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Mesosomes of *Staphylococcus aureus* were examined morphologically under the electron microscope. Three different methods of specimen preparation (thin section, freeze-fracture, and negative staining) were used to eliminate artifacts due to sample processing. Mesosomes were rarely seen in intact cells but were quite distinct in autolysed cells incubated in 1.2M sucrose -0.33M acetate buffer (pH 5.8) at 25 degrees C. The phospholipid content increased by 20% of the control as autolysis proceeded. Since the plasma membrane did not show any other significant changes, the development of mesosomes during autolysis was shown to be a real event. Most of the well-developed mesosomes were found at the septum in negatively stained specimens. Initial wall-lysis occurred at this site, suggesting a close relationship between autolysis and the development of mesosomes in *S. aureus*.

KEYWORDS: mesosomes, *S.-aureus*, autolysis, membrane synthesis, membrane phospholipids

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THE DEVELOPMENT OF MESOSOMES OF *STAPHYLOCOCCUS AUREUS* DURING AUTOLYSIS

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Abstract. Mesosomes of *Staphylococcus aureus* were examined morphologically under the electron microscope. Three different methods of specimen preparation (thin section, freeze-fracture, and negative staining) were used to eliminate artifacts due to sample processing. Mesosomes were rarely seen in intact cells but were quite distinct in autolysed cells incubated in 1.2M sucrose—0.33M acetate buffer (pH 5.8) at 25°C. The phospholipid content increased by 20% of the control as autolysis proceeded. Since the plasma membrane did not show any other significant changes, the development of mesosomes during autolysis was shown to be a real event. Most of the well-developed mesosomes were found at the septum in negatively stained specimens. Initial wall-lysis occurred at this site, suggesting a close relationship between autolysis and the development of mesosomes in *S. aureus*.

Key words : mesosomes, *S. aureus*, autolysis, membrane synthesis, membrane phospholipids.

Mesosomes are defined as intracellular membranous structures which are derived from plasma membrane, form lamellae, vesicles or tubules, and can be isolated from plasma membrane by the removal of cell wall. They have been found in most Gram positive bacteria (1). Their structure, however, is variable, depending on factors such as the growth phase of cells and the method of fixation (2). In fact, their very existence is controversial as it is not clear whether they are “real” or only “artificial false structures caused by specimen processing for electron microscopic observation.”

Staphylococcal mesosomes have been isolated and characterized (3, 4). Their isolation, however, usually requires the use of wall lytic enzymes with incubation at 37°C for several hours in a buffer at optimal pH for lytic enzyme activity. The isolation technique itself, therefore, may be the cause of structural and functional modifications in the cells.

The author studied the morphology of *Staphylococcus aureus* during wall lysis because cell membrane reconstruction had been suggested by marked changes in phospholipid composition during autolysis (10). Preliminary results indicated a

close correlation between mesosomes and autolysis. Clarification of the nature of these mesosomes was attempted, using thin section, freeze fracture, and negative staining to minimize artifacts due to processing of the samples for electron microscopy.

MATERIALS AND METHODS

Strain and growth conditions. *Staphylococcus aureus* 209P (FDA) was used in all experiments. The bacteria cultured in nutrient broth (Nissui Tokyo Japan) at 37°C with reciprocal shaking (40–50/min). The growth rate was followed by measuring optical density at 660 nm with an S. P. 20A (Shimazu, Japan). Cultures were harvested at logarithmic growth phase (O. D. =0.8). The cells collected by centrifugation at 8,000×g, for 10 min, then subjected to autolysis as follows.

Autolysis. Cells were washed twice with cold saline solution then resuspended in 1.2 M sucrose –0.33 M acetate buffer (pH 5.8, abbreviated as hypertonic buffer) at a concentration of 5 mg dry weight cells/ml (measured by optical density) (5). This suspension was incubated at 25°C with stirring. At intervals, aliquots of suspension were taken for determination of osmotic fragility and used for ultrastructural observation under electron microscopy.

Electron microscopy. Each sample was examined by three different methods; ultrathin section, negative staining and freeze-fracture. For thin sections, specimens were fixed in 2.5% glutaraldehyde (made in 1.0 M NaCl-0.01 M phosphate buffer, pH 7.2) for 60 min at 0–4°C, postfixed with 2% OsO₄ (made in the same buffer) for 3 h at 0–4°C, dehydrated in a graded ethanol series, embedded in Epon 812, thin sectioned, then stained with 2% uranylacetate in 50% ethanol and lead-citrate.

For negative staining, samples were fixed in buffered glutaraldehyde containing 10 mM MgCl₂ for 1 h, then stained with 2% ammonium molybdate, pH 7.0.

For freeze-fracture, specimens were used either without fixation or fixation in 1.0% glutaraldehyde for 1 h at 0–4°C. The pellet cells were suspended in cold 30% glycerin (made in 0.1 M phosphate buffer, with 1.0 M NaCl added for autolysed samples) and immediately frozen by immersion liquid nitrogen. Freeze-fracture was performed with an FD-2 (Hitachi, Japan) (6). Replicas were washed with diluted sodium hypochlorite solution.

Isolation of mesosomal vesicles and plasma membranes. Mesosomes were prepared by a modification of the procedure of Theodore *et al.* (3). Cells incubated for 4 h in hypertonic buffer were treated with DNase and RNase for 30 min at 37°C and centrifuged at 10,000×g for 20 min.

The pellet was suspended in hypertonic buffer, sonicated at 20 KHz for 5 min, and centrifuged at 10,000×g for 20 min. The supernatant of this sonicated sample was re-centrifuged at 100,000×g for 2 h and its sediment layered on a stepwise sucrose density gradient (55, 60, 65, 70, 75, 80%), which was then centrifuged at 100,000×g for 15 h. The 75% sucrose density band was used as the source of plasma membranes.

