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Effect of Temperature on the Distribution of Membrane Particles in *Streptococcus faecalis* as Seen by the Freeze-Fracture Technique

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When cells of *Streptococcus faecalis* ATCC 9790 were incubated at temperatures above 10 C before being frozen for freeze-fracture, a random distribution of particles was observed on the outer fracture face of the freeze-cleaved cell membrane. However, when cells were incubated below 10 C before freezing, particleless patches were seen on this membrane surface. The size of the patches produced on chilling could be increased by centrifugation or by storing the chilled cells overnight at about 3 C. Patch formation appeared readily reversible, since the medium and large patches that formed on chilling could not be observed in cells warmed for 10 s at 25 C. However, during the transition from the patch to patchless state, smaller patches not seen in the chilled cells were observed. This suggested that the smaller patches might have been intermediate forms produced by the fragmentation of larger patches on warming.

Assuming the interpretation of Pinto da Silva and Branton (13) and Nanninga (9) to be correct, it appears that during the freeze-fracture process the cytoplasmic membrane cleaves along an internal plane, producing two complementary fracture faces. Both faces usually show raised membrane particles; however, the outer, convex fracture face (OFF) of the membrane usually shows many more of these particles than does the inner, concave fracture face (IFF) (8). The particles appear to be about 7 nm in diameter (18) and are thought to be proteinaceous (14, 19). It has been shown that the distribution of particles observed on the OFF of bacterial membranes is subject to some variation. In some reports (6, 15) the particles appear evenly distributed over the OFF, whereas in others particleless areas or patches appear (1, 3).

Recent studies have shown that the distribution of membrane particles can be influenced by the environmental conditions to which membranes are subjected before they are frozen for freeze-fracture. For example, OFF particles appear to aggregate in human erythrocyte ghosts when incubated in buffers below pH 5.5 (12) or in tetrahymena and mycoplasma membranes incubated at low temperatures, i.e., below 15 C for tetrahymena alveolar membranes (17) and at 5 C for the mycoplasma membranes enriched with elaidate or myristate (22).

We studied the effect of such environmental factors on the distribution of the OFF particles in the bacterium *Streptococcus faecalis* ATCC 9790 and found that particleless patches are formed when these cells are incubated at temperatures of 10 C or below. The phenomenon of patch formation is interpreted in terms of a lipid transition, which has been physically and biochemically defined in other membrane systems as a transition in membrane lipids from a liquid-crystalline to a crystalline state on the lowering of the environmental temperature through a critical temperature (2, 10, 20).

MATERIALS AND METHODS

Cells of *S. faecalis* ATCC 9790 were grown at 37 C for at least 6 generations in a chemically defined medium (16) to a cell density equivalent to 4×10^8 per ml, or 320 μg per ml (dry weight). At this point the cells were either (i) transferred to a room maintained at 37 C and frozen immediately in liquid Freon cooled in liquid nitrogen, (ii) brought to 25 C by pouring 10 ml of culture into a 300-ml glass beaker kept at 25 C for 5 to 10 min and then frozen, or (iii) chilled and used as a source of experimental material as described below. Two methods of chilling were used. Either 8 to 10 ml of cell culture was (i) poured over an equal volume of ice made with distilled water, or (ii) poured into a prechilled 300-ml glass beaker that was swirled in an ice bath. These two methods are referred to herein as the ICE and GLASS methods of chilling (Table 1). After 5 to 10 min in an ice bath, the chilled cells were frozen directly in a 3 C cold room or warmed to a secondary incubation temperature before

freezing. All incubation temperatures shown in Table 1 were determined by directed measurements taken from the cultures during various periods of treatment.

In some experiments, chilled cells were concentrated by centrifugation (1,500 × *g* for 15 min at 3 to 5 C) in order that more cells could be observed in the final freeze-fracture replicas or so that the cells could be suspended in water or growth medium adjusted to pH 5.0 with 0.1 N HCl. Parallel thin-section studies were carried out on unchilled and chilled cells before and after centrifugation. The glutaraldehyde-osmium tetroxide fixation, Epon 812 embedding, and uranyl acetate-lead citrate staining procedures have been described before (5).

Effect of glutaraldehyde fixation on particle distribution. Glutaraldehyde (GA) solutions of 8% (stored under nitrogen until just before use, Polysciences Inc., Warrington, Pa.) were added to a final concentration of 3% in cultures maintained at 37, 25, or less than 1 C by GLASS methods of chilling and kept in an ice bath. Before addition, the GA solutions were pre-equilibrated to the desired fixation temperatures, and after 60 min of fixation cultures were either frozen or incubated at a second temperature for 5 to 10 min before freezing.

Reversibility of patch formation. ICE- or GLASS-chilled cells were incubated at 25 or 37 C for 5 to 10 min (either in a concentrated or unconcentrated state) before freezing. To determine the time at 25 C necessary for the disappearance of patches, droplets of GLASS-chilled, concentrated cells were placed with a micropipette on chilled gold specimen holders (Balzers 11-3054P1). The grids were maintained at less than 1 C by contact with a 2-kg brass block cooled in an ice bath. All operations were carried out in a 3 C cold room with prechilled instruments. After specimen preparation, the ice bath containing the brass block and samples was transferred to a 25 C room. Each sample was picked up with a pair of chilled forceps and transferred from the chilled block to another brass block equilibrated to 25 C. After various periods of warming, the samples were immediately frozen.

