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Changes in the Organization of the Outer Membrane of *Proteus mirabilis* During Swarming: Freeze-Fracture Structure and Membrane Fluidity Analysis

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Freeze-fracture studies of short, nonswarming *Proteus mirabilis* revealed the characteristic gram-negative profile of fractured inner membrane with densely packed particles and sectioned outer membrane with little or no fracture plane. Long swarming cells, however, fractured easily along both the inner membrane and a second membrane, probably the outer membrane. The inner membrane had a typical profile, whereas the outer membrane had fewer but more prominent particles. Isolation and purification of the inner and outer membranes of the short and long bacteria and examination of them with electron paramagnetic resonance measurements after spinlabeling supported the above observations. The outer membrane of swarmer cells allowed higher mobility of the spin label than did the outer membrane of the nonswarming short cells, which showed a typical rigid profile. These results suggest that regions of lipid bilayer appear in the outer membrane during swarmer formation. Previous observations of the behavior and biochemistry of *P. mirabilis* during swarming are discussed in light of these results.

When grown on many solid nutrient media, some *Proteus* species undergo an unusual cyclical morphology change. The normally short, sparsely flagellate bacteria at the edge of the growing colony develop into filamentous, highly flagellate organisms able to travel through the surface moisture of the agar in groups or rafts. After traveling about 1 cm in about 2 h, these swarmers slow down, eventually stopping and dividing into short, sparsely flagellate bacteria, which then grow and divide normally for a few hours before the swarm cycle is repeated (14).

Evidence has been accumulated that swarmers are in many ways different from their short parent cells. They are more vulnerable to hydrophobic antimicrobial agents (2) and have reduced synthetic metabolism (1) when compared with short cells.

The results of freeze-fracture electron microscopy and spin-label electron paramagnetic resonance (EPR) presented here suggest that there is physical reorganization of the outer membrane, creating areas of lipid bilayer and probably involving the need for an increase in long-chain lipopolysaccharides (LPSs) to stabilize the large numbers of flagella.

MATERIALS AND METHODS

Organism and growth conditions. The organism used throughout this study was *Proteus mirabilis* (designated P11), an isolate from University College Hospital, London.

Nonswarming bacteria were grown on Oxoid Nutrient Broth no. 2 with shaking to exponential phase. Swarmers were isolated during an active swarm phase from 25-cm square culture plates, each containing 250 ml of Oxoid nutrient broth no. 2 solidified with 1.75% agar (Difco Laboratories), and each plate was inoculated with eight separate colonies as previously described [1].

Freeze-fracturing. Actively swarming *P. mirabilis* and nonswarming bacteria were isolated into distilled water, washed, and pelleted. The pellets were fixed in 5% glutaraldehyde in 125 mM sodium cacodylate at pH 7.4 for 3 h before final immersion in 25% glycerol. Blocks of the pellets were frozen in liquid Freon 22 and fractured on a Balzers BAF 300 freeze-fracture apparatus at −100°C. The blocks were shadowed with carbon platinum at 45° succeeded by carbon at 90°. The replicas were examined on a Philips 300 electron microscope after digestion of the pellet with sodium hypochlorite (5).

Isolation of the inner and outer membranes. To avoid the confusion of overlapping spin-label signals from the inner and outer membrane of the bacteria, the two membranes were separated and purified by gentle cell lysis and sucrose-density gradient fractionation as previously described (10).

After removal of the sucrose by repeated high-speed centrifugation and identification of the membrane bands by the presence of ketodeoxyoconate for outer membrane fractions and cytochrome *b* in the inner membrane fractions (11), the membrane samples were

resuspended in 0.25 M NaCl to 2.5 μg of protein per ml. The membrane preparations were incubated (0.2 ml) with 0.5 ml of 2.5 mM 2-(10-carboxydecyl)-2-hexyl-4,4-dimethyl-3-oxaxolidinyloxyl methyl ester (5-doxylstearate) (Syva Co., Palo Alto, Calif.) in 5% BSA Fraction V for 5 min at 4°C before being washed three times and resuspended in 0.25 M NaCl by the method of Rottem et al. (12).

Samples were drawn into capillary tubes and examined in a Joel FEIX EPR spectrometer with the temperature maintained at 25°C. The molecular motion is reported as $2T_{11}$ (the hyperfine splitting), which has been previously reported to relate to the motion of the nitroxide radical (12). The smaller the values of $2T_{11}$, the greater the freedom of motion of the spin label.

RESULTS

EPR spectrometry. EPR spectra from isolated inner and outer membranes of long and short cells of *P. mirabilis* labeled with 5-doxylstearate are shown in Fig. 1. The spectra of the inner membrane of swarmers and nonswarmers are virtually identical with a relatively fluid profile, the hyperfine splitting $2T_{11}$ of the short and long cell membranes being 56G and 57G, respectively.

