Acta Medica Okayama

Volume 28, *Issue* 6 1974 *Article* 1

DECEMBER 1974

Freeze-etch studies on the bacterial cell surfaces: action of the cell wall lytic enzymes on the gram-positive cocci

Ken-ichi Tomochika* Yasuhiro Kanemasa†

*Okayama University,
†Okayama University,

Copyright ©1999 OKAYAMA UNIVERSITY MEDICAL SCHOOL. All rights reserved.

Freeze-etch studies on the bacterial cell surfaces: action of the cell wall lytic enzymes on the gram-positive cocci*

Ken-ichi Tomochika and Yasuhiro Kanemasa

Abstract

Using a freeze-etching method, the ultrastructure of cell surface of gram-positive cocci was studied by digesting cell wall with lytic enzyme. In M. lysodeikticus, the cell surface revealed a very simplified ultrastructure, i. e. a single cell wall layer and a single plasma membrane layer. On the contrary, the cell surface of S. aureus exhibited a unique structure composed of two cell wall layers and a single ploasma membrane layer. The wall layers were constituted of 160 -180 A particle layer (CWl) which was unsusceptible to the L-ll enzyme and amorphous layer (CW2) which was susceptible. These results suggested that 160-180 A particles in CWl consisted mainly of the teichoic acid.

*PMID: 4282000 [PubMed - indexed for MEDLINE] Copyright ©OKAYAMA UNIVERSITY MEDICAL SCHOOL

Acta Med. Okayama **28**, 381—389 (1974)

FREEZE-ETCH STUDIES ON THE BACTERIAL CELL SURFACES — ACTION OF THE CELL WALL LYTIC ENZYMES ON THE GRAM-POSITIVE COCCI —

Ken-ichi TOMOCHIKA and Yasuhiro KANEMASA

Department of Microbiology, Okayama University Medical School Okayama, Japan (Director: Prof. J. Tawara)

Receivd for publication, May 8, 1974

Abstract: Using a freeze-etching method, the ultrastructure of cell surface of gram-positive cocci was studied by digesting cell wall with lytic enzyme. In *M. lysodeikticus*, the cell surface revealed a very simplified ultrastructure, i.e. a single cell wall layer and a single plasma membrane layer. On the contrary, the cell surface of *S. aureus* exhibited a unique structure composed of two cell wall layers and a single plasma membrane layer. The wall layers were constituted of 160-180 Å particle layer (CW1) which was unsusceptible to the L-11 enzyme and amorphous layer (CW2) which was susceptible. These results suggested that 160-180 Å particles in CW1 consisted mainly of the teichoic acid.

Fine structures of bacterial cell surface, especially the outer membrane or cell wall, are well known in gram-negative bacteria which are generally complicated in their fine structures. On the other hand, almost all gram-positive bacreria possess very simplified structures of the cell wall as clarified by chemical analyses and electron microscopic observations.

To study fine structure, bacterial cell surfaces of gram-negative and gram-positive bacilli were treated with chemicals and lytic enzymes (1, 2, 3). These results demonstrated that cell walls of gram-negative bacilli were constituted with several layers as demonstrated in gram-positive bacillus (2). FUTAI *et al.* state that the cell wall of *Staphylococcus aureus* is composed of two morphologically distinct layers both of which are susceptible to the L-11 enzyme (4). This observation is very interesting in contrast to chemical compositions of the cell wall reported by ARMSTRONG *et al.* (5).

Recently, freeze-etching or freeze-fracture methods are employed in the analysis of the cell surface structure (6, 7, 8). These methods provide more effective ways for the elucidation of surface structures. For instance, the cell wall structure of *Escherichia coli* has been extensively clarified by this method (6).

The present freeze-etching studies were undertaken to establish the fine

Produced by The Berkeley Electronic Press, 1974

structures of the cell surface of gram-positive cocci, i. e. *Micrococcus lysodeikticus* and *S. aureus*.

MATERIALS AND METHODS

Cultures and Media: *Micrococcus lysodeikticus* IAM 1056 and *Staphylococcus aureus* 209P were cultured in a phosphate nutrient broth with gentle shaking at 37°C, and the cells which reached the late logarithmic phase were harvested by centrifugation at 6,000 xg for 10 min.

