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Periplasmic Structure of Frozen-Etched and Negatively Stained Cells of *Bacillus licheniformis* as Correlated with Penicillinase Formation

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Bacillus licheniformis strain 749/C (constitutive for penicillinase formation) and uninduced cells of strain 749 (penicillinase-inducible) were examined after freeze-etching. In the early stationary phase, strain 749/C organisms had clusters of vesicles (30 to 40 nm in diameter) on the outer surface of the plasma membrane. These are randomly distributed on the membrane, including the region of septum formation. The vesicles are not intimately associated with the plasma membrane, and their inner and outer surfaces are devoid of particles. Periplasmic vesicles were not detected by freeze-etching in strain 749 (uninduced) or in young cells of 749/C; however, the membrane of mid-logarithmic phase 749/C cells had a corrugated appearance. Negatively stained 749/C cells (logarithmic phase) also showed many vesicular and tubular bodies in the periplasm as well as septal and cytoplasmic mesosomes of typical morphology. The periplasmic structures appear to be formed either by evagination of plasma membrane or by migration of vesicular bodies from the membranous pockets of the cytoplasm. Stationary phase cells of 749/C still have many periplasmic vesicular bodies; however, the mesosomes are greatly reduced both in number and size. In sharp contrast, strain 749 organisms have very few structures similar to the periplasmic bodies of strain 749/C. These findings support our previous view that penicillinase-producing cells of 749/C have periplasmic membranous structures that are rare in the uninduced strain 749, though there is some lack of correspondence between freeze-etching, negative staining, and thin section data. These structures may be important for the retention or storage of penicillinase in the cell.

The ultrastructure of *Bacillus licheniformis* strains 749 and 749/C has been examined by Ghosh, Sargent, and Lampen (2) in relation to penicillinase induction and secretion. Thin sections and negatively stained cells of the penicillinase-magnoconstitutive strain 749/C showed tubules and vesicles in the periplasm. Comparable structures were also formed in the penicillinase-inducible strain 749 during induction. These tubules and vesicles were released during protoplast formation and contained 60% of the cell-bound penicillinase (11). The specific activity of penicillinase in the particles was six times greater than plasma membrane, and, unlike plasma membrane, these tubules and vesicles did not contain a significant amount of reduced nicotinamide adenine dinucleotide (NADH) oxidase. It was concluded that these structures probably constitute a special location of penicillinase in the cell, though the structure is certainly not essential for penicillinase secretion (12).

The kinds of artifacts that might be produced by the freeze-etching technique would almost certainly differ from those resulting from fixation procedures or negative staining. We have, therefore, examined *B. licheniformis* strains 749 (uninduced) and 749/C (constitutive for penicillinase) by freeze-etching and confirmed the presence of periplasmic vesicles in strain 749/C. Results obtained by freeze-etching have been correlated with the observations made by negative staining.

MATERIALS AND METHODS

Organism, inocula, and media. *B. licheniformis* strain 749 (a penicillinase-inducible strain) and 749/C (a penicillinase magnoconstitutive mutant of 749) were used throughout the investigation. The method of cultivation and the casein hydrolysate/salts medium (CH/S) have been described (2).

Organisms were grown as follows. Spores were maintained on agar slants containing per liter: potato extract, 10 g; Casitone, 10 g (Difco); yeast extract, 2 g (Difco); and salt mixture as used in CH/S medium (P. Hill, *personal communication*). (i) Spores were spread on agar plates containing CH/S medium and Andrade's indicator by the method of Lampen (3) and were incubated at 28 C for 10 hr (logarithmic phase) or 18 to 20 hr (stationary phase). In certain experiments, the cells were removed directly from the agar surface and treated for freeze-etching and negative staining. (ii) The growth from one agar plate was transferred to 150 ml of CH/S medium, and was incubated with shaking at 30 C. Samples were taken at various time intervals and centrifuged; the pellet was suspended in a small amount of distilled water and used for negative staining. Cells were taken directly from the pellet for freeze-etching. Organisms from three different growth phases were generally used: early logarithmic phase (2.5 hr growth), mid-logarithmic phase (5 hr growth), and early stationary phase (18 to 20 hr growth).