These results are similar to those described by Rottem et al (12). The spectra of the spin label in the isolated outer membrane of the long swarmer and short nonswarmer did, however, differ significantly. As expected, both gave a more rigid profile in comparison to the inner membrane spectra, but the outer membranes from the swarmers had significantly more fluid characteristics than did the nonswarmers. The hyperfine splitting of the swarmer outer membrane profile was 58G, whereas the nonswarmer outer membranes gave 60.6G, in agreement with previous work in broth-grown *P. mirabilis*. These results suggest that the spin label had some freedom of motion in the outer membrane of swarmer cells, and therefore there were probably regions of bilayer formed on swarming.

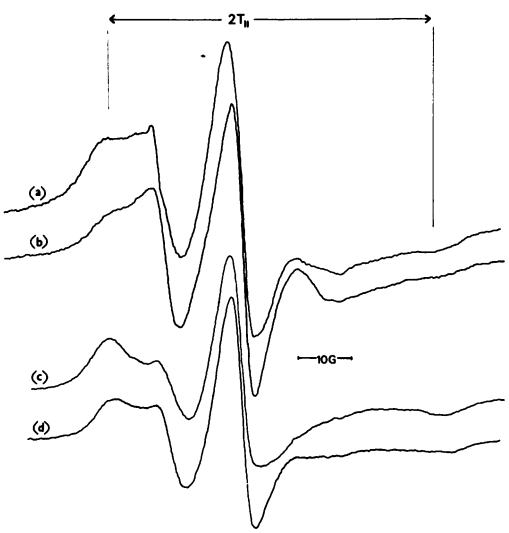

FIG. 1. Electron paramagnetic resonance spectra of isolated membranes of *P. mirabilis* labeled with 5-doxylstearate at 25°C. (a) Inner membrane from swarming cells; (b) inner membrane from nonswarming cells; (c) outer membrane from nonswarming cells; (d) outer membrane from swarming cells. $2T_{11}$ is the measure of the hyperfine splitting.

FIG. 2. Replicas of freeze-fractured whole cells of nonswarming *P. mirabilis* showing the fractured inner membrane (IM) and cleaved outer membrane (OM). The bar represents 0.5 μm.

Freeze-fracture. After freeze-fracture, short *P. mirabilis* had the typical gram-negative bacterial appearance. The outer membrane was sectioned but not cleaved in any of the preparations examined, whereas the inner membrane (IM on Fig. 2) showed the typical appearance of densely packed particles on one (convex) face and more sparsely arranged particles on the other concave face of the fractured membrane. The halo appearance (OM on Fig. 2) around the short cells was probably the result of sectioning through the outer membrane.

Figure 3 shows swarmers prepared and fractured in the same way as were nonswarming cells. These bacteria have been fractured along both the inner and the outer membranes. The inner membrane has a similar appearance to that of the nonswarmer, short cells with random,

