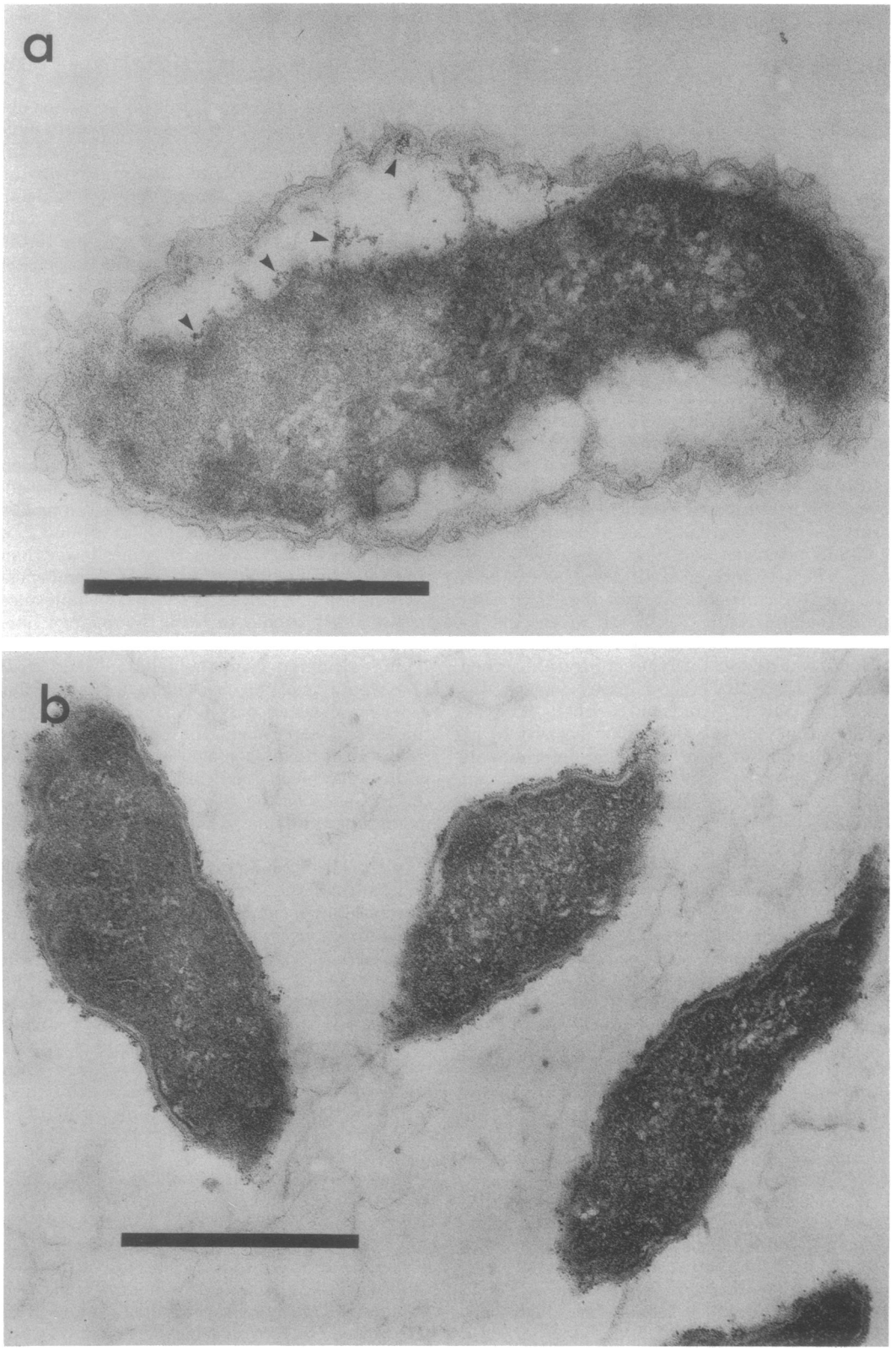

FIG. 5. *Thin-section micrographs of HN407 cells grown in YEN-NaCl, shifted to YEN, regrown, and labeled with the ferritin-conjugated antibody. (a, b) Cells were plasmolyzed and fixed by methods i and ii, respectively; (c, d) cells were plasmolyzed and fixed by method iii, as described in the text. (a, b) Cells grown for 20 min after the shift. (c, d) Cells