Electron microscopy. Freeze-etching has been carried out as previously described (8).

Negative staining was done with a 2% solution of phosphotungstic acid (PTA) in demineralized water adjusted with 0.1 N KOH to *p*H 6.1 to 6.2. A drop of cell suspension was placed on hydrophilic, carbon-coated, 400-mesh, copper grids (13); after 2 min the drop of cell suspension was removed with a piece of filter paper. Immediately, a drop of PTA was placed on the moist grid and removed by pieces of filter paper after 2 min of staining. These grids were placed upside down on bibulous paper to remove any traces of liquid and were finally dried in a vacuum evaporator for 5 min. It is particularly important to spread the stain uniformly since accumulation of a pool of stain in the cell walls and periplasm tends to mask any structures present. The method of drying the grids after specimen preparation is also critical. To prevent disorganization of biological structures from exposure to the electron beam in the presence of moisture, the stained specimen should be dried in an evaporator and examined immediately.

Freeze-etched specimens were examined by Philips EM 300 (Philips Electronic Instruments, Mt. Vernon, N.Y.) and JEM 120 (Japan Electron Optics Co., Medford, Mass.) electron microscopes at acceleration voltages of 60 and 80 kv, respectively. Negatively stained material was examined at an acceleration voltage of 120 kv by the JEM 120 electron microscope. Photographic records were made on Kodak electron image plates. Enlargements were made with a Durst enlarger model S-45 electron microscope (Durst Inc., Long Island City, N.Y.) having a point light source.

RESULTS

Structure by freeze-etching. Stationary phase cells of 749/C grown in liquid medium showed clusters of vesicles in the peripheral region between cell wall and plasma membrane (periplasmic space). These vesicles are 30 to 40 nm in diameter. Their limiting membrane (both inner and outer surface) is smooth and similar in appearance to the ground substance of the plasma membrane (Fig. 1–5). In a few cases there are particles present on the vesicle membrane (Fig. 1 and 4). The clusters of vesicles are randomly distributed on the outer surface of the plasma membrane, including regions of septum formation (Fig. 5). They do not penetrate the plasma membrane and, when removed, leave a depression on the membrane which, in this region, has the usual particulate appearance (Fig. 5). The imprint of a cluster of vesicles on the inner septal wall surface can be seen in Fig. 3, indicating that even at the septum the vesicles are periplasmic. No periplasmic vesicular bodies were detected in uninduced cells of strain 749 grown under identical conditions.

Early logarithmic phase organisms of strains 749 (uninduced) or 749/C showed no periplasmic structures (Fig. 7 and 8); however, in mid-logarithmic phase cells of 749/C, the inner surface of the plasma membrane had a highly corrugated appearance (Fig. 6). Uninduced strain 749 cells of this age did not show corrugations. Thus, the major differences between the two strains were seen in cells from the early stationary phase of growth (Table 1).

Structure by negative staining. The cells of strains 749 and 749/C stained with PTA have a sharply defined profile of cell wall and protoplast (Fig. 9–12). In the logarithmic phase (5-hr CH/S medium cells and 10-hr agar cells), both strains contain many mesosomes (Table 1), although the number varies widely from one cell to another. In a chain of organisms, one long cell may have 20 mesosomes, whereas the adjacent short one contains only 3 or 4 (Fig. 9).

The mesosomes can be classified into two groups which correlate with their location: (i) large mesosomes in the region of septum formation and extending into the center of the cell, which are lamellated structures composed of coiled tubules (Fig. 9, ml) or membranous sacs (Fig. 9, ma); (ii) mesosomes randomly distributed in the cytoplasm (Fig. 9, ms), which are much smaller and are composed of tightly coiled tubules. Some of the mesosomes are connected to the periplasm through a broad opening (Fig. 9 and 10), and there appears to have been migration of vesicular bodies from these mesosomal pockets into the periplasmic space (Fig. 10).