Determination of the minimum temperature for patch formation. GLASS-chilled cells were brought to temperatures ranging between 0.5 and 18 C by pouring about 8 to 10 ml of cell culture into 300-ml beakers which were swirled in water baths of the desired temperature. The bath temperatures were monitored during each experiment and were not allowed to vary more than +0.5 C. After 5 min of incubation, the cells were fixed with GA for 60 min at a given temperature. The cells were then concentrated by centrifugation and frozen.

Electron microscopy. The methods for the freezing, fracturing, etching, replicating, and clearing of samples were carried out according to Balzers BA360M instruction manual no. A11-3992e and are essentially the methods of Moor and Mühlethaler (7). The frozen samples were freeze-fractured and etched 1 min before replication with a Balzers BA360M freeze-etch microtome.

The samples were examined in a Siemens Elmiskop 1A electron microscope at instrumental magnifications of about 30,000.

Each experimental result reported in Table 1 was gathered from the study of at least 50 cells showing cleanly fractured cell membranes.

RESULTS

If exponential-phase cells were maintained at 37 or 25 C until they were frozen for freeze-fracturing, a fairly homogeneous distribution of particles was observed on the OFF (Fig. 1A). This distribution was seen in both early and late exponential-phase cultures. If particleless areas were noted, they were small and did not exceed about 25 nm in diameter. As observed in other bacteria (8), the particles of *S. faecalis* were found much more rarely on the IFF than on the OFF (Fig. 1A, insert) and ranged in diameter between 7 and 13 nm.

However, if cells were chilled to about 1 C and then subsequently kept at 3 C or below until they were frozen, particleless areas or patches of about 100 to 140 nm were then seen on virtually 100% of the OFF surfaces examined (Fig. 1B–E). The particles surrounding the patches did not appear to undergo any marked amount of aggregation, and the density of these particles seemed about the same as that observed before chilling.

Particleless areas could also be observed on the IFF (Fig. 1B, insert; Fig. 2B); however, in the absence of complementary fractures it is not known if such areas correspond to the patches seen on the OFF (Fig. 1B, 2B).

The OFF patches appeared regardless of whether the cells were chilled by being poured (i) over an equal volume of ice (ICE, Table 1), or (ii) in a precooled beaker swirled in an ice bath (GLASS, Table 1). The patches were not evenly distributed over the membrane surface (Fig. 1B), and the number of patches per unit area of OFF was found to be quite variable. Also, the size of the patches appeared to enlarge greatly when chilled cells were centrifuged (Fig. 2A; Table 1) or kept overnight at 3 C. The patches of these centrifuged cells occasionally covered large areas of the OFF, and the particleless areas on both the OFF and IFF surfaces often took on a wrinkled appearance (Fig. 2B). In parallel thin-section studies, "periplasmic gaps" not seen in the 25 or 37 C control cells (Fig. 3A) were found in many ICE- or GLASS-chilled cells (Fig. 3C). The frequency of gap observation was stimulated by either centrifuging the chilled cells or storing them overnight at 3 C. In addition, centrifugation also appeared to induce the appearance of multiple membrane