The supernatant was re-centrifuged at $100,000\times g$ for 2 h and its sediment layered on a stepwise sucrose density gradient which was then centrifuged as described above. The 60% sucrose density band was used as the source of mesosomes.

Polyacrylamide gel electrophoresis. Polyacrylamide slab gel electrophoresis was performed by the method of Laemmli and Faure (7). Isolated mesosomes and plasma membrane were dissolved in 1% SDS-mercaptoethanol – 50 mM Tris acetate (pH 8.4), boiled for 3 min, then subjected to electrophoresis on a 9% acrylamide gel and run at a constant current of 2 mA/cm. The run buffer was 50 mM Tris acetate (pH 8.4). The gels were stained with coomassie blue.

Extraction and analysis of phospholipids. Lipids were extracted directly from suspension by a modification of the procedure of Bligh and Dyer (8). Phospholipids were separated by two dimensional Silica-Gel G thin-layer chromatoplates (9), the spots scraped, and phosphorous assayed by a modification of the method of Kates (10).

RESULTS

Autolysis at various growth phase. Chart 1 shows the rate of autolysis at each growth phase. Autolysis was most marked in cells at middle log growth phase (O. D.=0.8) and was almost complete within 2 h. As growth proceeded, the rate of autolysis decreased compared with that during the middle log growth phase. Unless otherwise noted, cells at middle log growth phase were used.

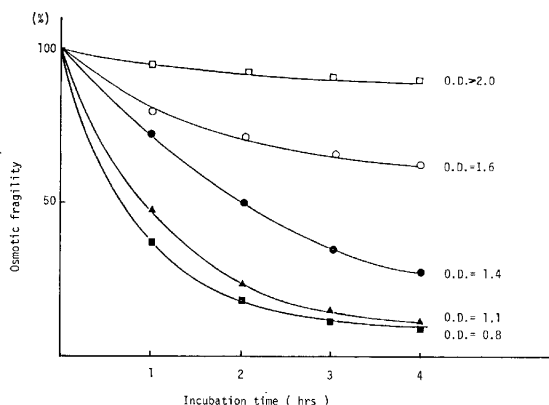


Chart 1. Time course of the development of osmotic fragility of *Staphylococcus aureus* at each growth phase. Cells were suspended in 1.2 M sucrose—0.33 M acetate buffer (pH 5.8) at a concentration corresponding to 5 mg dry weight/ml. Incubation was carried out at 25°C. At suitable intervals, 0.1 ml of the suspension was pipetted into 5 ml of 0.01 M phosphate buffer (pH 6.8) or 5 ml of the same buffer containing 1.2 M NaCl. Percentages of initial turbidity were measured as an index of osmotic fragility. Symbol; ■ middle log growth phase (O. D.=0.8), ▲ middle log growth phase (O. D.=1.1), ● late log growth phase (O. D.=1.4), ○ late log growth phase (O. D.=1.6), □ stationary phase (O. D.>2.0).

Mesosomes of intact S. aureus. Intact cells had few mesosomes in thin sectioned and freeze-fractured specimens. Fig. 1 shows cells at middle log growth phase. The inner structure of the cell was not clear with negative staining because ammonium molybdate did not penetrate the cell wall; however, a very low contrast lattice or linear structure was noted on the wall (Fig. 1-c). When the temperature exceeded above 4°C during the sample processing, mesosomes were increased in number.

Mesosomes in autolysed cells. Fig. 2 shows cells after suspension in hypertonic buffer for 15 min. Many mesosomes are present and appear as single vesicles or packs of small vesicles in thin sections (Fig. 2-b). Vesicles were 30 m μ to 150 m μ in diameter and consisted of two leaflets of membrane 120Å in thickness. The membrane was continuous with the inner electron-dense layer of the periphery of the cell but was of higher contrast. In freeze-fractured specimens, mesosomes appeared as single vesicles about 100 m μ in diameter (Fig. 2-a). Other ring-like structures were also seen. Mesosomes were equally located at the septum and around the circumference. By negative staining, however, most of the mesosomes appeared to be located at the septum (Fig. 2-c). These were of similar size and shape to those seen in thin sections and were mostly vesicles. Tubular forms were rare. Mesosomes of cells in late log growth phase or in stationary phase after 15 min incubation were also examined (Fig. 3). The number and size of these mesosomes were less than those in middle log growth phase cells. The result was consistent with the rate of autolysis (Chart 1) at each growth phase. Fig. 5 shows cells after 1 h incubation in hypertonic buffer. There are two different types of mesosomes: one is the same mesosomes in cells incubated for 15 min, the other is bigger and consists of sacs and vesicles (Fig. 4-a). The development was more clearly seen by freeze-fracture (Fig. 4-b). Each mesosomal sac was made of infolded plasma membrane. The electron density of the mesosomal membrane was the same as that of the plasma membrane. Cell wall lysis was not evident at this stage. Fig. 5 shows cells after 2 h incubation in hypertonic buffer. Cell wall damage is marked in thin sections (Fig. 5-a, b). Cell wall rupture was evident at the site of the mesosomes and some mesosomes had been extruded, indicating on-going autolysis. The majority of these extruded mesosomes were vesicular. Some appeared as rods in thin sections, but as tubular or linked beads in negative staining. Fig. 6 shows cells after 4 h incubation. Cell walls are detached in places with bulging of the underlying plasma membrane. Complete protoplasts (no remaining cell wall) were rare (Fig. 6-a). Some mesosomes were still within the cells, although most had been extruded.

There was no difference in the samples for freeze-fracture prepared with and without fixation with 1% glutaraldehyde indicating that the fixation was not responsible for artificial modifications of the mesosomes.