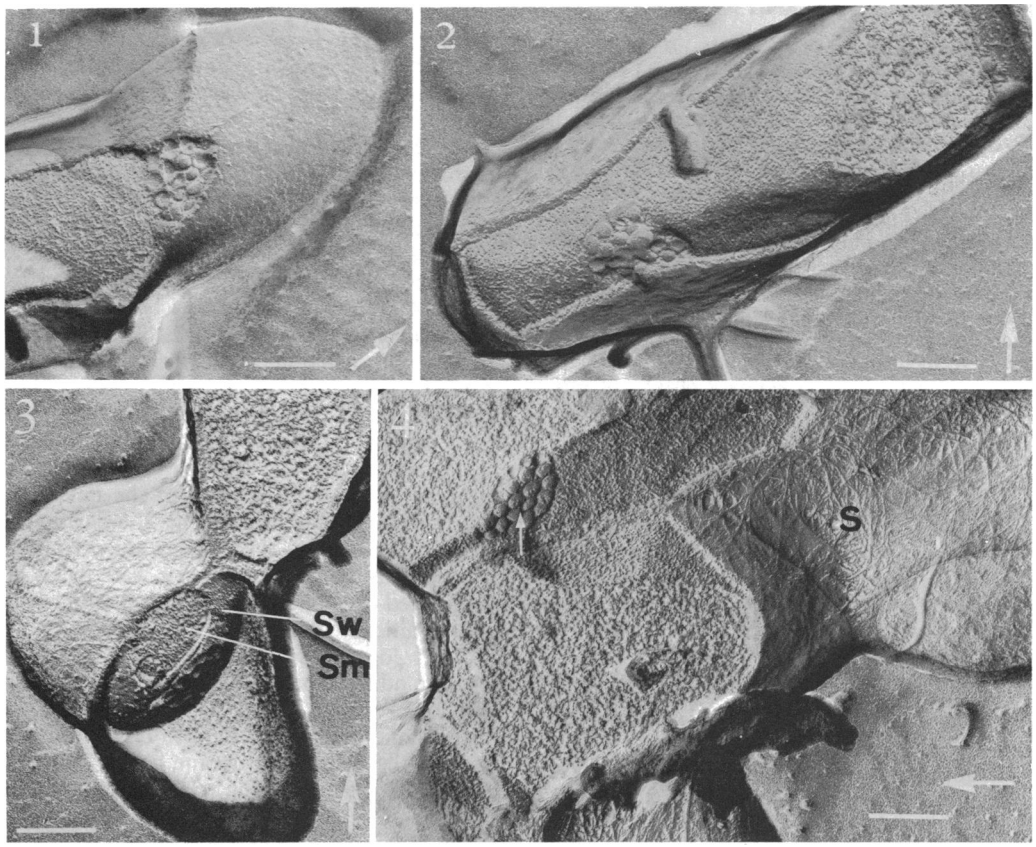


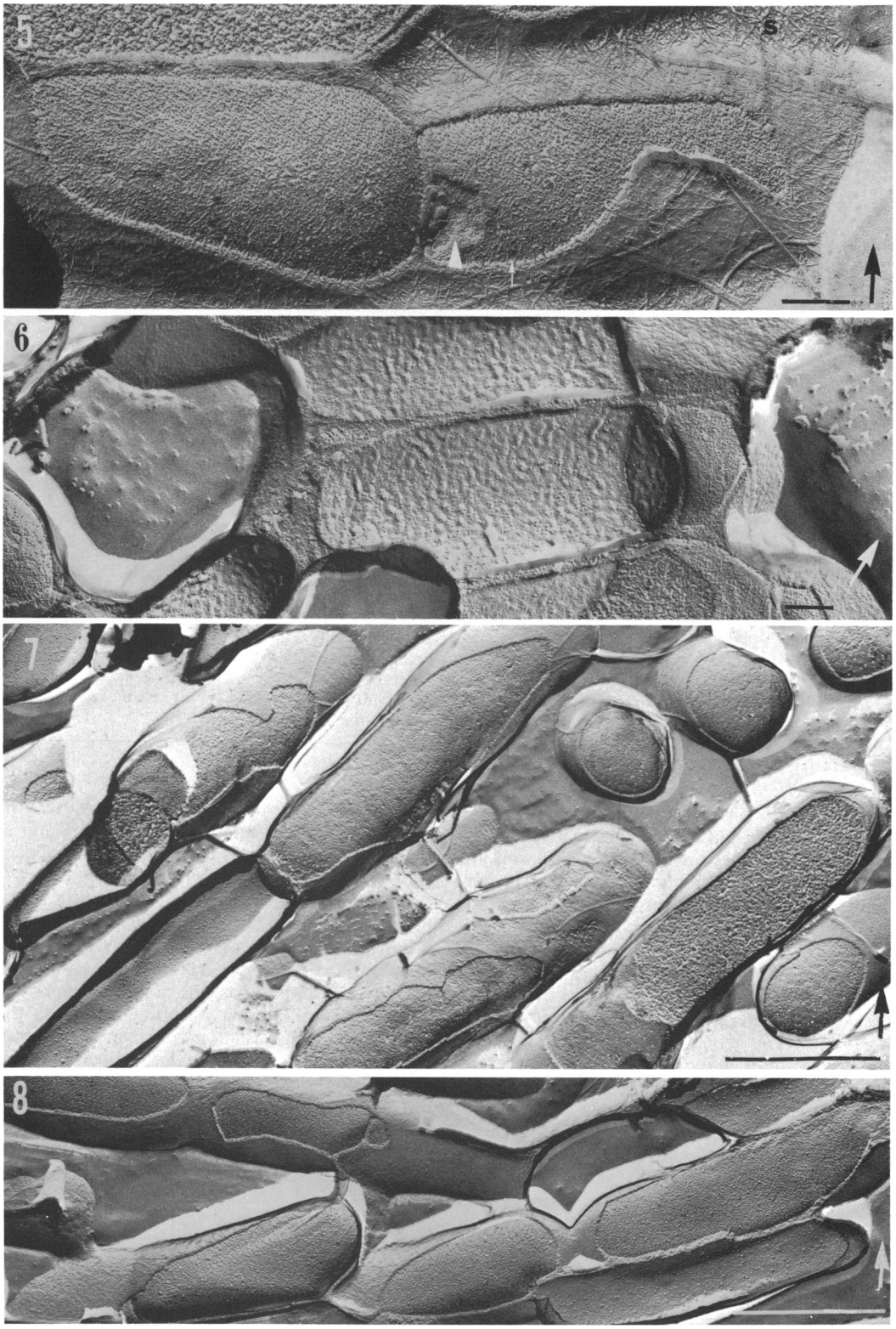
FIG. 1–4. *Freeze-etched preparations from strain 749/C cells grown in CH/S medium for 20 hr. Clusters of vesicles are presented on the outer surface of plasma membrane. The vesicles pile up in the periplasmic space (Fig. 1) and do not extend into the cytoplasm (Fig. 4). The inner and outer surfaces of the vesicles are smooth, but the latter may have a few particles (Fig. 4, arrow). A fracture through the septum exposes the septal membrane (Fig. 3, Sm) and wall (Fig. 3, Sw). The septal wall shows the imprints of vesicles removed during fracturing (Fig. 3). The smooth outer surface of cell wall is occasionally covered with fibrillar slime material (Fig. 4, S). Marker denotes 2 μm. Arrows in the right-hand corners indicate the direction of shadow.*

The periplasm of 5-hr CH/S medium cells and 10-hr agar cells (mid-logarithmic phase) of strain 749/C contains many complex vesicular and tubular bodies. Similar negative stains of the uninduced strain 749 show a few smaller and less complex structures (Fig. 13 and 14). The periplasmic bodies of strain 749/C appear to arise as an evagination of the plasma membrane (Fig. 11). Some are vesicles (Fig. 12), whereas others are lamellated structures apparently formed by folding and refolding of membrane (Fig. 11). The cell wall is pushed out, probably as a result of growth of the membranous material into the periplasmic space (Fig. 11).

