Morphological Phenomena Associated with Penicillinase Induction and Secretion in *Bacillus licheniformis*

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Cells of uninduced *Bacillus licheniformis* (strain 749) in mid-logarithmic phase have no extensive intracytoplasmic membrane. After induction with cephalosporin C, characteristic organelles that contain tubules and vesicles with single-layered membranes and no visible internal substructure can be seen in thin sections in the periplasm. A magnoconstitutive penicillinase producer (749/C) contains similar structures. It is suggested that they represent a penicillinase secretory apparatus. In the first 15 min after induction, negatively stained preparations of induced 749 show large intracellular vesicles without individual contact with the cell surface. Negatively stained 749/C and fully induced 749 contain invaginations comparable to the structures seen in thin sections. When protoplasts of induced 749 and of 749/C are prepared, vesicles and tubules similar to those seen in thin sections of whole cells are liberated from the cell. Growing protoplasts of induced 749 show massive convolutions of the peripheral membrane, multiple layers of membrane, and characteristic long, slender tubules extending from the protoplast surface. These phenomena are not observed in uninduced 749 except for the production of a relatively small number of tubules. In 749/C, there were fewer convolutions than in induced 749, although tubule production was similar. Multiple layers of membrane were not observed in 749/C. The relation of the penicillinase secretory structures to mesosomes and to secretory structures of other organisms is discussed.

Investigations of mammalian systems have revealed a general requirement for specific structures in the transport of macromolecules across the permeability barrier (8, 20, 21). Lampen (11) suggested that the mesosome or other invaginations of the plasma membrane may have a comparable role in the production and release of exoenzymes in bacteria. Well-organized mesosomes have been demonstrated in a strain of *Bacillus licheniformis* by Ryter and Landman (28).

We have selected *B. licheniformis* for morphological studies of penicillinase secretion in conjunction with more biochemical approaches. A magnoconstitutive strain (749/C) derived from an inducible parent (749) by a single mutation is available in which 1.5% of the cell protein is penicillinase (11). The behavior of a range of penicillins as inducers, substrates, and antibiotics has been studied extensively (22, 23).

The constitutive (749/C) and inducible (749) penicillinase-producing strains of *B. licheniformis*

liberate up to 50% of the total enzyme into the medium (23). The cell-bound enzyme is thought to be covalently bound to the membrane (12). It is freely accessible to substrate (11), partially accessible to antibodies to exopenicillinase, and it can be liberated as free exoenzyme by exposure to trypsin in hypertonic sucrose (10). There is strong evidence that the enzyme is located outside the permeability barrier (10, 11), and the cell-bound enzyme probably is an intermediate in exoenzyme formation (3, 11).

During protoplast formation at pH 6.5, about two-thirds of the enzyme is liberated and the rest remains with the protoplast. The bulk of the penicillinase liberated is excluded from Sephadex G-75 and is, in fact, particulate (30).

We have examined thin sections and negatively stained preparations of cells and protoplasts by electron microscopy and we have monitored the time courses of induction and of protoplast formation by morphological examination.

MATERIALS AND METHODS

Organisms, inocula, and media. *B. licheniformis* strains 749 and 749/C were maintained as spores on soil extract agar. Inocula were prepared and cultures were inoculated as described by Lampen (12). Cells were grown at 30 C in 500-ml flasks that contained 150 ml of a modified CH/S medium (23). The medium contained a casein hydrolysate (Casitone, Difco), 1%; 0.02 M phosphate buffer (*p*H 7.5); and the salts used by Pollock (23). Growth and cell concentrations were determined by measuring turbidity at 540 nm in a Klett-Summerson colorimeter and then converting to milligrams (dry weight equivalent) by a standard curve.

Induction of penicillinase. The growth from one plate culture of strain 749 was washed into 100 ml of CH/S medium to give a cell density of 0.04 mg per ml, and the mixture was shaken at 30 C. After 2 hr, when the growth had reached 0.12 mg/ml, cephalosporin C (a gift from Eli Lilly & Co., Indianapolis, Ind.) was added to the medium (1 μg per ml) and the shaking was continued. The time of addition of inducer constituted zero-time for the experiment. At certain intervals, samples were collected for turbidity measurement, fixation, negative staining, and penicillinase estimation. Penicillinase was determined by the method of Sargent (29).