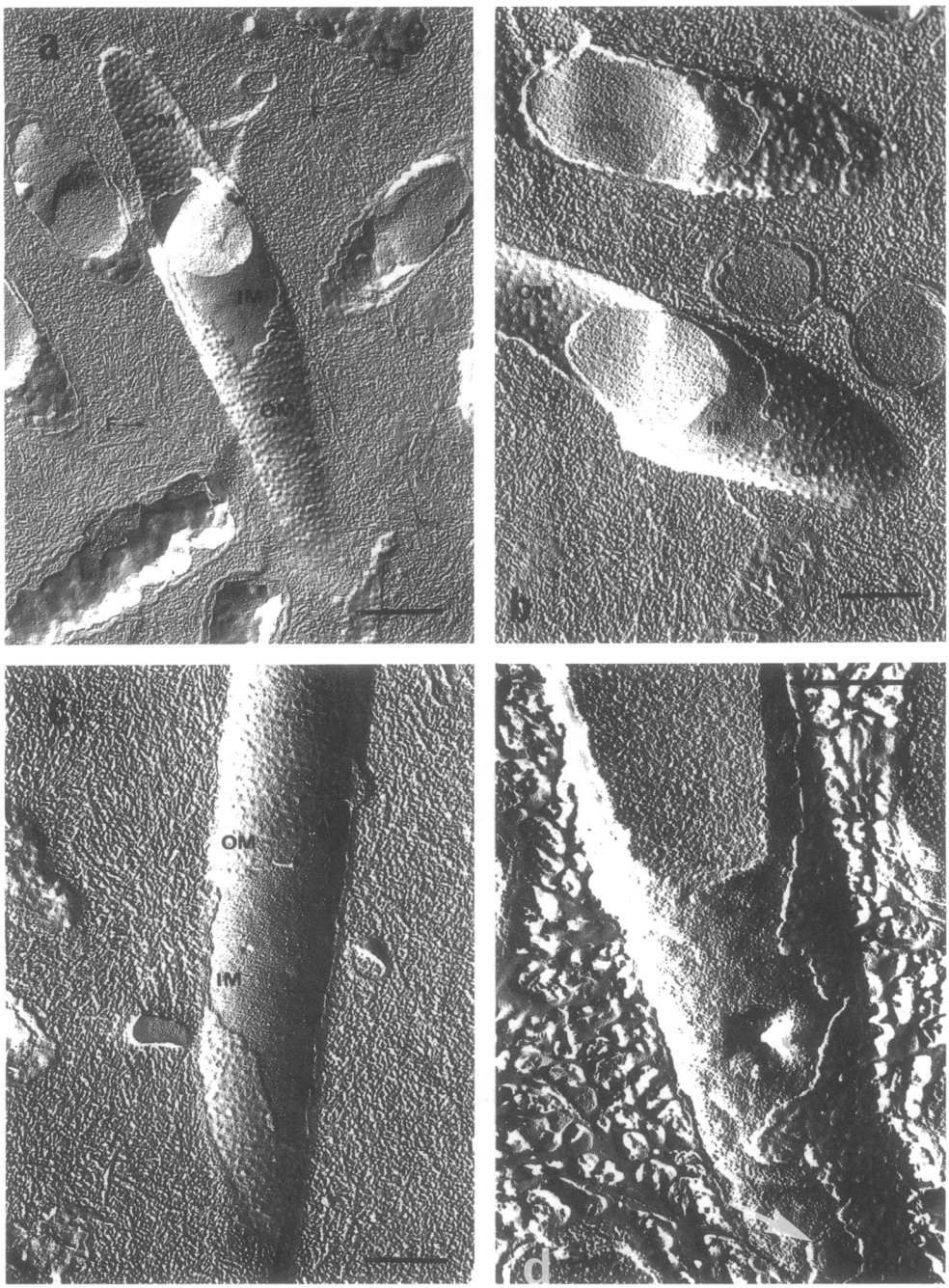

FIG. 3. Freeze-fractured whole swarming cells of *P. mirabilis*. All sections show fractured inner membrane (IM) and outer membrane (OM). Fig. 3a and b also show fractured flagella bundles (F), and Fig. 3d shows the outer surface of the inner membrane (arrow). The bar represents 0.5 μm.

densely packed, small particles on the convex face and sparsely arranged, small particles on the concave face. The other membrane, presumably the outer membrane, had also cleaved, resulting in most cells having at least three different profiles. In many fractures both sides of the outer membrane were revealed. The convex face shows small particles evenly arranged and much larger, apparently raised regions, whereas the concave face has few particles but

large indentations. In some cases a shelf can be seen beween the inner and outer membrane cleaved planes (Fig. 3d), which may be a short cleavage along the outer surface of the inner membrane.

The appearance of the background in the fracture of swarmer cells was most probably caused by the very large number of flagella on swarmer cells, 50 per unit cell wall area as opposed to 1 per unit area of short cells. In some fractures flagella bundles are clearly visible (Fig. 3a and b; F).

DISCUSSION

Hydrophobic antimicrobial agents are normally unable to penetrate gram-negative bacteria because of the hydrophilic nature of the surface created by the calcium cross-linking of the LPS sidechains protruding from the outer surface of the outer membrane. This makes the gram-negative bacteria resistant to bile salts, actinomycin D, low concentrations of detergents, etc. (9). The cross-linking and asymmetry of the outer membrane with its high percentage of LPS and low concentration of phospholipid makes it difficult under normal circumstances to cleave by using freeze-fracture, there being apparently little lipid bilayer weakness to break (6, 8). For the same reason spin labels are usually more rigid in the outer membrane than in the inner membrane, where the regions of lipid bilayer allow rotation of the lipid polar heads and flexing of the nonpolar tails.

Short nonswarming cells of *P. mirabilis* presented the same freeze-fracture and EPR spin-label profile as normal, wild-type, gram-negative bacteria. They had a rigid outer membrane that did not cleave during freeze-fracture and allowed little freedom of movement to spin label.

Swarmers cells, however, behaved very differently, cleaving along both the inner and the outer membrane and revealing more freedom of motion of spin labels in isolated outer membrane than did their nonswarming equivalents. Similar results have been reported for deep-rough mutants and sugar LPS auxotrophs of *Escherichia coli* (8, 13). Both of these systems show either cleavage of the outer membrane or freedom of motion of spin labels. In both of these cases the change in character of the outer membrane has been attributed to the alterations in the LPS composition of the outer membrane. Deep-rough mutants lack the hydrophilic polysaccharide side chains, making them sensitive to hydrophobic antibiotics and unable to develop flagella. The galactose-starved *E. coli* were also unable to produce LPS side chains with a similar effect. The loss of the hydrophilic protective layer appeared to allow the formation of regions of lipid bilayer.