Procedures for the Enzyme Treatment: The harvested cells of *M. lysodeikticus* were washed twice with physiological saline solution, and then rewashed with 0.1 M phosphate buffer (pH 6.0). Cells were resuspended in the same buffer containing 0.5 M sucrose for stabilizing protoplast. Egg lysozyme (Boehringer and Soehne Gmbh Mannheim), a bacteriolytic enzyme, was added onto the cell suspensions at the rate of 20 μg per 1 mg wet cells.

Staphylococcal cells were washed twice with physiological saline solution and rewashed twice with 0.01 M tris (hydroxymethyl) aminomethane (Tris)-HCl buffer (pH 7.2). The washed cells were suspended in the same buffer with 0.8M sucrose. Crude L-11 enzymes, that were kindly supplied by Prof. S. KOTANI, Osaka Univ., were purified by the method of KATO *et al.* (9, 10), and purified L-11 enzymes were added at the rate of 0.2 unit per mg of washed cell. The treatments with both enzymes were performed under the condition of gentle shaking at 37°C and were checked for a decrease in the optical density which was measured at 650 nm in a Coleman spectrophotometer. The treatments were stopped by chilling in an ice bath at an appropriate time. The treated cells were collected by centrifugation at 12,000 xg for 30 min.

Freeze-Etching: *M. lysodeikticus* was washed and immersed with 20% glycerol containing 0.5 M sucrose and *S. aureus* with 20% glycerol containing 0.8 M sucrose, respectively. After the penetration of glycerol into the cells at 4°C for 5 hr, cell pellet was placed on a copper block and immediately frozen in Freon-12 cooled by liquid nitrogen. The freeze-etching was carried out in an EE-FED freeze-etch apparatus (Japan Electron Optics Laboratory Co. Ltd, Tokyo). The specimens were freeze-cleaved at −120°C, etched 2 min at −100°C, shadow-casted with platinum-carbon and replicated with carbon in the high vacuum at 4 to 10×10^{-5} Torr. The replicas were cleaned in 70% H_2SO_4, rinsed in distilled water and mounted on a bare copper grid. Specimens were examined with an electron microscope, model JEM-7 (JEOL Ltd). In all micrographs shadows appear white, and shadow directions are given by the encircled arrows. The scales indicate 1 μm except for insert in Fig. 6 that has 100 nm scale.

RESULTS

Enzyme Treatments on M. lysodeikticus: *M. lysodeikticus* was used for the comparative studies because the cell wall of this organism is, for the most part, composed of peptidoglycan. The cell wall lysis with enzyme treatment was shown as an abrupt decrease in optical density, which reached a plateau

Tomochika and Kanemasa: Freeze-etch studies on the bacterial cell surfaces: action of the

by 20 min (Fig. 1).

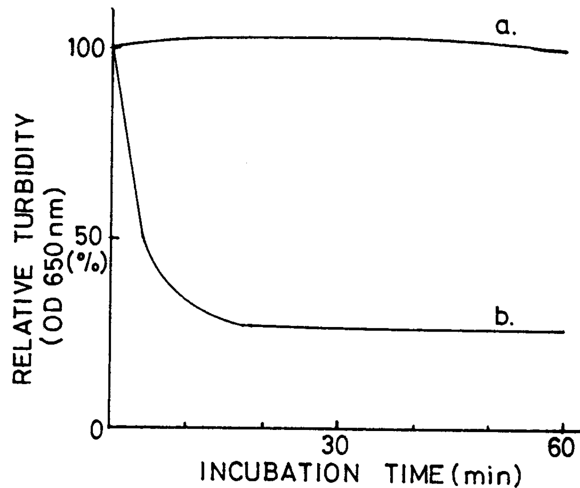

Fig. 1. Action of lysozyme on *M. lysodeikticus*.
a. Enzymatically treated. Condition: incubated in 0.1 M phosphate buffer (pH 6.0) with 0.5 M sucrose.
b. Enzymatically treated. Condition: incubated in the above described buffer containing lysozyme (20 μg/mg wet weight cells).

Freeze-etch appearances of untreated *M. lysodeikticus* are shown in Fig. 2. In cross fracturing faces the cell wall is seen to be an amorphous and diffusible single layer which is about 500 Å in thickness. There can observed no distinct differences in the surface pattern between the convex face ($\overline{PM}$) and the concave face ($\overline{PM}$) of the plasma membrane. The cave-in figures are observed on the fractured face that appeared inside the plasma membrane. These figures seem to be of scooped mesosomal vesicles (ME).