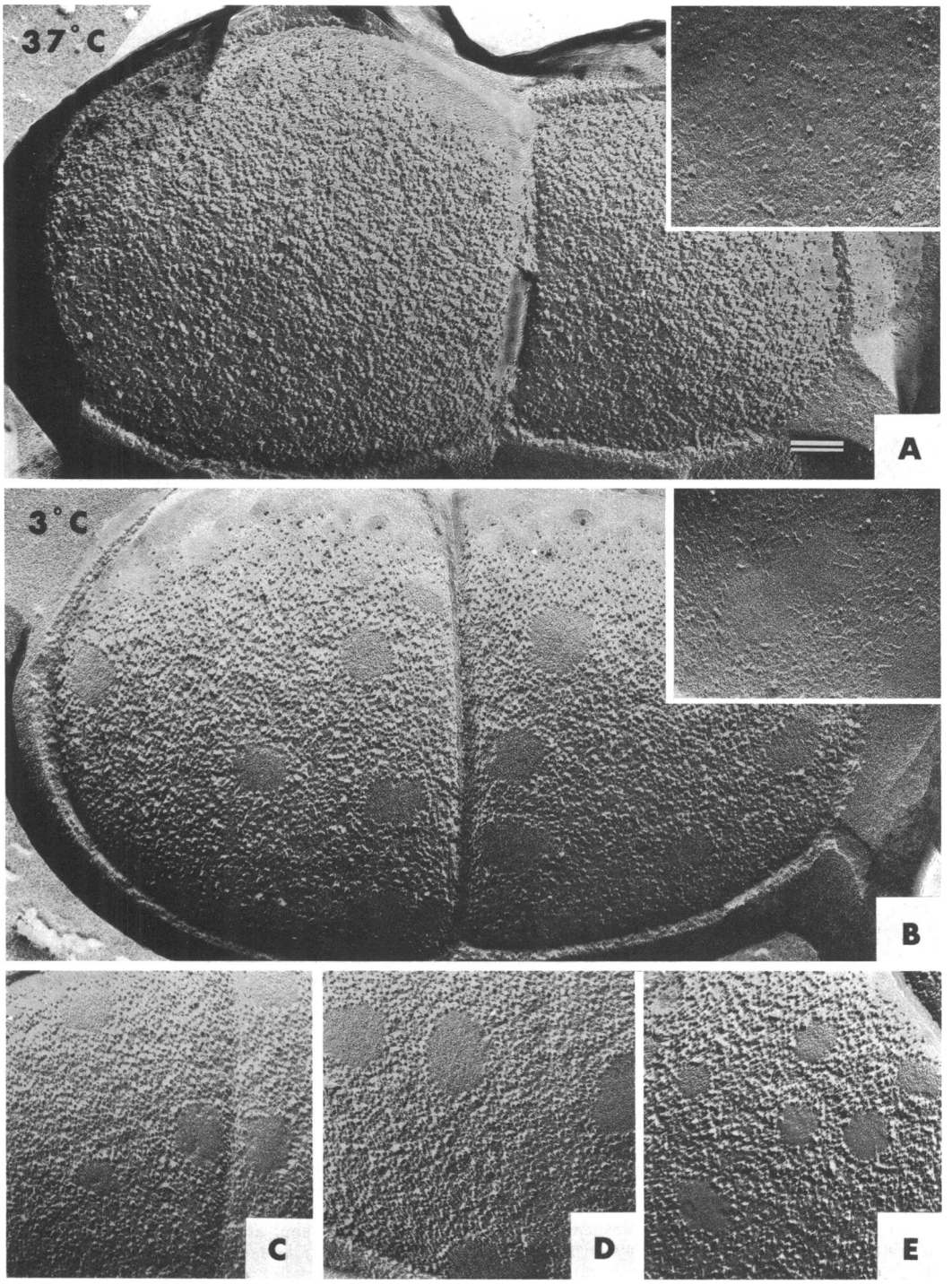

FIG. 1. *Freeze-fractures of the cell membrane of S. faecalis incubated at 37 and 3 C before being frozen. (A) OFF and IFF (insert) of cell membranes kept at 37 C before freezing. (B) Same fracture faces shown in (A) except that cells were chilled and maintained at 3 C before freezing. (C) to (E) Some additional OFF membranes of chilled cells. The bar in (A) applies to all and equals 0.1 μm.*

pockets (Fig. 3B). These pockets were not seen in cells fixed at 25 or 37 C, or after GLASS or ICE chilling, or upon overnight storage at 3 C. Therefore, it is conceivable that the corrugated OFF surface shown in Fig. 2B may be induced by membrane rearrangement which may be

TABLE 1. *Distribution of OFF particles under various environmental conditions*

Type of test and expt no.[a]	Method of chilling and treatment[b]	Temp of incubation (C)	Time of incubation (min)	Temp of room used for sample freezing (C)	Patch size[c]
Temperature[d]					
1				37	N
2				25	N
3	ICE			3	M
4	GLASS			3	M
5	GLASS-CENTR			3	M-L
	ICE-CENTR			3	M-L
6	GLASS-overnight			3	M-L
Wash[e]					
7 (Water)	ICE-CENTR	<1	5-10	3	M-L
8 (Water)	ICE-CENTR	37	5	25	N
9 (pH 5)	ICE-CENTR	<1	5-10	3	M-L
10 (pH 5)	ICE-CENTR	37	5	25	N
Reversibility[f]					
11	ICE-CENTR	<1	5-10	3	M-L
12	ICE-CENTR	25 or 37	5	25 or 37	N
13	ICE	25 or 37	5	25 or 37	N
14	GLASS-CENTR	25	0	3	M-L
15	GLASS-CENTR	25	1/2	25	N, S, M, L
16	GLASS-CENTR	25	1/6, 1/3, 1/2, 1, 2	25	N, S
Fixation[g]					
17	GA			25	N
18	GA	<1	5-10	3	N
19	GLASS-GA			3	M-L
20	GLASS-GA	25	5-10	25	M-L
21	GLASS-GA	37	5-10	37	M

[a] With the exception of treatments 7 to 10, which were based on a single experiment, all other results reported here are based on the analysis of at least 50 cells from two experiments.

[b] Cells were chilled (i) over an equal volume of ice (ICE), or (ii) in a precooled glass beaker which was swirled in an ice bath (GLASS). After 5 to 10 min of incubation, the cells were centrifuged at 1,500 × *g* for 15 min at 3 to 5 C (CENTR) and then frozen immediately, except as otherwise stated.

[c] The size of the particleless patches on the OFF of cell membrane was ranked as follows: N, no patches observed, or occasional particleless areas no larger than about 25 nm; S, small, regularly occurring patches greater than 25 nm and less than 100 nm; M, medium patches averaging about 100 to 140 nm; L, large patches of greater than 140 nm. The indicated appearance of OFF represents better than 95% of the membrane surfaces examined.