Protoplast formation. Mid-logarithmic phase cells of 749 and 749/C were used for protoplast formation. The cells were centrifuged and washed; then they were suspended in the protoplasting medium. The incubation mixture had the following composition: 0.75 M sucrose; 0.02 M phosphate buffer, *p*H 6.5; 0.001 M MgCl₂; special salt mixture (as used in CH/S medium), 0.1 ml/100 ml; enzymatic hydrolysate of casein (Calbiochem, Los Angeles, Calif.), 210 mg/100 ml; and lysozyme, 20 mg/100 ml. The protoplasts that were formed grew in this medium. The zero-time sample was taken before addition of lysozyme; further samples were taken at intervals after addition of lysozyme.

Fixation of whole cells. Cells from the mid-logarithmic phase of growth were prefixed in buffered 3% glutaraldehyde; then they were fixed for 12 to 15 hr in Kellenberger's fixative (9). The fixation and all subsequent steps were conducted at room temperature (28 C). Commercial supplies of glutaraldehyde (50%; Fisher Scientific Co., Pittsburgh, Pa.) were diluted with an equal volume of 0.1 M tris(hydroxymethyl) aminomethane (Tris) buffer (*p*H 7.0) that contained 0.01 M MgCl₅ (Tris-Mg). This was added to the culture medium to give a final concentration of 3% glutaraldehyde. After prefixation, the cells were centrifuged (8,000 × *g*) and washed three times with Tris-Mg to remove excess glutaraldehyde. The washed cells were then mixed with 2% Noble agar dissolved in Tris-Mg (45 C). This was spread in a layer 1 mm thick on a clean glass slide, solidified on ice, cut into 1-mm cubes, and dropped into Kellenberger fixative.

For protoplasts, all fixation steps were carried out using the protoplasting medium, described above, as a basal medium rather than Tris-Mg used for whole cells. After 12 to 15 hr, the blocks were treated with 0.5% uranyl acetate in Kellenberger buffer (*p*H 6.8)

for 2 hr, and they were then dehydrated in alcohol (30% alcohol, 30 min; 50%, 30 min; 75%, 30 min; 95%, 30 min; absolute alcohol, 30 min, two changes; propylene oxide, 30 min, two changes). The dehydrated blocks, in small vials, were suspended in 50% Epon (7 volumes of mixture A plus 3 volumes of mixture B) in propylene oxide (13). These were shaken by swirling motion for 6 to 8 hr to allow infiltration. The infiltrated blocks were immersed in freshly prepared Epon mixture in Beem capsules. The blocks were polymerized by successive 12-hr incubations at 37 C, 45 C, and 60 C. They were stored at room temperature for 24 hr before sectioning. Sections (silver-grey interference color) were cut with a Sorvall MT-1 ultramicrotome (Ivan Sorvall, Inc., Norwalk, Conn.) that used a diamond knife. They were collected on 75/300 mesh grids coated with 1% Formvar, and then they were generally stained for 20 to 30 min with lead citrate (25). Longer periods (up to 45 min) of staining were required for very thin sections. To avoid precipitation, sections were washed first with 0.01 M NaOH and then with demineralized CO₂-free water. The stained sections were examined with an Akashi Tronoscope model 50 E 1 microscope (Akashi Seisakusho, Ltd., Marunochi, Tokyo, Japan) at an accelerating voltage of 50 kv. The initial instrument magnification was 5,000 to 20,000. Pictures were taken on 35 mm Kodak fine grain positive film. They were further enlarged 10 to 12 times in a Durst M-35 (Durst, Inc., Long Island City, New York) photographic enlarger.

Measurements of the various structures were made on negatives that had been magnified 14-fold by the Durst enlarger. Clearly focussed images of 10,000 instrumental magnification were chosen, and the structures were measured in 5 different areas of 10 random sections.