Lysozyme-treated cells on incubation for three min. reveal a figure in which the cell wall materials have been removed completely (Fig. 3). In addition, globular arrangements are observed on the plasma membrane which is constituted of the particles (arrow) ranging 150–170 Å in diameter on cross fracture face and of a convex face of plasma membrane.

When the cell treatment was prolonged more than 20 min, osmotically fragile cells (Fig. 4a) and membrane fragments (MF) (Fig. 4b) derived from the osmotically fragile cells were observed.

Enzyme Treatment on S. aureus: A bacteriolytic enzyme called the L-11 enzyme prepared from *Flavobacterium sp.* was used throughout this experiments. When *S. aureus* was exposed to L-11 enzyme, there occurred a gradual

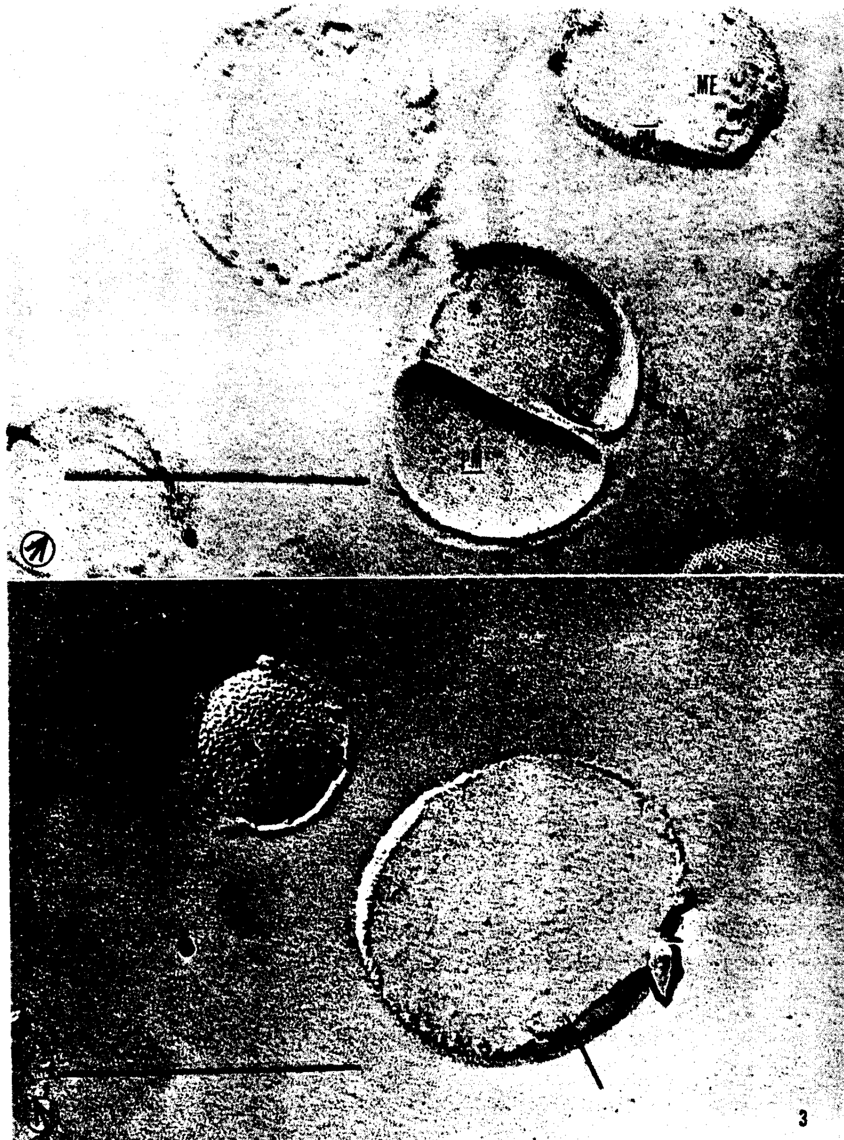

Fig. 2. Replica of freeze-etched intact *M. lysodeikticus*. CW: cell wall, PM: convex face of plasma membrane, PM : concave face of plasma membrane, and ME: mesosomal vesicles.