[d] Cells were grown as described and then either (i) maintained at 37 C (experiment 1), (ii) brought to 25 C (experiment 2), or (iii) ICE- or GLASS-chilled. The cells were kept at these temperatures for 5 to 10 min until they were frozen or concentrated by centrifugation and then frozen. The cells in experiment 6 were incubated in an ice bath overnight.

[e] Cells were ICE-chilled, centrifuged, and resuspended in cold distilled water or chilled medium adjusted to pH 5 and then incubated at the stated temperature and time prior to being frozen.

[f] Cells were chilled, centrifuged (except experiment 13), and either kept at the stated temperature until freezing (experiments 11 to 13) or were transferred to chilled gold specimen holders placed in contact with a chilled brass block (experiments 14 to 16). In the latter case, grids were subsequently transferred to a second 25 C brass block for the stated secondary incubation times before being frozen.

[g] Cells were either brought to 25 C (experiments 17, 18) or GLASS-chilled. The cells were then fixed by adding a glutaraldehyde solution (GA) adjusted to 25 or 1 C to a final concentration of 3% for 60 min. With the exception of experiment 17, the cells were centrifuged after fixation and incubated at the stated temperature until they were frozen.

reflected in the thin-section observations by the stimulation of "periplasmic gap" formation or induction of multiple membrane pockets, or both. However, no direct correlation could be made between the formation of the corrugated OFF surfaces, mesosome arrangement, and multiple membrane formation, for the areas of multiple membrane formation were (i) not observed in cells stored overnight at 3 C, (ii) frequently located far away from the septal mesosome attachment site of this organism, and (iii) appeared to coexist in cells that also contained septal mesosomes. However, some mechanism by which partial mesosome eversion followed by a general rearrangement of the membrane resulting in a wrinkled surface is still open.

In addition, it appeared that patch formation (i) did not require a specific component of the growth medium, or (ii) was not induced by a slight decrease in pH that could occur during growth, for cells suspended in either water or medium adjusted to pH 5.0 did not form patches until they were chilled (Table 1).

The process of patch formation seemed readily reversible, for patches were not observed after cells had been warmed for 5 min at 25 or 37 C (Fig. 4A; Table 1). The minimum time for