RESULTS

Structure of uninduced B. licheniformis strain 749. Thin sections of organisms harvested in mid-logarithmic phase are presented in Fig. 1–7. The cells have a trilamellar wall, each layer of which is amorphous. The average thickness of cell walls of strains 749 and 749/C was 3.25 and 2.50 pm, respectively. In many instances, there is no clear distinction between cell wall and plasma membrane. This may indicate close association of cell wall and plasma membrane, or it may be a consequence of the "angle of tilt" (26). Clear definition of plasma membrane is possible if the washed cells are slightly autolyzed by incubating them in saline at room temperature for 3 to 4 hr. (Fig. 7). The plasma membrane is of the unit membrane type with a total thickness of 0.8 to 1.0 pm. In a sharply focused picture, it is possible to discern a separate layer between the wall and the plasma membrane that appears to be composed of regularly spaced spike-like structures originating either from the inner layer of cell wall or from the outer layer of plasma membrane (Fig. 4, 5, and 27).
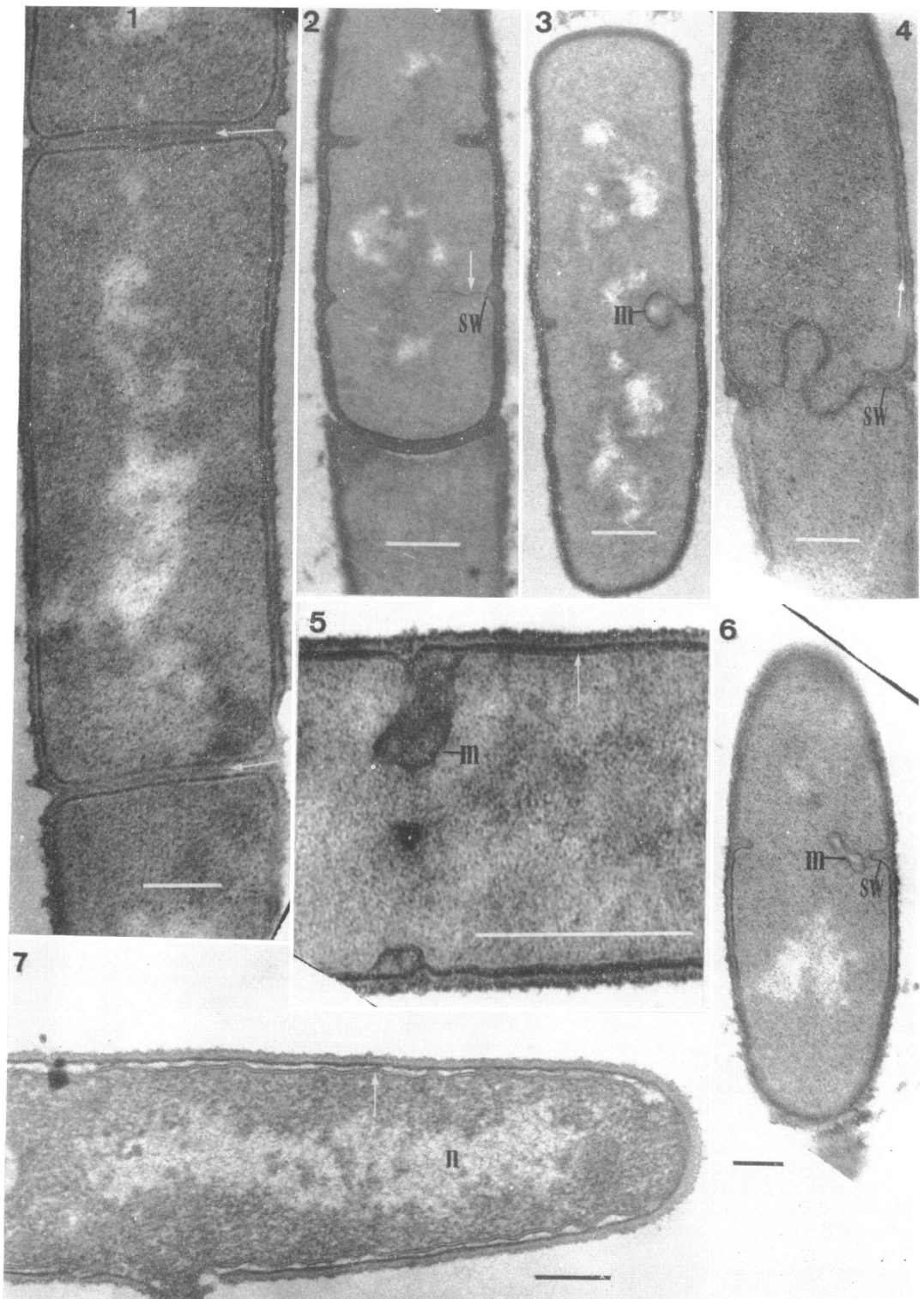
FIG. 1–7. *Thin sections of strain 749. General structure of cells showing trilamellar cell wall (Fig. 1, 5, and 6); fibrillar nuclear material (Fig. 7n); cytoplasmic granules which cannot be seen in preparations not stained with lead (Fig. 2); completed septum with a layer in the middle of the septum (Fig. 1, arrow); cross-bridged layer formed by spike-like structures between cell wall and plasma membrane (Fig. 4 and 5, arrow); unit membrane type of plasma membrane (Fig. 7, arrow); septal mesosome of various stages of development—the structure begins as a thin membranous invagination (Fig. 2, arrow) and grows to complete septation by membrane only (Fig. 4). The invaginated membrane may round up to form vesicular structure (Fig. 3, 5, and 6m). Deposition of cell wall takes place after the formation of septal mesosome (Fig. 2–6sw). The section, excepting Fig. 2, is stained with Reynold's lead stain (26). The marker in all micrographs denotes 0.2 μm unless otherwise stated.*