Fig. 3. Replica of freeze-etched *M. lysodeikticus* treated with lysozyme for 3 min. Cell wall layer was removed entirely. An arrow indicates the cross fracture face of the plasma membrane.

decrease in turbidity without a lag and the turbidity reached plateau (60% in relative turbidity) within 30 min.

The freeze-etch appearances of non-treated *S. aureus* are shown in Fig. 6. It is demonstrated that the surface layer seems to be composed of plasma

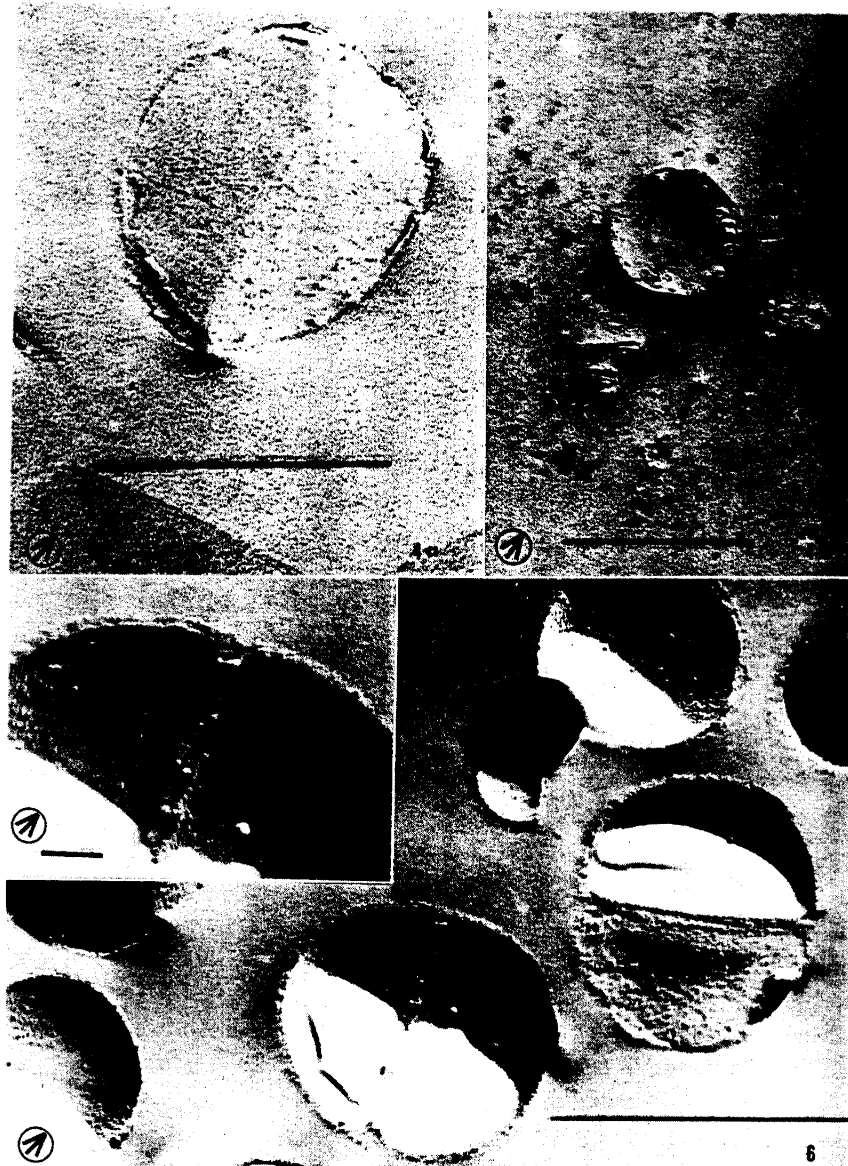

Fig. 4. Replica of freeze-etched protoplast (a) and membrane fragments (b) derived from *M. lysodeikticus* treated with lysozyme for 20 min.

Fig. 6. Replica of freeze-etched non-treated *S. aureus*. Insert: cross fracture face of surface layer at a high magnification.

membrane and cell wall layers, 360–410 Å in total thickness. More precisely, the inner-most layer is a plasma membrane formed by an array of 80–130 Å particles. The cell wall layer is seen to consist of two layers; the outer layer (CW1) contains 160–180 Å particles in proper intervals and the inner layer is amorphous, existing between the plasma membrane layer and CW1 (Insert in Fig. 6).