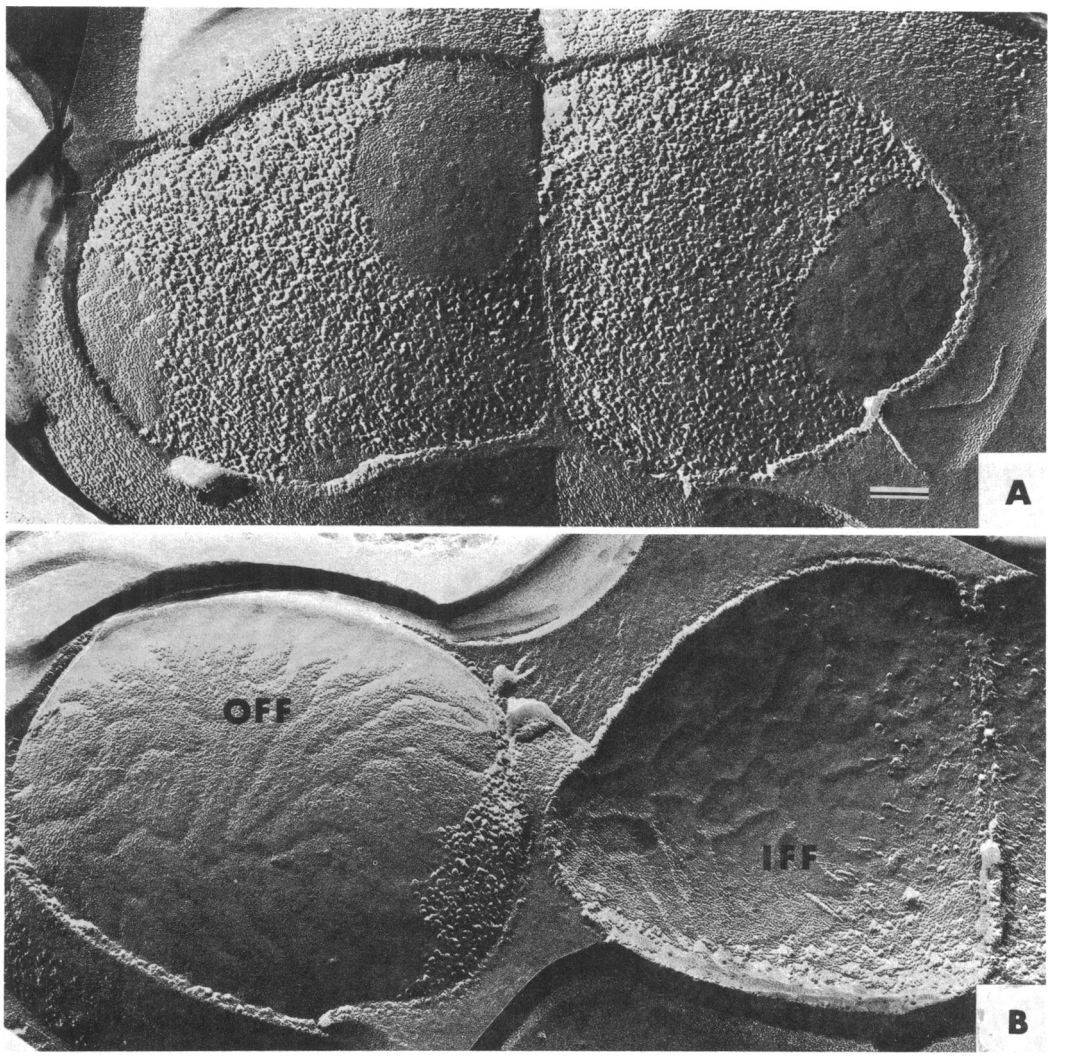

FIG. 2. *Freeze-fractures of cell membranes of chilled cells that have been centrifuged before being frozen. On centrifugation the patch areas increased in size over that observed after chilling alone (A). Occasionally the patches may cover large portions of the OFF (B); however, no increase in particle density on the IFF could be observed. Bar in (A) applies to (B) and equals 0.1 μm.*

this reversal was determined by rapidly raising the temperature of the chilled cells to 25 C for various periods before freezing. This was done by transferring specimen holders containing chilled cells from a brass block maintained at about 0.5 C to a second brass block equilibrated to 25 C.

After 10 s at 25 C, none of the large and medium-sized patches observed in the chilled samples could be seen (Table 1); however, some smaller patches like those seen in Fig. 4B now appeared. Other than in size, these smaller patches differed from those formed on chilling in that the smaller patches appeared evenly distributed over the OFF. The small patches decreased in frequency of appearance and size with increasing periods of incubation until they were no longer observable after 120 s at 25 C. This suggested that the small patches might have been intermediate forms caught in the transition from the patch to patchless membrane state.

In the past, we have observed similar evenly distributed small patches in our freeze-fractures. It was found that the small patches appeared when chilled cells were quickly transferred in a 25 C room to specimen holders attached to glass slides (Fig. 4B). Apparently, the slower warming of these cells on the glass surfaces accounted for the predominance of the small patches observed with this preparative method.

GA fixation preserved the particle distribution observed in unfixed membranes incubated at 3, 25, and 37 C (Table 1). Accordingly, chilled cells fixed with chilled GA showed patches, whereas those fixed at 25 or 37 C showed no patches. These distributions were not altered by temperature shifts after fixation. Thus, cells fixed at 3 C and then incubated at 25 C before freezing still showed patches (Fig. 5B), whereas those fixed at 25 C and incubated at 3 C had no patches (Fig. 5A; Table 1).

The ability of GA to "freeze" particle distributions was used to study the minimum temperature at which patches formed. Cells were incubated for 5 min at temperatures between 0.5 and 18 C before GA solutions (equilibrated to the same temperatures) were added. In three experiments no patches were seen in cells that had been incubated before and during fixation above 10 C (Table 2). As the incubation temperatures were reduced below 10 C, an increasing frequency of cells showed patches. Thus, 28% of the cells incubated at 10 C showed patches, whereas patches were seen in 100% of the cells incubated at 3 C (Table 2).

DISCUSSION

The results presented indicate that particleless patches form on the OFF when exponential-phase cells of *S. faecalis* are incubated at temperatures below 10 C. This is consistent with a pushing aside of membrane particles on cooling due to a solidification of the membrane lipids, induced by a liquid crystalline-crystal-

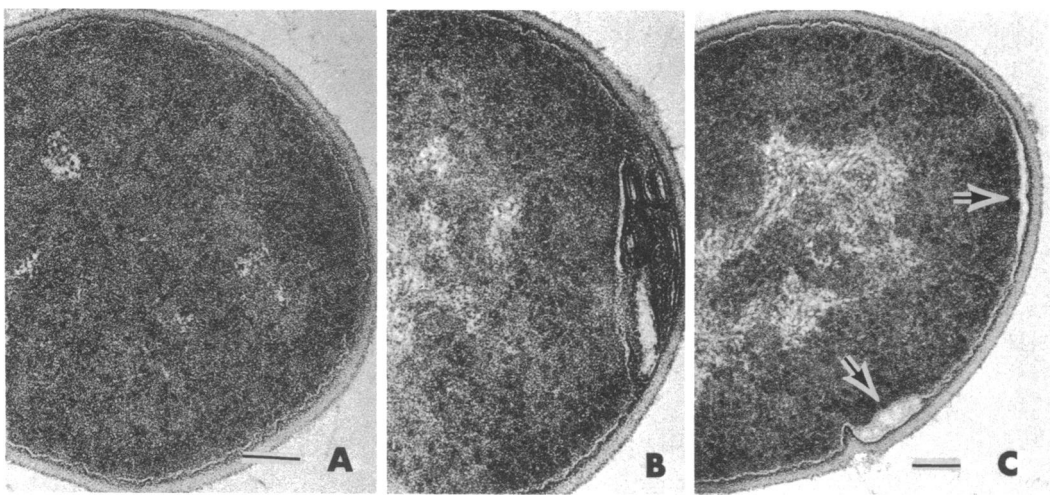

FIG. 3. *Thin sections of the cells of S. faecalis fixed at 37 C (A) and 3 C (B, C). Only a portion of each cell section has been included. On centrifugation chilled cells were observed to form multiple membrane pockets absent in the 37 C control cells. (B) Multiple membrane formation in a GLASS-chilled cell after centrifugation (1,500 × g for 15 min at 3 to 5 C). "Periplasmic gaps" also not observed in the control cells were seen in ICE- or GLASS-chilled cells (C, see arrows). These gaps increased in frequency on centrifugation or overnight chilling. Bars in (A) and (C) equal 0.1 μm. Bar in (A) applies to (B).*