The cytoplasm contains tightly packed granules, and filamentous material is distributed throughout the cytoplasm and may be closely associated with the granules. The nuclear material of cells prefixed with glutaraldehyde shows less tendency to aggregate than that of nonprefixed cells. Examination of large numbers of random sections revealed that the mid-logarithmic phase organism has only a very simple type of septal mesosome. In this strain, mesosome structures arise from thin membranous invaginations (Fig. 2–4) that become vesicular (Fig. 5 and 6). In cells where septation is complete, a layer appears that divides the septum. This may be concerned with the separation of daughter cells (Fig. 1 and 2).

Figures 8 and 9 illustrate the appearance of cells of strain 749 after negative staining with phosphotungstic acid (PTA) and ammonium molybdate, respectively. As in thin sections, little or no mesosomal structure is evident. The cell wall and protoplast are clearly defined by ammonium molybdate staining (Fig. 9). This may be due to slight retraction of the protoplast from the cell wall. In the space between cell wall and plasma membrane, small projections can be seen at intervals (Fig. 9). These may be points at which the cell wall and protoplast membrane are in particularly intimate contact.

Structure of penicillinase-constitutive mutant 749/C. Thin sections of strain 749/C cells are illustrated in Fig. 10–16. The basic structure is similar to that of strain 749, but unusual clusters of vesicles and tubules can be seen in the periplasmic area (16). The plasma membrane is invaginated so that the periplasm is enlarged. These regions contain large numbers of vesicles or tightly coiled tubules. The vesicles and tubules appear to have single-layered limiting membranes about 0.35 to 0.4 pm thick (Fig. 11–13). The vesicles either contain amorphous material or are empty (Fig. 12 and 13) and they do not appear to contain ribosomes. These clusters may be located in any region of the cell periphery. Figure 16 shows the distribution of this structure in a section of a chain of four cells. Spike-like structures similar to those in 749 cells connect cell wall and plasma membrane (Fig. 10, 12, and 13). However, the wall and membrane are more
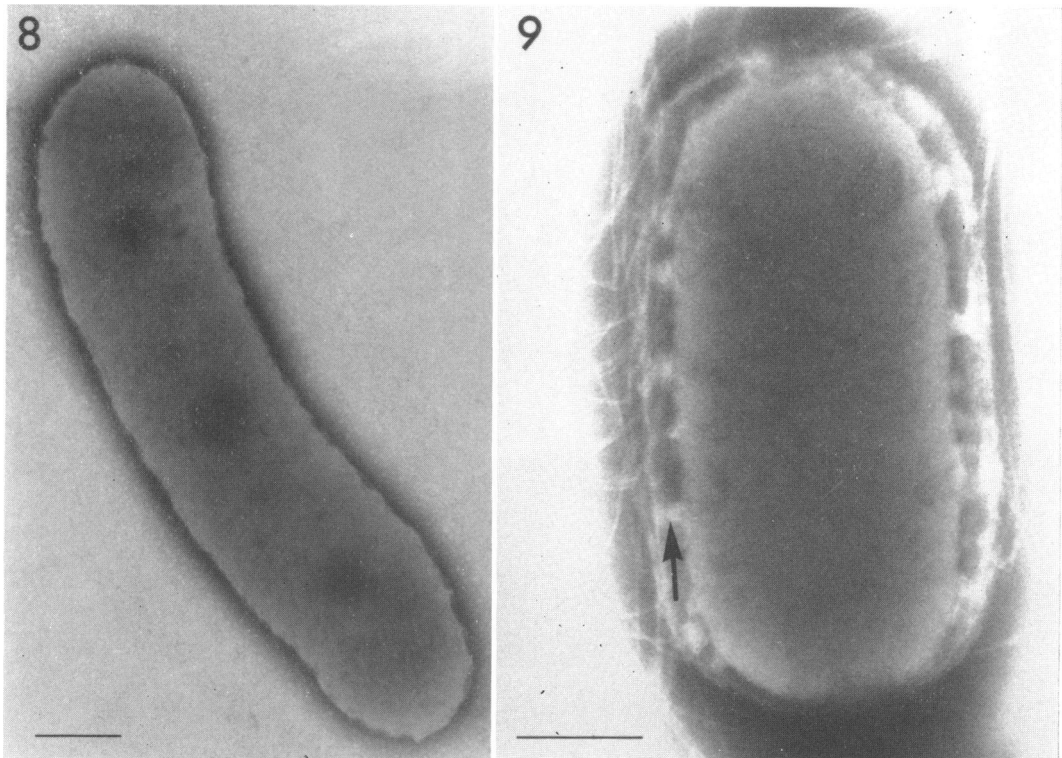