The isolated mesosomes. Mesosomes were isolated by sucrose gradient from the supernatant of cells prepared after 4 h incubation in hypertonic buffer. Isolated mesosomes were small polymorphic vesicles 40 m μ to 150 m μ in diameter. They were similar in size to intracellular mesosomal vesicles (Fig. 7). Two kinds of isolated vesicles were seen in thin sections: one contained electron dense substances and had no definite unite membrane structures; the other had a well-definite unite structure of the vesicle membrane but without any recognizable substance within the vesicles. Most isolated mesosomes had smooth surfaces on negative staining resembling that of cells after 2 h incubation (Fig. 5-d). The polypeptide patterns on SDS-acrylamide gel of mesosomal vesicles and plasma membrane are shown in Fig. 8. They are similar.

Changes in phospholipid composition during autolysis. During autolysis, the phospholipid content increased by 20% in terms of the amount of total phospholipid per the amount of cells. The increase plateaued in 1 h. The cardiolipin content increased to 50% of, and phosphatidylglycerol (the precursor of cardiolipin) decreased to 20% of, the total phospholipids (Chart 2).

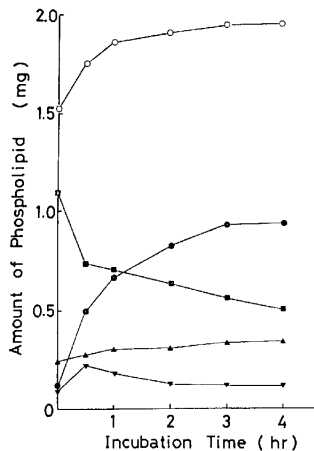


Chart 2. Changes in total and individual phospholipid during autolysis in hypertonic buffer. Samples were incubated in hypertonic buffer at 25°C. At intervals, lipids were extracted directly from 10 ml of incubated suspension by a modification of the method of Bligh and Dyer. The ordinate represents the lipid count in 10 ml of suspension. Symbols: ○ total phospholipid, ● cardiolipin, ■ phosphatidylglycerol, ▲ lysylphosphatidylglycerol, ▼ phosphatidic acid.

DISCUSSION

This study demonstrates the development of mesosomes in *S. aureus* during autolysis. Electron micrographs taken by three different methods (thin sections, negative staining and freeze-fracture) concur in terms of the structure and num-

ber of mesosomes in cells. The freeze-fracture is generally believed to preserve more a 'close to life' structure than any other method for electron microscopic examination. Nanninga (11), Fooke-Achterrath *et al.* (12), and Higgins *et al.* (13), reported that mesosomes were seldom seen in unfixed freeze-fractured cells, but were visible in chemically-fixed-freeze-fractured specimens. They concluded that mesosomes were some sort of modified structure induced by chemical fixatives such as osmium and glutaraldehyde.

In this report, the development of mesosomes is clearly shown in freeze-fractured specimens both of cells fixed with 1% glutaraldehyde and of unfixed ones. Glycerol treated-unfixed cells were reported to have more mesosomes than glycerol non-treated ones prepared by freeze-fracture (14). In the present study, however, as shown in Fig. 2-b, mesosomes were very rare in freeze-fractured specimens treated with 30% glycerol.

Analysis of the plasma membrane phospholipid during autolysis showed that the total phospholipid content increased by 20% of the control as autolysis proceeded. The development of mesosomes during autolysis is shown to be a real event both biochemically and morphologically, since the plasma membrane did not show any significant changes other than the development of mesosomes during that period. This result is supported by the fact that the development of mesosomes was observed not only in freeze-fracture, but also in the other two methods of electron microscopy. Autolysis occurring during osmium fixation in hypertonic buffer seemingly reduces the number of mesosomes observed in thin sections and freeze-fracture (14, 15). This is perhaps due to the use of a hypotonic, rather than a hypertonic, buffer for osmium fixation. Cells with mesosomes may rupture resulting in an apparent reduction in number of mesosomes.

Mesosomes developed within 30 min of the initiation of incubation in hypertonic buffer, although both autolysis and the development of mesosomes were well advanced after only 15 min. When the temperature during sample preparation for electron microscopy exceeded 4°C, mesosomes were frequent in intact cells, suggesting that the mesosomes developed as soon as autolysis began. Nanninga and Fooke-Achterrath's preparation, therefore, were possibly modified by autolysis during fixation. Fixative only penetrated slowly because of rigid thick cell walls. During fixation, autolysis occurred possibly before cell death, resulting in the development of mesosomes. Higgins reported that glutaraldehyde strongly inhibited cell autolysis (15). Glutaraldehyde might act on cell walls during initial fixation and inhibit autolysis, so that it takes longer for the fixative to reach the plasma membrane, thus allowing the formation of mesosomes. Rensen also showed mesosomes in unfixed freeze-fractured cells (16), but it was not clear whether the samples were treated so as not to cause autolysis. Mesosomes have been demonstrated clearly by both Ryter-Kellenberger's fixation at

room temperature (17, 18) and single fixation with osmium tetroxide (19). These conditions seem to allow autolysis long enough for the development of mesosomes. For the isolation of mesosomes, cells are usually suspended in hypertonic buffer and incubated at room temperature for more than 1 h with lytic enzyme or with the addition of the enzyme at the end of incubation (3, 20, 21, 22). During incubation, autolysis occurs with the development of mesosomes. This may be the reason for the improved yield of mesosomes and the convenience of isolation.