grown for 15 and 30 min, respectively, after the shift. Arrows in (a) indicate ferritin molecules in the periplasmic space. Arrows in (c) and (d) show ferritin molecules located over the sites of adhesion of the outer and inner membranes. Scale markers correspond to 0.5 μm.*

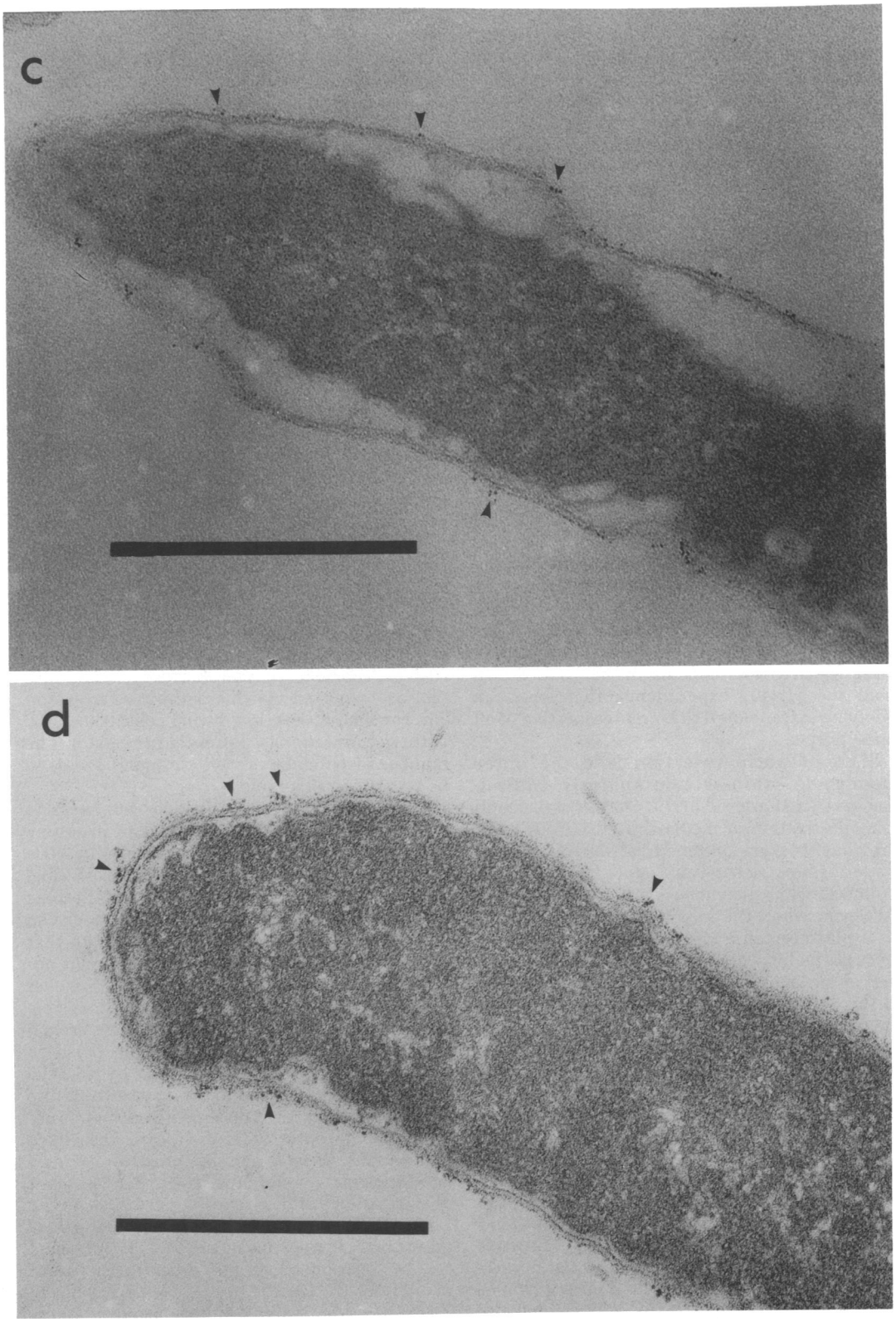

FIG. 5 c–d

do not diffuse any significant distance away from their site of insertion.