FIG. 5. *Freeze-etched preparation from 749/C cells grown in CH/S medium for 20 hr. Vesicles are present in the region of septum formation. When vesicles are removed during fracturing, they leave a shallow depression on the plasma membrane; this region of membrane has the usual particulate appearance (arrow head). Microfibrils are visible on the plasma membrane and sometimes continue into the cell wall (arrow). Note the slime layer (S) outside the cell. Marker denotes 2 μm. Arrow in the right hand corner indicates the direction of shadow.*

FIG. 6. *Freeze-etched preparation from 749/C cells grown on agar medium for 10 hr. The inner surface of the plasma membrane has a corrugated appearance. Marker denotes 2 μm. Arrow in the right hand corner indicates the direction of shadow.*

FIG. 7 and 8. *Frozen-etched young cells of strains 749 (Fig. 7) and 749/C (Fig. 8) grown in CH/S medium for 2.5 hr. No appreciable difference can be detected; note the presence of a smooth cell wall, the absence of slime layer on the cell wall, and particles on inner and outer surfaces of plasma membrane. Markers denote 10 μm.*



FIGS. 5 to 8

TABLE 1. *Periplasmic structures and mesosomes of Bacillus licheniformis cells from cultures of different ages*

Electron microscopy technique	Strain	Age of culture		
		2.5 hr	10 hr	20 hr
Freeze-etching	749/C	No structure	Corrugation of plasma membrane	Vesicles (30–40 nm) in periplasm
	749	No structure	No structure	No structure
Negative staining	749/C	Very few vesicles and tubules in periplasm Invaginations of plasma membrane with broad opening to periplasm Septal mesosomes	1. Large number of vesicles and tubules in periplasm 2. Invaginations of plasma membrane with broad opening and containing many vesicles which migrate to periplasm Septal mesosomes	Vesicles mainly in periplasm Invaginations of plasma membrane almost gone Septal mesosomes almost gone
	749	Very few vesicles and tubules in periplasm Invaginations of plasma membrane with broad opening to periplasm Septal mesosomes	Structures comparable to (1) and (2) of 10 hr 749/C cells are rarely found Septal mesosomes are similar to those of 749/C	Very few periplasmic structures present
Ultrathin section[a]	749/C	Not done	Complex septal mesosomes Invaginations of plasma membrane and the resultant pocket full of vesicles and tubules (periplasmic body)	Not done
	749	Not done	Simple septal mesosomes No periplasmic structures	Not done

[a] Data from B. K. Ghosh et al. (2).

In stationary phase 749/C cells (20-hr CH/S medium) the periplasm contains many vesicles, but the mesosomes inside the cytoplasm are greatly reduced in number and size and are ill-defined (Fig. 12). Septal mesosomes have essentially disappeared from these older cells.

The structure of uninduced 749 cells grown on agar medium for 20 and 10 hr are shown in Fig. 13 and 14, respectively. Ten-hour cells have many cytoplasmic mesosomes which are similar to those in strain 749/C (*compare* Fig. 9, 10, and 14). The periplasm of uninduced 20-hr cells of strain 749 contains some small vesicles, but many fewer than in 749/C cells. Also, strain 749 did not show the lamellated periplasmic membranous structures characteristic of strain 749/C (Table 1).

DISCUSSION

In this paper we have demonstrated by freeze-etching that early stationary phase cells of strain 749/C have clusters of vesicles on the outer surface of the plasma membrane. Comparable types of vesicles have been detected in *B. subtilis* by Remsen (9) and by Nanninga (7). However, the vesicles of *B. licheniformis* strain 749/C differ from those of *B. subtilis* in the following respects. (i) None were seen in young cells (2 to 5 hr). (ii) Clusters of vesicles covered by a thin membranous envelope were not found in the cytoplasm. (iii) Vesicles could not be detected at the inner surface of plasma membrane. (iv) Vesicles are situated loosely on the plasma membrane. The regions of plasma membrane carrying the vesicles have the usual particulate appearance, whereas such areas of *B. subtilis* membrane are smooth and contrast sharply with plasma membrane (7).