Physiologically, mesosomes are thought to be the site of wall synthesis, the place of membrane depotion, the location of electron transfer chain or the initiation point of DNA duplication (2, 23). As shown in this autolysis study and others (15), mesosomes were most frequent at the cell septum region where wall lysis began, and their development was most prominent at middle log growth phase (Chart. 1), a time when cell division is most active. These facts suggest that mesosomes are involved in the synthesis and lysis of the cell wall. Higgins proposed that formation of mesosomes by fixation was due to either contraction of the cell wall or to existence of precursors of mesosomes (13). In view of the development of mesosomes, the increase of phospholipid and the rapid formation of mesosomes during autolysis, the author supports this theory of some kind of cytosol precursors, which develop into mesosomes in response to environmental stresses. This study indicates that the formation of mesosomes and/or plasma membranes is important in autolysis and wall lysis.

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Legends to Figures

Fig. 1. Electron micrographs of intact cells of *Staphylococcus aureus* at middle log growth phase (O.D.=0.8) (a, thin sectioned; b, freeze-fractured, c, negative stained) a, b, Intact *S. aureus* has no mesosomes. c, A lattice or linear structure as a very low contrast material. Cell envelope at the peripheral portion observed clearly as four layers (outer dense layer, inner dense layer and inner non dense layer from outer to inner layer, respectively). b, plasmic face (PF) with many particles, and external plasmic face (EF) with a few particles. Bar equals 0.5 μ m.

Fig. 2. Electron micrographs of cells after 15 min incubation in hypertonic buffer. (a, freeze-fractured; b, thin sectioned; c, negative stained) Mesosomes appear as a single vesicles. b, Ring-like structures; c, Note that mesosomes, especially the well developed ones, are mostly located at the cell septum. Bar equals $0.5\ \mu\text{m}$.

Fig. 3. Thin sections of cells a late log growth phase after 15 min incubation in hypertonic buffer. Mesosomes are fewer than Fig. 2. Autolysis is not uniform and intact cells and cells with detached walls are present in the same specimen.

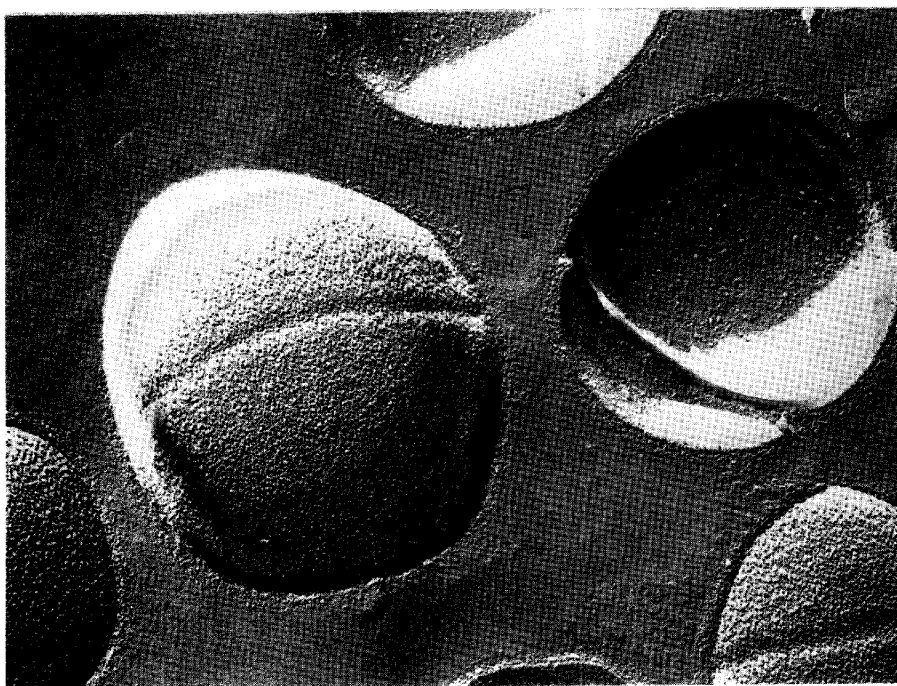
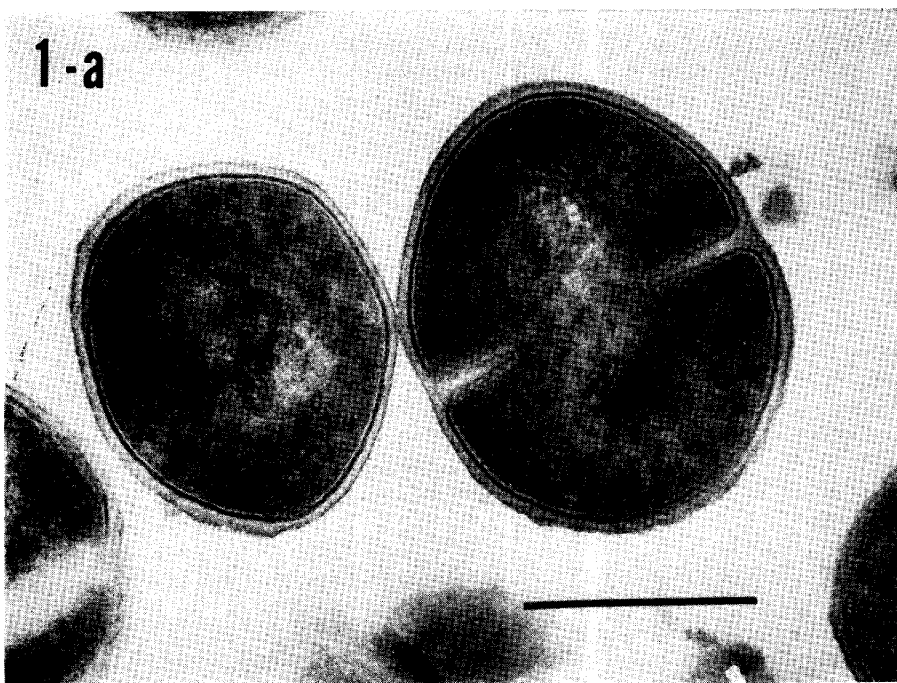
Fig. 4. Electron micrographs of cells after 1 h incubation in hypertonic buffer. (a, thin sectioned; b, freeze-fractured) a, Mesosomes are bigger and of two types, similar to Fig. 3, and a sac with the component vesicles. The sac membrane continues from the inner dense layer. Bar equals $0.5\ \mu\text{m}$

Fig. 5. Electron micrographs of cells after 2 h incubation in hypertonic buffer. (a and b, thin sectioned; c, freeze-fractured; d, negative stained) a and b, Damage of cell walls. a, Extruded mesosomes from vesicles or rods. d, vesicles, rods and linked-beads. Bar equals $0.5\ \mu\text{m}$.