However, there are alternative interpretations of these results. For example, it could be that ferritin antibody oligomers, by virtue of having more antibody attached, have a higher avidity for the surface of the cell. But the proportion of higher oligomers with more than three ferritin molecules was perhaps 5% of the number of units in the ferritin-antibody preparation used (see Methods). Most clusters of particles in Fig. 3a, however, contained far more than 3 or 4 particles, an observation inconsistent with the interpretation described above. Furthermore, antibody-induced aggregation of porins seems unlikely since similar clusters were seen in experiments with strain HN202, in which antibody labeling was performed at 0°C, a temperature known to retard the antibody-induced, artifactual "patching" of membrane proteins even in the highly fluid lymphocyte membrane (17).

Labeling studies of strain HN202, containing all three porins, gave results similar to the results obtained with HN407. Since the antibody population was shown to contain antibodies specific to 34K and 36K porins as well (see Results), what was visualized in the HN202 experiments was the appearance of newly made porins of all three species. The conclusion we have drawn from the HN407 experiments, therefore, can safely be extrapolated to be representative of all three porins.

Sites of porin insertion into the outer membrane—thin-section analysis. Difficulties were encountered in the process of plasmolysis and fixation of media-shifted HN407 cells. Previous workers obtained satisfactory results by performing plasmolysis before fixation (6), or a simultaneous plasmolysis and fixation (8, 29). However, when the media-shifted HN407 cells were plasmolyzed and then fixed (procedure i), most of the ferritin molecules disappeared from the cell surface (Fig. 5a). This is possibly because in the cells with newly inserted porins, which are not yet firmly attached to peptidoglycan, the regions of the outer membrane with ferritin-labeled porins get pulled into the periplasm by the plasmolysis-induced stretching of the adhesion regions.

When the media-shifted cells were plasmolyzed and fixed with 4% glutaraldehyde simultaneously (procedure ii), no plasmolysis was seen (Fig. 5b). Since glutaraldehyde destroys the permeability barrier of biological membranes (28), procedure ii produces a "race" between plasmolysis mediated by the differential permeability of the membranes and the destruction of these barrier properties by glutaraldehyde. Thus, it is possible that in cells with few porins the rate of sucrose influx is slow enough so that the glutaraldehyde-induced destruction of membrane permeability wins the race, and no visible plasmolysis occurs.

Thus, a new condition for plasmolysis fixation using a lower concentration of glutaraldehyde was devised (procedure iii, Methods), and with this method it was shown that the newly made porins were exported at sites of fusion between the outer and inner membranes (Fig. 5c and d). Even 30 min after the osmotic shift, most of the ferritin still appeared over the fusion regions (Fig. 5d). Eventually, however, this relationship was terminated, since in fully labeled (unrepressed) cells there was a lack of close correlation between the location of ferritin on the surface and the location of the fusion regions (Fig. 5a).

Mode of outer membrane growth in enteric bacteria. We shall now briefly discuss our main conclusions in relation to the results of other workers.

(i) Porin insertion in the regions of membrane adhesion. The observation that porins are inserted at the regions of membrane adhesion is consistent with the results of Bayer and co-workers, as well as Mühlradt et al., who have demonstrated that outer membrane components such as "capsular" polysaccharides (35) and LPS (29) are exported via this pathway. Bayer and Thurow found that, in growing cells, 200 to 400 of these connections are present per cell (8). The number of patchlike arrays we found seems to be roughly in this range.