FIG. 8. *A cell of uninduced strain 749 negatively stained with PTA.*

FIG. 9. *Preparation of uninduced strain 749 negatively stained with ammonium molybdate. Note projection connecting cell wall and protoplast (arrow).*
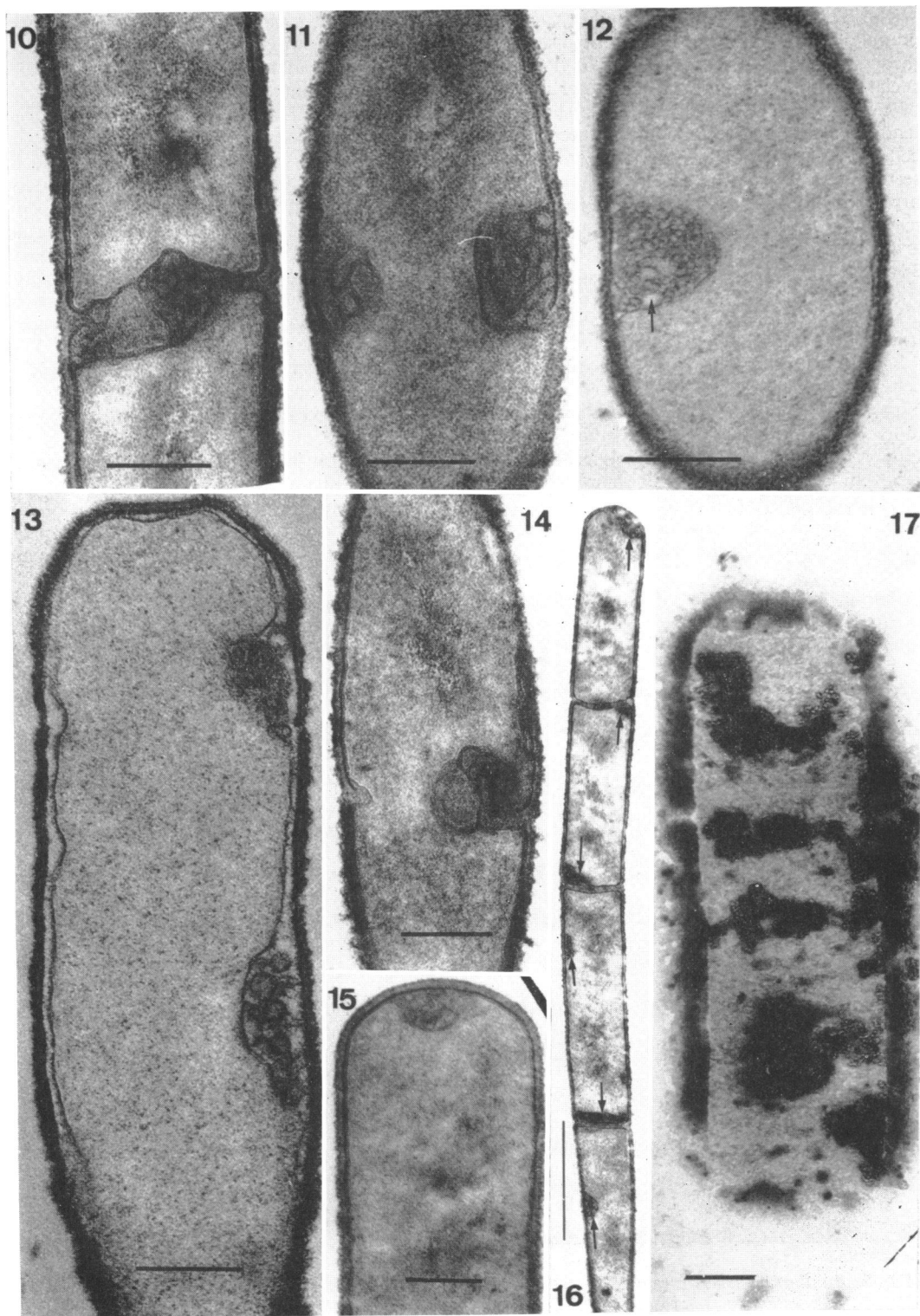
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Fig. 10–17