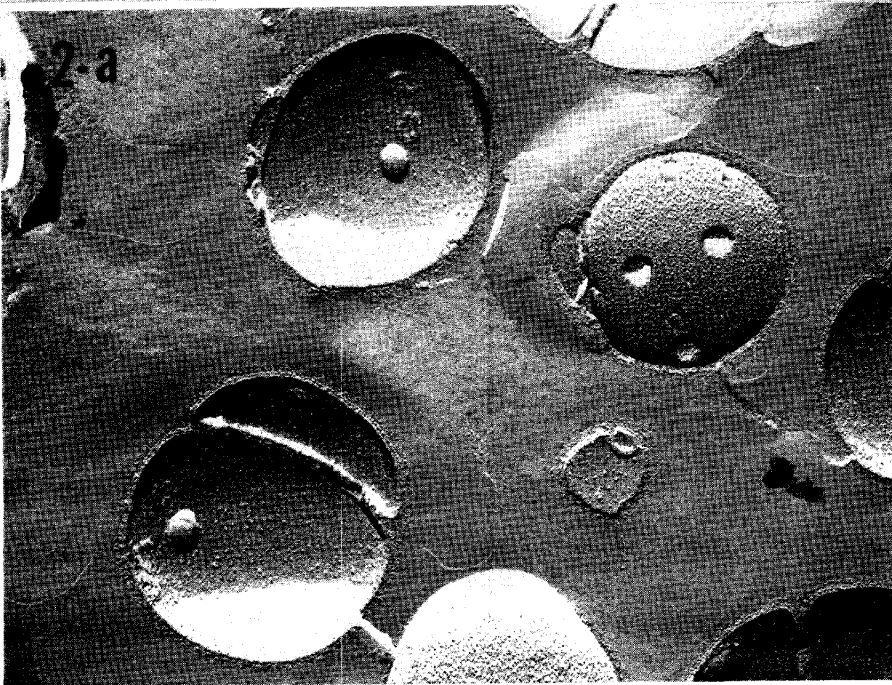
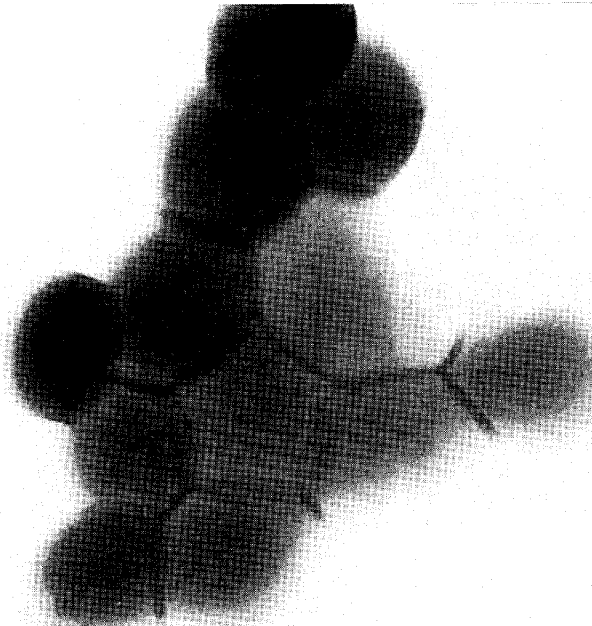
Fig. 6. Electron micrographs of cells after 4 h incubation in hypertonic buffer. (a and b, thin sectioned; c, freeze-fractured; d, negative stained) a, Protoplasts; b, Mesosomes still in a cell. c and d, Plasma membranes bulge at regions where is the cell wall detached. Bar equals $0.5\ \mu\text{m}$.

Fig. 7. Mesosomes isolated by sucrose density centrifugation. (a, negative stained; b, thin sectioned) a, Small vesicular mesosomes with polymorphysm. Most of the vesicles have a smooth surface. Some have a rough surface. Bar equals $0.5\ \mu\text{m}$.