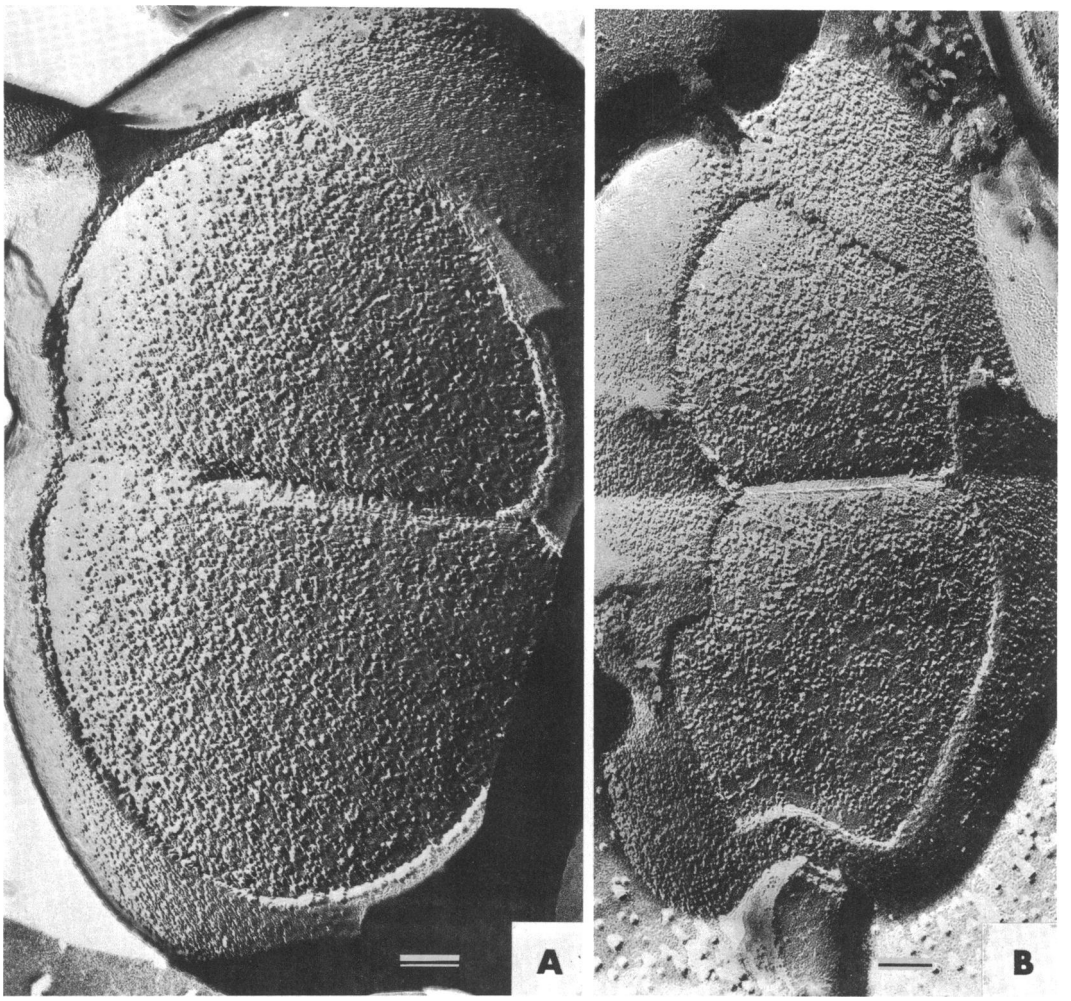

FIG. 4. *OFF of a cell membrane that has been chilled and then rapidly (A) or slowly (B) warmed to 25 C before being frozen. The rapid warming was carried out by transferring cells in chilled specimen holders to a 25 C brass block, whereas the slower warming took place by transferring chilled cells in a micropipette to specimen holders attached to a glass slide at 25 C. Bars in (A) and (B) equal 0.1 μm.*

line phase transition. A similar explanation was given for the aggregation of membrane particles observed on incubating tetrahymena (17) or mycoplasma (22) membranes at low temperatures. However, these observations differed from ours in that the patches formed on the chilling of *S. faecalis* were well-defined oval or circular areas formed without any gross aggregation of surrounding membrane particles. Thus, if particle aggregation does take place in *S. faecalis* to make room for patch formation, it must be a subtle process, or, alternatively, space for patch formation may be accommodated on cooling by some membrane particles shifting out of the usual plane of fracture. The last possibility is especially likely after centrifugation or long-term chilling, where patch areas sometimes cover large portions of the OFF (Fig. 2B). If some particles do shift out of the normal fracture plane, it appears that they do not go preferentially to the IFF, for no enrichment of IFF particles could be seen in any of the long-term chilled or centrifuged cells (Fig. 2B).