The newly inserted porin does not seem to diffuse away rapidly from the site of insertion, but rather to create a patch that may be little larger than the adhesion zone underneath (Fig. 3a and 5d). In view of the fact that porins become noncovalently associated with the peptidoglycan layer (41, 47), it is tempting to speculate that this interaction is involved in the apparent immobilization of newly exported porins. Whatever the underlying mechanism, it seems plausible that the adhesion zones may have to be broken or moved laterally to allow for the porin to assume the function of providing a hydrophilic channel through the outer membrane to the periplasm. Thus, the continued insertion of porins into the outer membrane over the entire surface may be a result of formation of new adhesions and possibly the lateral displacement of existing adhesions.

(ii) Diffuse intercalation versus zonal growth. Several studies have been published on the location of growth zones in the outer membrane (9, 10, 15, 16, 21, 26, 45). Some of them, however, suffer from the use of markers with significant lateral mobility, such as phos-

pholipids, and will not be discussed further. In contrast, Begg and Donachie (10) examined the distribution of a presumably less mobile marker, T6 receptor protein, in a strain that was temperature sensitive for its synthesis and concluded as follows. (i) Newly synthesized T6 receptors are inserted uniformly all over the cell surface. (ii) If cells are shifted from a permissive to a nonpermissive temperature, one or both of the cell poles becomes devoid of the T6 receptor; this is interpreted to mean that the framework or the matrix of the outer membrane is being added only at the pole(s), thus pushing the preexisting "old membrane" area (containing the T6 receptors) to the central region.

The immobile framework postulated by Begg and Donachie is probably made up of a closely packed protein matrix, close to 50% of which corresponds to porins. The Begg-Donachie hypothesis thus predicts that the newly made porins will get inserted only at cell poles, and our results are thus at variance with this prediction. In view of this discrepancy, we have carefully looked for cells with heavy labeling at cell poles, but could not find even a single case among many hundreds examined after "derepression" (HN407) or "antibody blocking" (HN202). We believe that the conclusions of Begg and Donachie are incorrect. A major problem in their study is that there is no strong evidence that newly synthesized T6 receptors are inserted at all at the poles. Under all conditions, there were fewer T6 receptors at cell poles (10); this might indeed be due to the dilution caused by the polar synthesis of the framework, but it could equally well be due to the failure of insertion of T6 receptors into the polar regions. If the latter mechanism is correct, it is obviously impossible to extract any information on the site of framework growth from the absence of the receptor in cell poles.

Whatever the nature of the deficiency in the experimental setup of Begg and Donachie, our approach clearly produces more reliable information on the location of framework growth, since we followed directly the insertion of the most predominant, or major, protein of the outer membrane matrix, whereas in the Begg-Donachie experiment the mode of addition of matrix was deduced indirectly from the behavior of a minor protein marker. Our results thus provide very strong evidence that the cell surface or outer membrane framework of *Salmonella*, and most probably *E. coli*, grows predominantly by diffuse intercalation occurring all over the cell surface.

The same mode of insertion was also found for LPS (9, 15). Such studies have often been criticized on the basis of the possible lateral mobility of LPS. However, we now know that the diffusion of LPS across the length of the cell is likely to take a much longer time than the doubling time of *E. coli* or *Salmonella* (see above). The conclusions of earlier workers (9, 15, 16) are thus probably correct, and indeed Mühlradt et al. (29) have shown directly the insertion of newly made LPS at sites distributed all over the cell surface by making observations only a few minutes after the shift into the permissive conditions.

It thus appears quite certain that two of the major components of the outer membrane, porins and LPS, are added to the cell surface by the diffuse intercalation process. In contrast to this predominant mode of outer membrane growth, however, another outer membrane protein, λ-receptor, is known to be preferentially inserted into the septal regions (42). Possibly this is related to the fact that this protein becomes synthesized only during a short period before cell division (42; M. Ohki and H. Nikaido, manuscript in preparation), in contrast to most other outer membrane proteins (including porins) that are synthesized and inserted throughout the cell cycle.

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