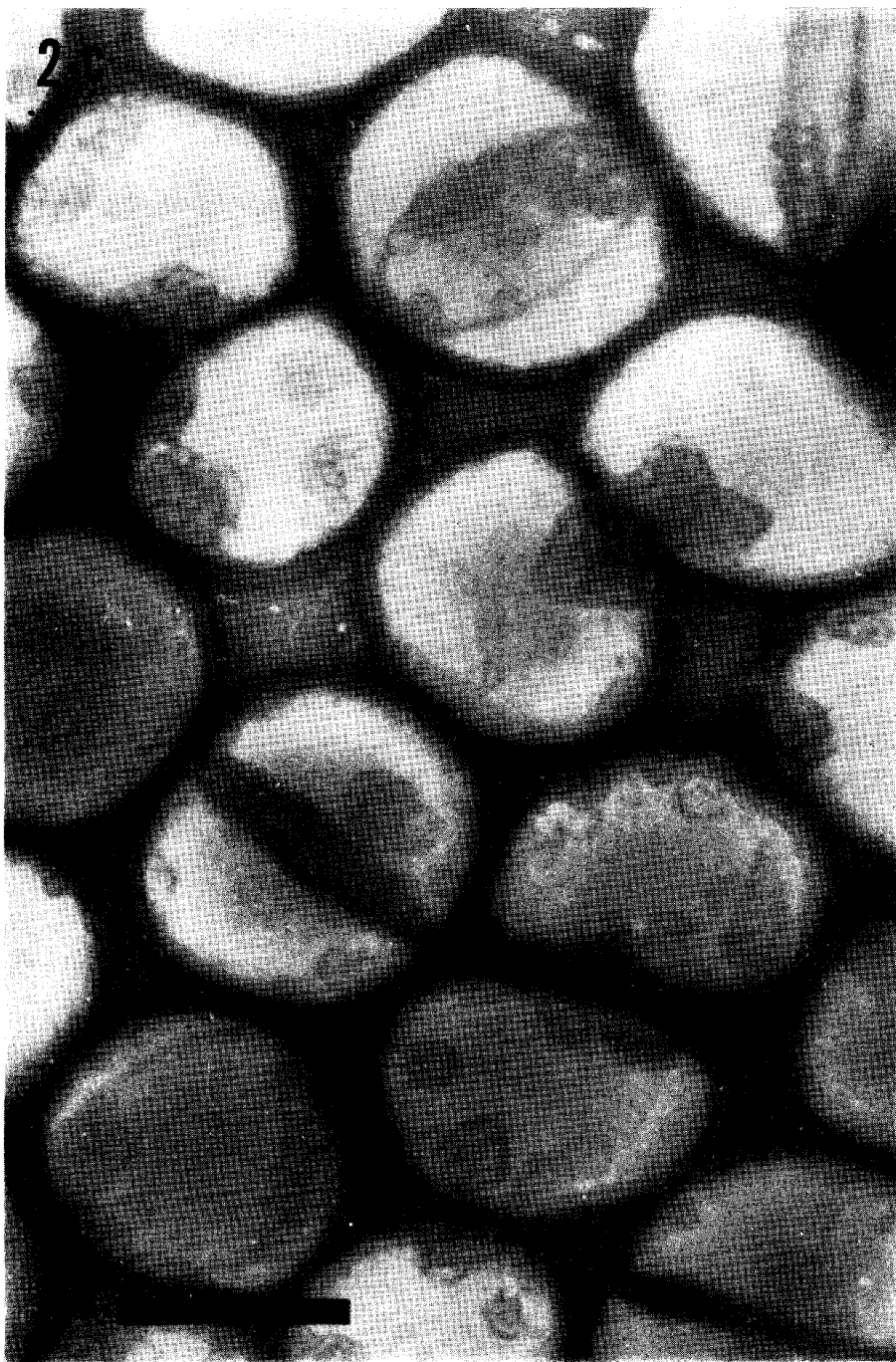
Fig. 8. Polyacrylamide-slab gel electrophoresis in 1% sodium dodecyl sulphate of plasma and mesosomal membranes. (a; mesosomal membrane; b, plasma membrane).