In a previous study of strains 749 and 749/C with negative staining and thin sectioning (2), we reported that strain 749 lacked extensive intracytoplasmic membranes and that the predominant morphological features of 749/C were large invaginations of the plasma membrane containing vesicles and tubules. By using a better microscope and an improved negative staining method, we now report that intracytoplasmic

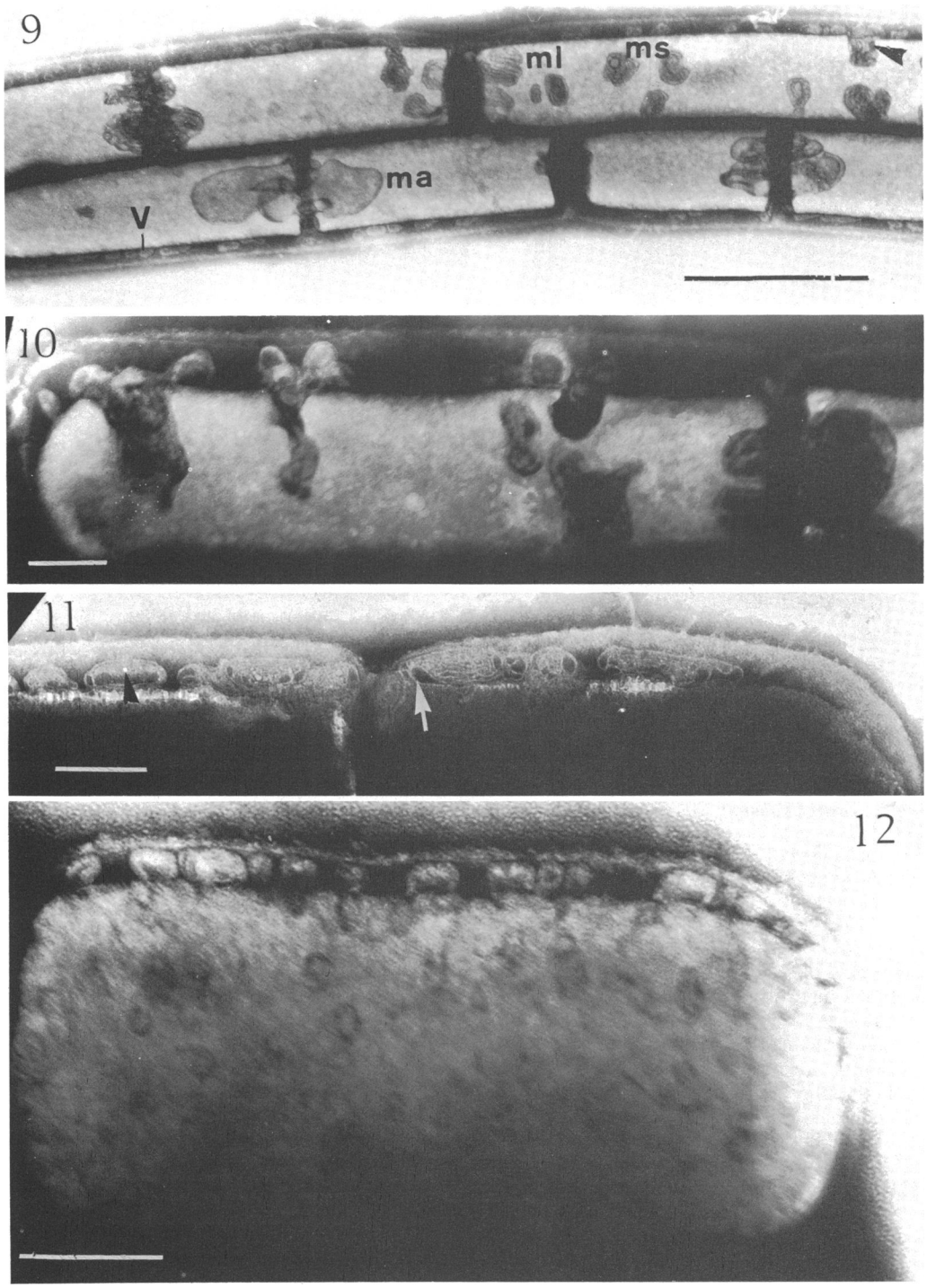


FIG. 9. *Negatively stained (PTA) cells of strain 749/C grown on agar for 10 hr. Septal mesosomes in the form of membranous bags (ma) or lamellated structures (ml), small lamellated mesosomes (ms) randomly distributed in the cytoplasm, mesosomal contents released into the periplasm (arrow head), and many vesicles in the periplasm (V). Marker denotes 2 μm.*