loosely associated than in strain 749 because the process of fixation, which did not cause separation of wall and plasma membrane in 749 cells, occasionally caused a distinct separation in strain 749/C (Fig. 13).

In negatively stained 749/C cell (Fig. 17), large invaginations of the plasma membrane are evident and they appear to contain randomly organized membrane material. These structures are probably similar to those seen in thin sections.

The septal structure of strain 749/C is also more complex than in 749 cells (Fig. 10 and 14). Figure 10 shows a late stage in cell septation. In this region, clusters of vesicles can be seen. The vesicular structures occasionally found in the polar region of divided cells (Fig. 15) may originate in this way.

Structure of strain 749 cells after induction. Figure 18 illustrates growth and penicillinase synthesis after the addition of cephalosporin C (1 μg per ml) to a culture of strain 749. The growth curve (Fig. 18A) indicates only a 5% decrease in the average rate of growth after addition of the inducer. The rate of growth returned to the normal value 4 hr after addition of the inducer. Penicillinase synthesis started about 60 min after induction (Fig. 18B). The specific activity of the whole culture reached a peak 6 hr after induction. About 50% of the enzyme was then extracellular. During the first 3 hr of induction, changes in cellular morphology were monitored by electron microscopy with negative staining and thin sectioning.

A negatively stained 749 cell, 15 min after induction, is shown in Fig. 19. It contrasts sharply with uninduced cells (Fig. 8 and 9) in that it contains many sharply defined electron-dense vesicles. Connections with the surface are relatively rare; therefore, it is unlikely that the vesicles are formed by infolding of the peripheral membrane. They may be interconnected by tubules (Fig. 19) because stain penetrates to many vesicles in spite of the lack of individual connection to the surface.

A negatively stained specimen of strain 749, 180 min after induction, is shown in Fig. 20. Vesicles similar to those in early stages of induction can be seen, but many are greatly enlarged and show obvious connections with the surface. The general appearance of the organism at this stage is approaching that of negatively stained 749/C.

Pictures of thin sections of 749 cells at 10 min (Fig. 21 and 22), 30 min (Fig. 23), 60 min (Fig. 24 and 25), and 180 min (Fig. 27) after induction show significant changes in the ultrastructure. These can be described as follows. (i) Electron-dense structures within the cell wall (Fig. 21, arrows) can be observed in cells 10 min after induction. These electron-dense areas probably represent lesions in the wall due to the action of cephalosporin. (Detailed investigations of the role of this damage phenomenon are being undertaken in our laboratory.) Damaged cell walls in the presence of high concentration of cephalosporin C (50 μg/ml) are shown in Fig. 26. (ii) Invagination of peripheral membrane can be seen 10 min and 30 min after induction (Fig. 22 and 23), and the resulting periplasmic space seems to contain undifferentiated agranular cytoplasmic material (Fig. 22). At 60 min after induction (Fig. 24), rolled up membranous bodies can frequently be observed in the cytoplasm. Large invaginations of membrane are very commonly found 60 min (Fig. 25) and 180 min (Fig. 27) after induction. The periplasmic space at this stage contains some vesicles or tubules, or both, as well as some undifferentiated cytoplasmic material. These have close similarity to the periplasmic structures of 749/C and are located randomly in any part of the cell periphery (Fig. 27). They have no apparent relationship to the cell septum.