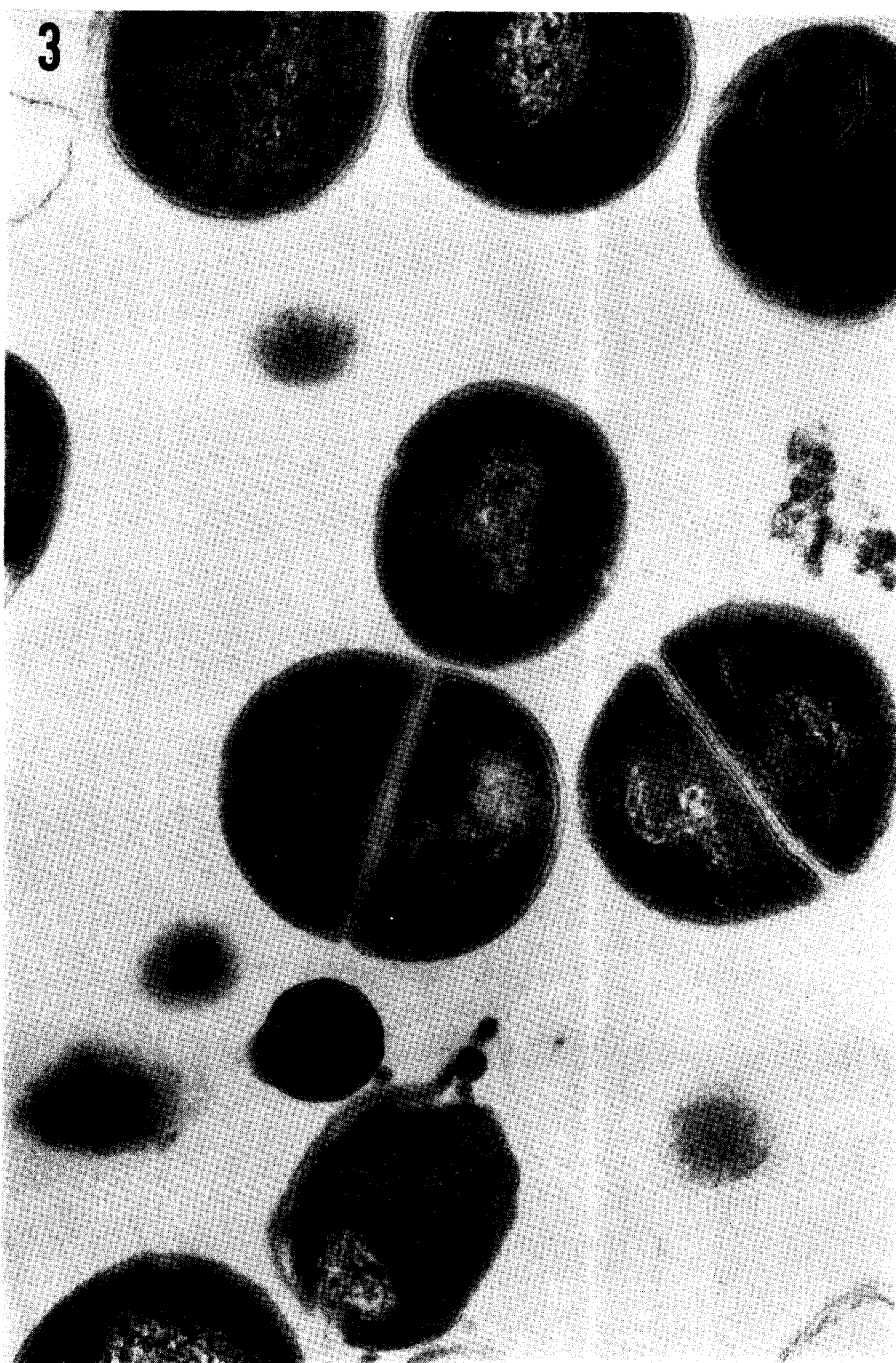


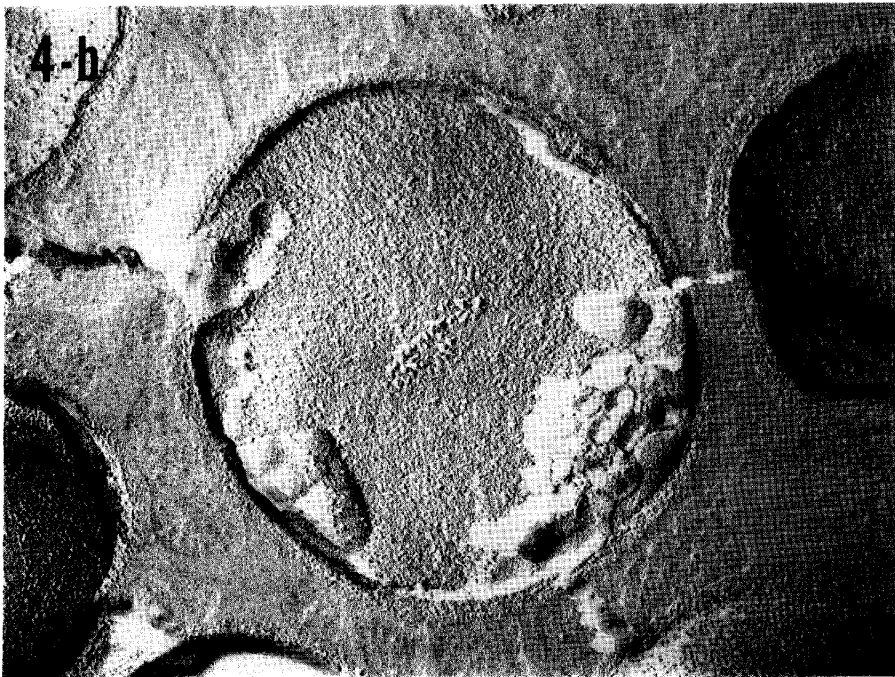
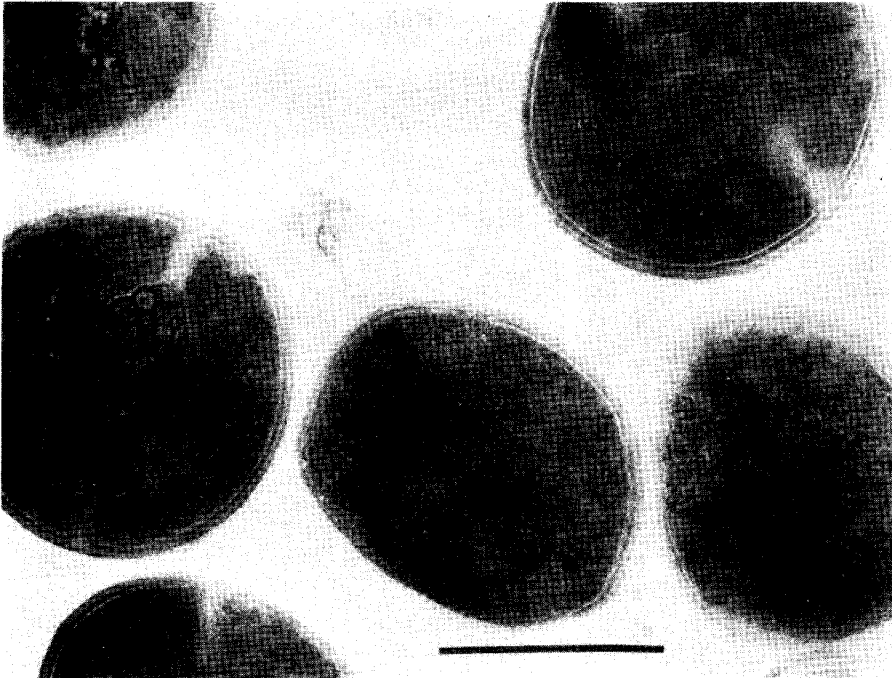
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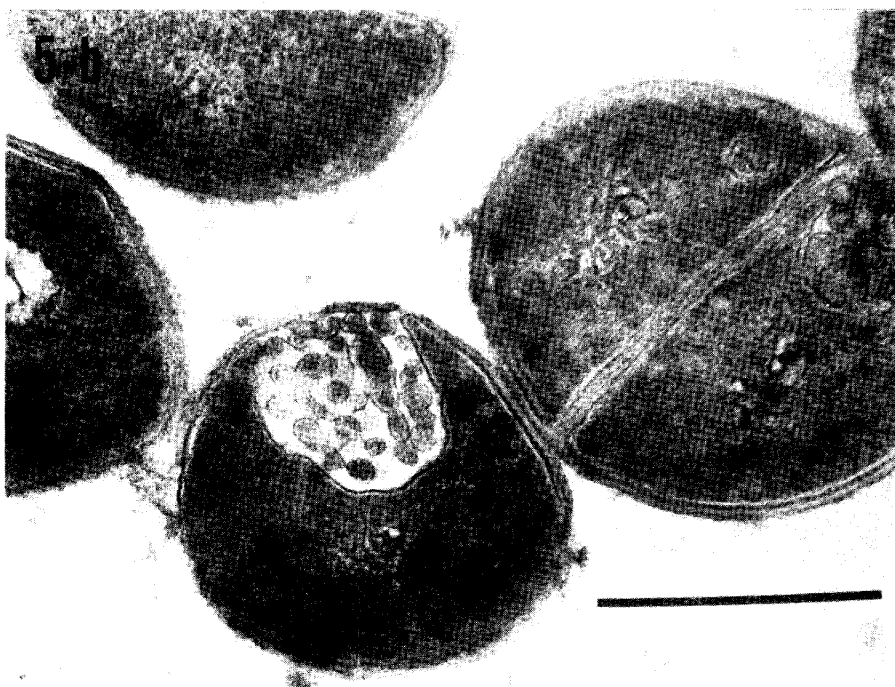
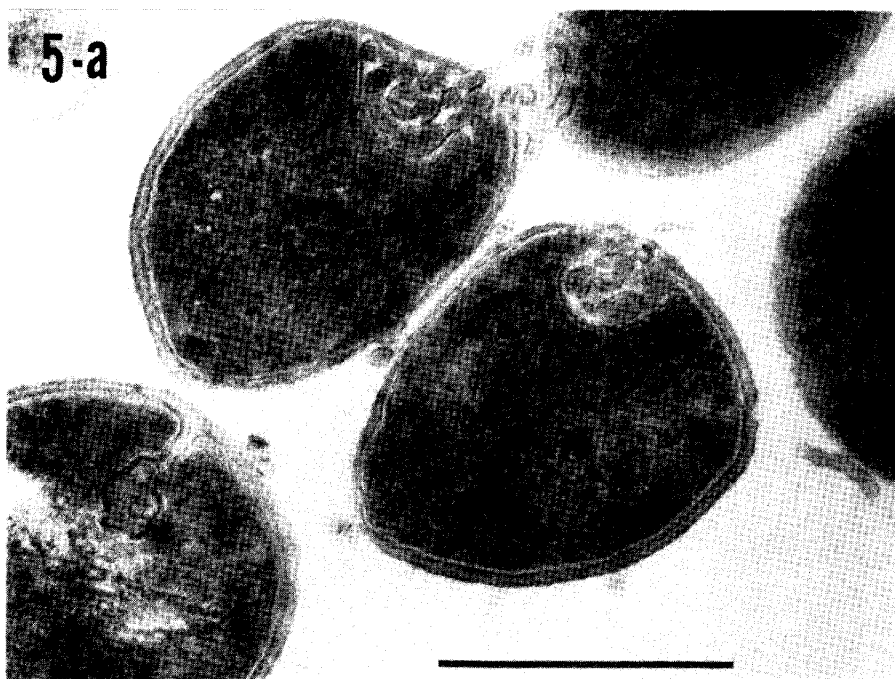