Swarmer cells of *P. mirabilis* have been shown to be sensitive to hydrophobic antibiotics in the same way as deep-rough mutants of *Salmonella* (3), suggesting an alteration in the hydrophilic protective surface layer. After performing biochemical analyses, however, I found that a higher percentage of isolated LPS from swarming *P. mirabilis* had longer side-chain polysaccharide than did LPS from the nonswarming cells (4), which in theory should have resulted in a more hydrophilic surface. Unfortunately, the actual amount of LPS in the bacterial envelope was impossible to calculate accurately because of the technical problems of isolation from a small population of agar-grown cells.

At the onset of swarming, the number of flagella on a *P. mirabilis* cell increases 50-fold per unit area (3, 7). The lack of flagella on deep-rough mutants suggests that LPS has a fundamental role in the growth of flagella, probably in providing a stable anchor in the outer membrane. It would therefore be expected that an increase in flagella per unit surface would require an equivalent increase in the proportion of LPS committed to stabilizing the flagella, hence the increase in long side-chain LPS. It is likely that the dramatic increase in flagella would cause a rearrangement of the LPS within the outer membrane with a very high proportion of the LPS grouped and cross-linked around the flagella, exposing the areas of lipid bilayer responsible for the motion of the spin labels, sensitivity to hydrophobic agents, and freeze-fracture cleavage.

At the end of swarming, when metabolism returns to the nonswarming state and the swarmers stop swimming, the flagella are lost before division recommences. This loss of flagella probably allows the rearrangement of the LPS and the insertion of new short side-chain LPS before septation occurs.

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LITERATURE CITED

1. **Armitage, J. P.** 1981. Changes in metabolic activity of *Proteus mirabilis* during swarming. J. Gen. Microbiol. **125**:445–450.
2. **Armitage, J. P., R. J. Rowbury, and D. G. Smith.** 1975. Indirect evidence for cell wall and membrane differences between filamentous swarming cells and short nonswarming cells of *Proteus mirabilis*. J. Gen. Microbiol. **89**:199–202.
3. **Armitage, J. P., and D. G. Smith.** 1978. Flagella development during swarmer differentiation in *Proteus mirabilis*. FEMS Microbiol. Letts. **4**:163–167.
4. **Armitage, J. P., D. G. Smith, and R. J. Rowbury.** 1979. Alterations in the cell envelope composition of *Proteus*

mirabilis during the development of swarmer cells. Biochim. Biophys. Acta **584**:389–397.
5. **Gabella, G., and D. Blundell.** 1978. Effect of stretch and contraction on caveolae of smooth muscle cells. Cell Tissue Res. **190**:255–271.
6. **Hasin, M., S. Rottem, and S. Razin.** 1975. The outer membrane of *Proteus mirabilis*: isolation and characterisation of the outer and cytoplasmic membrane fractions. Biochim. Biophys. Acta **375**:381–394.
7. **Hoeniger, J. F. M.** 1967. Development of flagella by *Proteus mirabilis*. J. Gen. Microbiol. **40**:29–42.
8. **Irvin, R. T., J. Lam, and J. W. Costerton.** 1979. Structural and biochemical examination of ghosts derived from a deep-rough (heptose-deficient lipopolysaccharide) strain and a smooth strain of *Escherichia coli*. Can. J. Microbiol. **25**:436–446.
9. **Nikaido, H.** 1976. Outer membrane of *Salmonella typhimurium*. Transmembrane diffusion of some hydrophobic substances. Biochim. Biophys. Acta **433**:118–132.
10. **Oltmann, L. F., and A. H. Stouthamer.** 1973. Purification of cytoplasmic membrane and outer membrane from *Proteus mirabilis*. Arch. Microbiol. **93**:311–325.
11. **Osborn, M. J., J. E. Gander, E., Parisi, and J. Carson.** 1972. Mechanism of assembly of the outer membrane of *Salmonella typhimurium*. Isolation and characterisation of cytoplasmic and outer membrane. J. Biol. Chem. **247**:3962–3972.
12. **Rottem, S., M. Hasin, and S. Razin.** 1975. The outer membrane of *Proteus mirabilis*. II. The extractable lipid fraction and electron paramagnetic resonance analysis of the outer and cytoplasmic membranes. Biochim. Biophys. Acta **375**:395–405.
13. **Rottem, S., and L. Leive.** 1977. Effect of variations in lipopolysaccharide on the fluidity of the outer membrane of *Escherichia coli*. J. Biol. Chem. **252**:2077–2081.
14. **Williams, F. D., and R. H. Schwarzhoff.** 1975. Nature of the swarming phenomenon in *Proteus*. Annu. Rev. Microbiol. **32**:101–122.