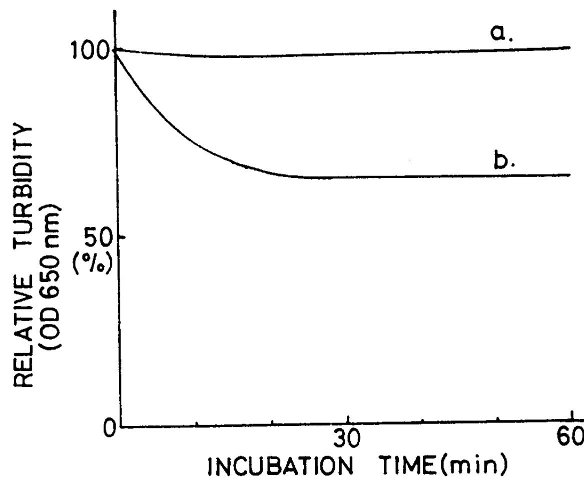

Fig. 5. Action of L-11 enzyme on *S. aureus*.
a. Enzymatically treated. Condition: incubated in 0.01 M Tris-HCl buffer (pH 7.2) with 0.8 M sucrose.
b. Enzymatically treated. Condition: incubated in the above described buffer containing L-11 enzyme (0.2 unit/mg wet weight cells).

On treating with L-11 enzyme for 5 min (Fig. 7), there appeared remarkable differences in the contour of the cell wall layers from the wall layers of non-treated cell. CW1 remained to be observed separately from the plasma membrane, because the cell wall layeı was partially digested and CW2 became wider. This phenomenon may indicate that the particles constituting CW1 are not affected by enzyme treatment. Convex face of plasma membrane $\overline{\text{(PM)}}$ reveals the particles which range from 80 to 130 Å, and in cross fracturing face of plasma membrane its particles appear to be uniform particles measuring 150 Å (Fig. 7).

The prolonged treatment (30 min) gave the protoplast from which cell wall materials were entirely removed (Fig. 8a, and 8b). Convex face of the plasma membrane $\overline{\text{(PM)}}$ appeared to be smooth, whereas concave face $\underline{\text{(PM)}}$ was densely covered with 80–130 Å particles.

Fig. 7. Replica of freeze-etched *S. aureus* treated with L-11 enzyme for 5 min. An arrow indicates the particle layer (CW1) of cell wall.

Fig. 8. Replica of freeze-etched *S. aureus* treated with L-11 enzyme for 30 min. Cell wall materials entirely disappeared.

DISCUSSION

In the present investigation, we studied the fine structure of cell surface in the gram-positive cocci, i. e. *M. lysodeikticus* and *S. aureus*, using a freeze-

etching method.

Chemically, the cell wall of gram-positive cocci consists of the peptidoglycan only or both the peptidoglycan and teichoic acid (5, 11). The cell wall of *M. lysodeikticus* contains about 90–93% peptidoglycan by dry weight and lacks teichoic acid (11). Consequently, these gram-positive cocci were treated with lysozyme having the activity of N-acetyl-muramylhydrolase that forms protoplasts easily, and the freeze-etching figures of enzymatically non-treated or treated cells agree reasonably well with the simplified structure of this organism (Fig. 9a).

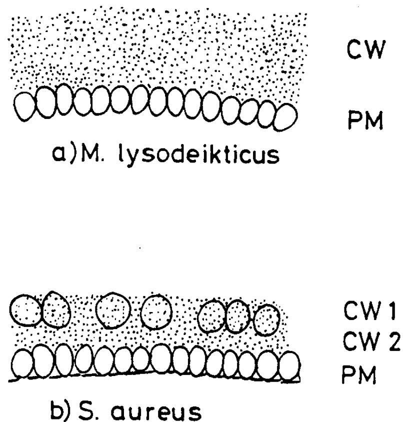

Fig. 9. Diagrams of the surface layers of *M. lysodeikticus* (a) and *S. aureus* (b).