Stages in formation of protoplasts of uninduced strain 749. Thin sections of washed cells of strain 749 suspended in protoplasting medium (without lysozyme) have been examined. The cells showed no sign of the plasmolysis in protoplasting

FIG. 10–16. *Thin sections of strain 749/C.*

FIG. 10. *Complex organization of vesicular structure in the region of septum formation. Here, cross-membrane formation is complete but deposition of wall material is not complete. Membranous vesicles are seen outside the plasma membrane; therefore, they are essentially periplasmic.*

FIG. 11–13, 15, and 16. *Organization and distribution of periplasmic structure. The peripheral plasma membrane is invaginated to surround groups of tubules and vesicles, and the space between cell wall and plasma membrane is enlarged. Vesicles or tubules have single-layered membranes and may be full or have no contents (Fig. 12, arrow; electron-lucid vesicles). Separation of cell wall and plasma membrane by usual methods of fixation can be found occasionally (Fig. 13). Tightly coiled tubular structures can be seen in Fig. 11. Periplasmic structure in the polar region of the cell (Fig. 15).*

FIG. 14. *Complex organization of septal mesosome characteristic for 749/C cells.*

FIG. 16. *Normal mid-log cell is found as a chain of 4 or 5 cells. Sections through such a chain show the random distribution of periplasmic structure in the cell periphery (arrows). Marker indicates 1 μm.*

FIG. 17. *Preparation negatively stained with PTA. Note large invaginations of membrane and randomly organized contents of invaginated area.*

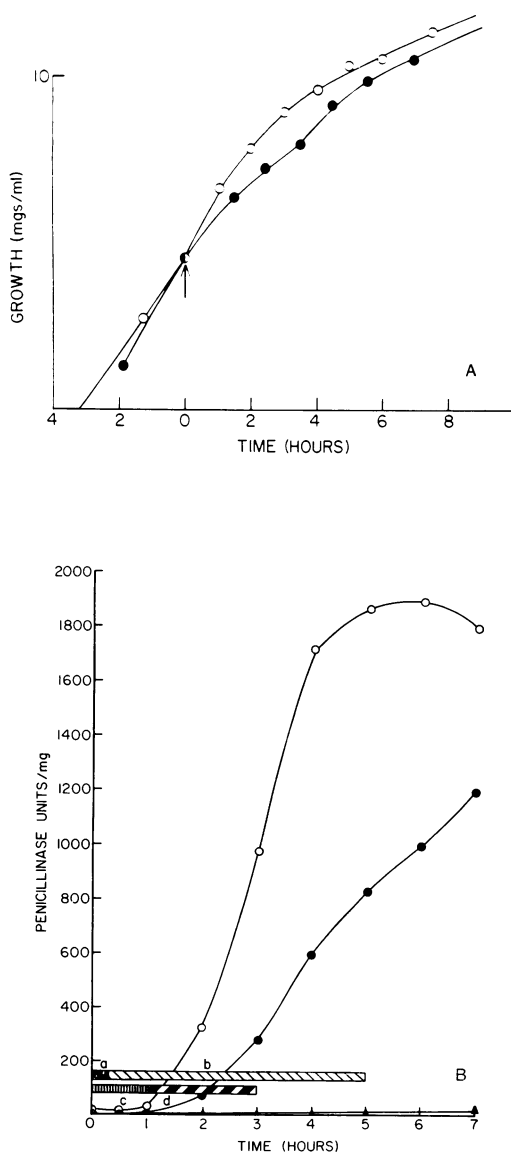medium that is reported so frequently in *Bacillus* species (5, 32).

The changes in fine structure of the 749 cell at various time intervals after lysozyme treatment are as follows. (i) Within 3 to 5 min after adding lysozyme (Fig. 28) the cell wall and protoplast separate. This may be the result of contraction of the protoplast or of expansion of the cell wall, or both. In either case, it indicates that the cell wall and the protoplast must be held under tension in the untreated cells. (ii) Fibrils connecting the cell wall and the plasma membrane become more visible (Fig. 28). (iii) Small numbers of tubules or vesicles, or both, appear in this expanded periplasmic area. The tubules originate from the protoplast and may form vesicles (possibly by fragmentation). (iv) The cell wall trilamellar structure remains largely (although temporarily) intact, which suggests that an early site of action of lysozyme may be in the inner layer of cell wall or on a structure joining the cell wall and the plasma membrane. The rod shape of the protoplast is still maintained except for the appearance of a few convolutions.

After a 15-min treatment with lysozyme, the cell wall has disintegrated and the protoplast then gradually undergoes transformation to a spherical shape. After 30 to 60 min, most protoplasts have irregular profiles; by 180 min, they are essentially spherical (Fig. 32). The densely packed cytoplasmic granules and well-organized nuclear material indicate that osmotic swelling has not occurred.