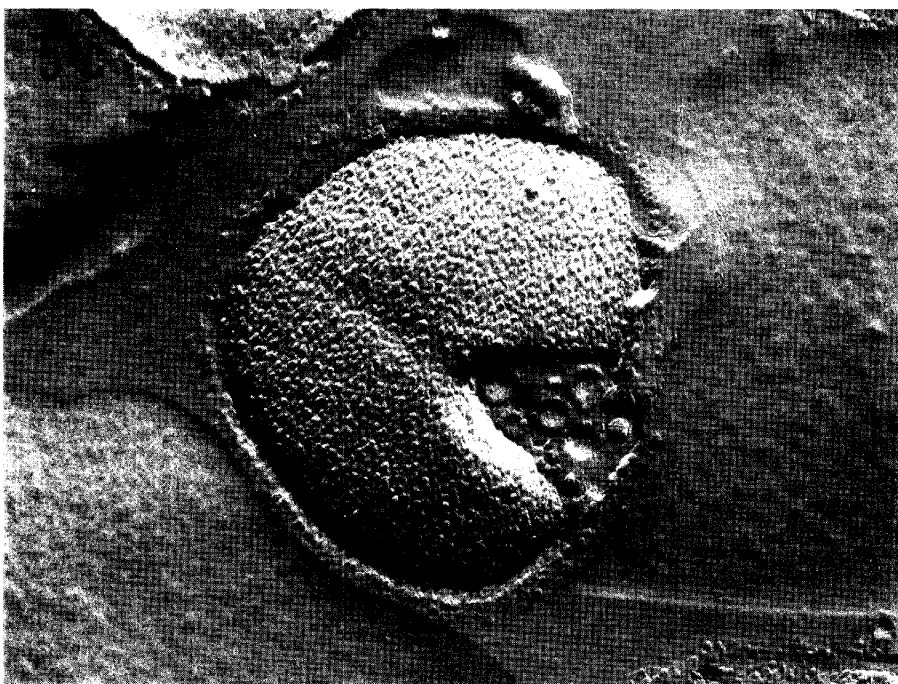












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