As compared with *M. lysodeikticus*, the cell wall of *Staphylococcus* consisting approximately of peptidoglycan and teichoic acid in an equal amount (12), has a unique structure (Figs. 6 and 9b) by showing distinguishable particle and amorphous layers. Fine structures of cell walls on *S. aureus* were previously reported by SUGANUMA (13) and FUTAI *et al.* (4) by thin sectioning methods and MORIOKA *et al.* (14) by freeze-etching. SUGANUMA (13) suggested that cell walls of *S. aureus* were composed of three layers, of which the two outer layers are relatively rigid and the innermost layer is fused to plasma membrane. FUTAI *et al.* (4) drew a conclusion that a cell wall of *S. aureus* was composed of two layers by thin sectioning method using L-11 enzyme. However, their conclusion differs slightly from SUGANUMA's report (13). On the freeze-etching studies of *S. aureus*, MORIOKA *et al.* (14) have described that the cell wall exhibits multiple layers on the fracture plane. However, the fine structure of cell wall has not been revealed clearly.

From the evidence obtained after the treatment with L-11 which is known to hydrolyze amide and peptide linkages, it is appropriate to consider that CW1 is composed of teichoic acid and peptidoglycan, and CW2 is composed of peptidoglycan only or peptidoglycan-rich materials which are digested by L-11 enzyme.

From these results, it seems most reasonable to conclude that the fine structures of cell surface are evidently of stratiform as shown in Fig. 9b.

Acknowledgement: The authors wish to thank Mr. Nobuo HAYASHI for his helpful technical advice.

REFERENCES

1. SCHNAITMAN, C. A.: Effect of ethylendiamine-tetraacetic acid, triton X-100, and lysozyme on the morphology and chemical composition of isolated cell walls of *Escherichia coli*. *J. Bact.* **108**, 553-563, 1971
2. NERMUT, M. V. and MURRAY, R. G. E.: Ultrastructure of the cell wall of *Bacillus polymyxa*. *J. Bact.* **93**, 1949-1965, 1967
3. MARTIN, H. H.: Bacterial protoplasts. A review. *J. Theor. Biol.* **5**, 1-34, 1963
4. FUTAI, M., OKABAYASHI, K. and MIZUNO, D.: Electron microscopic studies on the outer layers of *Staphylococcus aureus* using a lytic enzyme from *Flavobacterium*. *Japan. J. Microbiol.* **16**, 341-350, 1972
5. ARMSTRONG, J. J., BADDILEY, J., BUCHANAN, J. G., DAVISON, A. L., KELEMEN, M. V. and NEUHAUS, F. C.: Composition of teichoic acids from a number of bacterial walls. *Nature* **184**, 247-248, 1959
6. VAN GOOL, A. P. and NANNINGA, N.: Fracture faces in the cell envelope of *Escherichia coli*. *J. Bact.* **108**, 474-481, 1971
7. FILL, A. and BRANTON, D.: Changes in the plasma membrane of *Escherichia coli* during magnesium starvation. *J. Bact.* **98**, 1320-1327, 1969
8. WATSON, S. W. and REMSEN, C. C.: Cell envelope of *Nitrosocystis oceanus*. *J. Ultrastructure Res.* **33**, 148-160, 1970
9. KATO, K., KOTANI, S., MATSUBARA, T., KOGANI, J., HASHIMOTO, S., CHIMORI, M. and KAZEKAWA, I.: Lysis of *Staphylococcus aureus* cell walls by a lytic enzyme purified from culture supernatants of *Flavobacterium* species. *Biken J.* **5**, 155-179, 1962
10. KATO, K. and STROMONGER, J. L.: Structure of the cell wall of *Staphylococcus aureus*. IX. Mechanism of hydrolysis by the L_{11} enzyme. *Biochemistry* **7**, 2754-2761, 1968
11. PERKINS, H. R. and ROGERS, H. J.: The products of the partial acid hydrolysis of the mucopeptide from cell walls of *Micrococcus lysodeikticus*. *Biochim. J.* **72**, 647-654, 1959
12. ACHIBALD, A. R., BADDILEY, J. and HECKELS, J. E.: Molecular arrangement of teichoic acid in the cell wall of *Staphylococcus lactis*. *Nature New Biol.* **241**, 29-31, 1973
13. SUGANUMA, A.: Fine structure of *Staphylococcus aureus*. *Ann. N. Y. Acad. Sci.* **128**, 26-44, 1965
14. MORIOKA, H., SUGANUMA, A., YOKOTA, Y. and TAWARA, K.: Ultrastructure of Staphylococci after freeze-etching. *J. Electronmicroscopy* **22**, 255-266, 1973