FIG. 10–12. *Negatively stained (PTA) cells of strain 749/C grown on agar for 10 hr (Fig. 10 and 11) and 20 hr (Fig. 12). Note the vesicles in the periplasmic space and continuation of mesosomal material into the periplasm (Fig. 10); the plasma membrane seems to evaginate and fold into lamellated structure (Fig. 11, white arrow) in the periplasm; this membrane has many attached particles (arrow head). There are vesicles in the periplasm of 20-hr cells (Fig. 12), but mesosomal pockets are not seen. Markers denote 2 μm.*

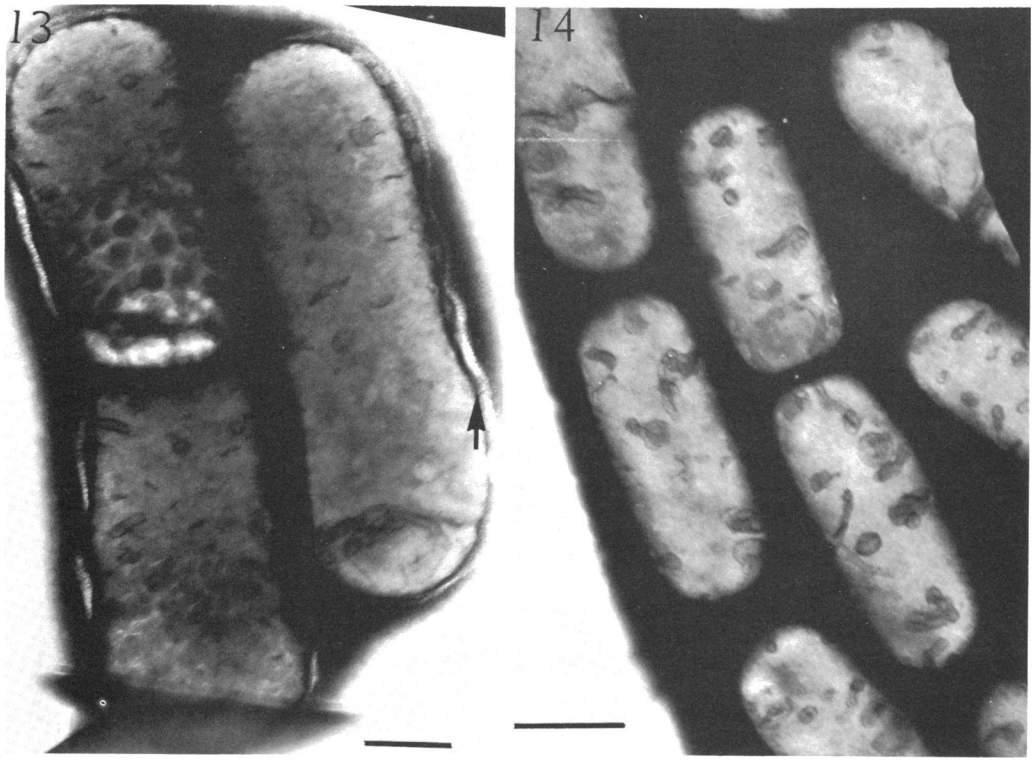


FIG. 13 and 14. *Negatively stained (PTA) uninduced 749 cells grown on agar. The periplasm of 20-hr cells (Fig. 13) contrasts sharply with 749/C cells (Fig. 12) in having only a few ill-defined vesicles; usually the cell wall of 749 cells remains closely apposed to the protoplast and no vesicles are seen (Fig. 13, arrow). In 10-hr 749 cells (Fig. 14), mesosomes deep in the cytoplasm are noted as in 749/C cells (Fig. 9). Though the periplasm is not visible in Fig. 13 due to heavy accumulation of stain, 749 cells did not usually show evaginated membrane, vesicles released from mesosomal pockets, or lamellated membranous tubules in the periplasm as observed in 749/C cells. Markers denote 1 μm (Fig. 13) and 5 μm (Fig. 14).*

membranes of 749 and 749/C are more highly developed than we thought previously.