Studies on the membrane lipid composition of two strains of *S. faecalis* indicated that about 40 to 50% of the membrane lipids contain *cis*-monounsaturated hydrocarbon chains (4, 11). Other investigations of *Escherichia coli* have suggested that an enrichment of membrane lipids with *cis*-ethylenic hydrocarbon chains results in a transition temperature of

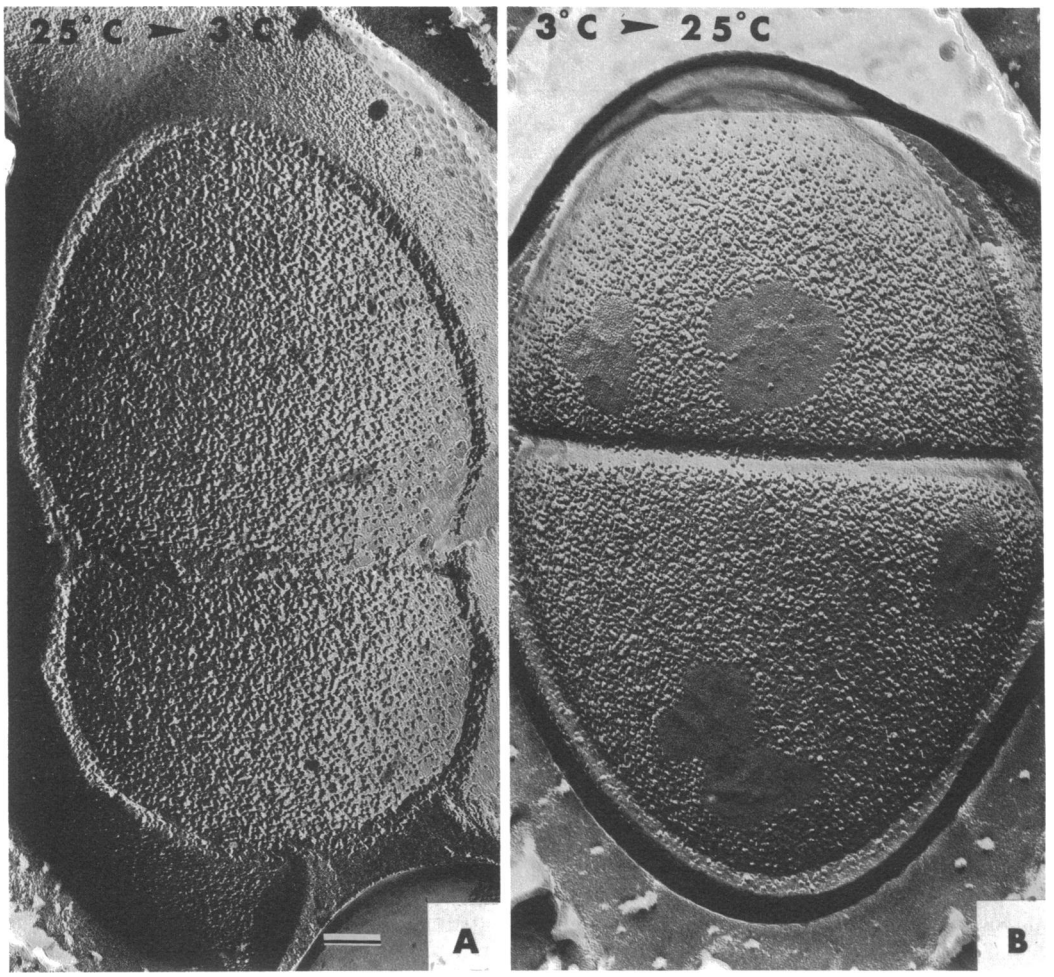

FIG. 5. *Effect of glutaraldehyde fixation on the distribution of OFF particles. (A) Cell was fixed at 25 C and then chilled to 3 C before freezing. (B) Cells were fixed at 3 C and then warmed to 25 C before freezing. Bar in (A) applies to (B) and equals 0.1 μm.*

TABLE 2. *OFF patch formation as a function of temperature*[a]

Temp (C)	Patch size[b]	Frequency of appearance (%)
0.5	M	100
3	M	100
6	M	83
9	M	50
10	M	28
12	N	0
14	N	0
18	N	0

[a] Cells were GLASS-chilled and warmed to the stated temperature by pouring 6 to 8 ml of the chilled cultures into prewarmed 300-ml beakers which were swirled in water baths of the appropriate temperature for 5 min. After this period, cells were fixed by the addition of GA solutions also previously equilibrated to the water bath temperature. After 60 min of fixation (in 3% GA), the cells were centrifuged and frozen.

[b] See footnote *c* in Table 1.

about 14 C (10). Thus, in accordance with these observations in *E. coli*, the induction of patch formation in *S. faecalis* at about 10 C would be consistent with its membrane-lipid composition and a possible phase transition occurring in its membranes at approximately this temperature.