Figures 9–14 illustrate three types of membranous structures. The septal and cytoplasmic mesosomes are present in both 749 and 749/C (Table 1); the periplasmic bodies are found in large numbers in 749/C but very infrequently in 749. These bodies consist of an abundance of vesicular and tubular structures lying loose in the periplasm or confined to broad-mouthed pockets in the plasma membrane. The vesicles may be derived from the plasma membrane by evagination or by migration of vesicles included in the mesosomes into the periplasmic space. We feel that they are located in the periplasm rather than in the cell wall, since they can be seen in a zone of electron-dense PTA which has accumulated between the profile of the protoplast and an electron-lucid material to the outside which is probably the cell wall (Fig. 9).

It is particularly interesting to note the changes in membranous structures during growth (Table 1). In mid-log-phase cells of 749/C, all three types of structure can be seen; by 20 hr, however, the cytoplasmic and septal mesosomes have disappeared, whereas the periplasmic vesicles have substantially increased in number (Fig. 12). Thus, the state of the periplasmic vesicles seems to vary independently of the mesosomes, suggesting strongly that penicillinase is not associated with what may be termed "the classical mesosome" (6).

Several aspects of the freeze-etching data contrast with the data for negative staining; thus, we have not found cytoplasmic or septal mesosomes in freeze-etched, log-phase cells of 749 and 749/C. In addition, the periplasmic vesicles seen in log-phase cells by negative staining are not seen in freeze-etched preparations. Corrugation of the plasma membrane is, however, evident in 749/C, yet it is absent from 749. Cells from early stationary phase cultures correspond best

by the two methods, yet it is at this time that the difference between 749 and 749/C is most marked. Lastly, the periplasmic structures themselves have a different appearance by the two methods. Freeze-etching gives highly regular clusters of spherical vesicles. By negative staining the form is much more irregular; vesicles are visible, but tubules and irregular structures of various sizes can also be seen. These differences require further exploration. It is possible that the freeze-etching method as used here is strongly biased against exposing the cytoplasmic mesosome during a fracture, and, in fact, less than 10% of the fractures do pass through the cytoplasm, though clearly this will not explain the absence of periplasmic bodies from 10-hr cells, nor their irregularity by negative staining.

Negative staining is subject to the criticism that PTA may interact with the specimen and alter the structure; also, because the unfixed specimen is exposed to the electron beam, secondary modifications in structure are possible. There is also a problem in interpreting the micrographs obtained by negative staining because of the superimposition of images.

Freeze-etching, on the other hand, is open to entirely different criticisms. In particular, the effect of freezing on the cell should be considered seriously. Although it has been shown that the microorganisms retain their viability after freezing for the freeze-etching technique (5), it is also known that to retain viability it is necessary for unbound water to leave the cell before it freezes (4). This flux of water is powered by the higher chemical potential of the internal water achieved in the cell after the external medium has frozen, but before the internal medium has frozen. Therefore, the effect of nonlethal freezing may be similar to the effect of a hypertonic medium and this is known to be expressed by the eversion of mesosomes (14). Similarly it is known that low temperatures (albeit above freezing point) cause mesosome eversion (1). There have been other reports illustrating discrepancies between freeze-etching and conventional methods (10; M. E. Bayer and C. C. Remsen, *in press*).

We conclude, therefore, that the results obtained by freeze-etching support our original view that certain periplasmic structures are present in the penicillinase constitutive strain 749/C but are absent from the uninduced inducible parent strain 749. This view, however, is qualified to the extent that it is not possible to correlate in detail structures seen by negative staining and freeze-etching.

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