The irregular distribution and rapid formation of the patches seen in chilled *S. faecalis* cells are indicative of the rapid lateral movement of membrane components that have been suggested on the basis of biochemical-biophysical investigations of other membranes (see reference 21 list for current references). The observation in *S. faecalis* of smaller and more evenly distributed patches after short periods of warming (Fig. 4B; Table 1) is also indicative of rapid lateral movement and further suggests that the large- and medium-sized patches formed on chilling may break up into smaller zones on warming. In addition, the fact that medium-sized patches aggregate to form larger patches on centrifugation or long incubation at 3 C implies that the membrane still maintains partial fluidity and mobility even at 3 C.

These observations may account for some of the variation in particle distribution previously reported in freeze-fracture studies of bacterial membranes and indicate that preparative conditions must be carefully studied and reported in investigations of cell membrane by the freeze-fracture technique.

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LITERATURE CITED

1. Bayer, M. E., and C. C. Remsen. 1970. Structure of *Escherichia coli* after freeze-etching. J. Bacteriol. **101**:304-313.
2. Esfahani, M., A. R. Limbrick, S. Knutton, T. Oka, and S. J. Wakil. 1971. The molecular organization of lipids in the membrane of *Escherichia coli*: phase transitions. Proc. Nat. Acad. Sci. U.S.A. **68**:3180-3184.
3. Fiil, A., and D. Branton. 1969. Changes in the plasma membrane of *Escherichia coli* during magnesium starvation. J. Bacteriol. **98**:1320-1327.
4. Fischer, W., H. R. Landgraf, and J. Herrman. 1973. Phosphatidyl-diglucosyl diglyceride from *Streptococci* and its relationship to other polar lipids. Biochim. Biophys. Acta **306**:353-367.
5. Higgins, M. L. 1973. A fixation and embedding procedure for thin-sectioning bacteria. CRC Handb. Microbiol. **1**:686-689.
6. Holt, S. C., and E. R. Leadbetter. 1969. Comparative ultrastructure of selected aerobic spore-forming bacteria: a freeze-etching study. Bacteriol. Rev. **33**:346-378.
7. Moor, H., and K. Mühlethaler. 1963. Fine structure in frozen-etched yeast cells. J. Cell Biol. **17**:609-628.
8. Nanninga, N. 1968. Structural features of mesosomes (chondroids) of *Bacillus subtilis* after freeze-etching. J. Cell Biol. **39**:251-263.
9. Nanninga, N. 1971. Uniqueness and location of the fracture plane in the plasma membrane of *Bacillus subtilis*. J. Cell Biol. **49**:564-570.
10. Overath, P., and H. Träuble. 1973. Phase transitions in cells, membranes, and lipids of *Escherichia coli*. Detection by fluorescent probes, light scattering, and dilatometry. Biochemistry **12**:2625-2634.
11. Pieringer, R. A., and R. T. Ambron. 1973. A method for the specific labeling of the glycerol in glyceride-containing lipids of *Streptococcus faecalis* ATCC 9790. J. Lipid Res. **14**:370-372.
12. Pinto da Silva, P. 1972. Translational mobility of the membrane intercalated particles of human erythrocyte ghosts. pH-dependent, reversible aggregation. J. Cell Biol. **53**:777-787.
13. Pinto da Silva, P., and D. Branton. 1970. Membrane splitting in freeze-etching. Covalent bound ferritin as a membrane marker. J. Cell Biol. **45**:598-605.
14. Pinto da Silva, P., and D. Branton. 1972. Membrane intercalated particles; the plasma membrane as a planar fluid domain. Chem. Phys. Lipids **8**:265-278.
15. Remsen, C., and D. G. Lundgren. 1966. Electron microscopy of the cell envelope of *Ferrobacillus ferrooxidans* prepared by freeze-etching and chemical fixation techniques. J. Bacteriol. **92**:1765-1771.
16. Shockman, G. D. 1963. Amino acids, p. 567-673. *In* F. Kavanagh (ed.), Analytical microbiology. Academic Press Inc., New York.
17. Speth, V., and F. Wunderlich. 1973. Membranes of *Tetrahymena*. II. Direct visualization of reversible transitions in biomembrane structure induced by temperature. Biochim. Biophys. Acta **291**:621-628.
18. Tillack, T. W., and V. T. Marchesi. 1970. Demonstration of the outer surface of freeze-etched red blood cell

membranes. J. Cell Biol. **45**:649-653.
19. Tourtellotte, M. E., and J. S. Zupnik. 1973. Freeze-fractured *Acholeplasma laidlawii* membranes: nature of particles observed. Science **179**:84-86.
20. Träuble, H. 1972. Phase transition in lipids. Biomembranes **3**:197-227.
21. Tsukagoshi, N., and C. F. Fox. 1973. Transport system assembly and the mobility of membrane lipids in *Escherichia coli*. Biochemistry **12**:2822-2829.
22. Verkleij, A. J., P. H. J. Ververgaert, L. L. M. Van Deenen, and P. F. Elbers. 1972. Phase transitions of phospholipid bilayers and membranes of *Acholeplasma laidlawii* B visualized by freeze fracturing electron microscopy. Biochim. Biophys. Acta **288**:326-332.