The protoplast membrane is clearly of the unit membrane type; the outer layer appears thicker than the inner layer. Slender tubules are also attached to the protoplasts and their contents are mostly amorphous, in contrast to the granular cytoplasm.

Strain 749 cells (induced) at various stages of protoplast formation. Sections of cells that were harvested 2 hr after induction show clusters of vesicles in the periplasm particularly in the region of septum formation (Fig. 27).

Protoplast formation appears to proceed as in uninduced cells, although the cell walls seem to be much more sensitive to lysozyme than those of uninduced cells, there being substantial digestion of the trilamellar structure of the cell wall within 3 to 5 min of lysozyme treatment

FIG. 18. (*A*) *Growth curve of strain 749 with 1 μg of cephalosporin C per ml* (●) *and without cephalosporin C* (○). *Cephalosporin C (inducer) was added 2 hr and 15 min after inoculation (arrow). Results expressed as mg (dry weight) per ml. (B) Time course of penicillinase synthesis and secretion after induction with cephalosporin C (1 μg/ml). Results are expressed as specific activity of penicillinase [penicillinase units/ mg (dry weight) of cell]. Symbols:* ○, *activity in cell plus supernatant with inducer;* ▲, *activity in cell plus supernatant without inducer;* ●, *activity in cell with inducer. Horizontal bars indicate the time sequence of the appearance of characteristic structures during induction of 749. (a) Small intracellular vesicles that have no obvious connection to the surface (demonstrated*

by negative staining). (b) Large vesicles of irregular shape, many of which are connected to the surface by invagination of peripheral membrane (demonstrated by negative staining). (c) Small electron-dense pockets in the cell wall (demonstrated in thin sections). (d) Clusters of tubules and vesicles in the periplasm (demonstrated in thin sections).
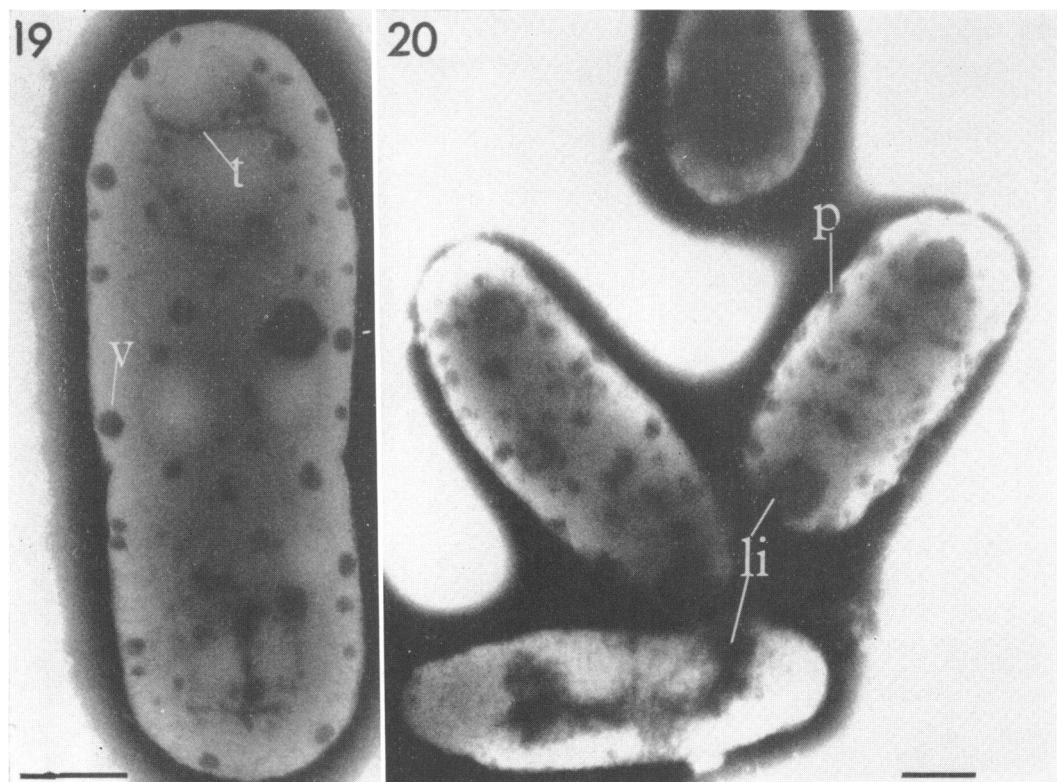
Fig. 19 and 20. *Negatively stained preparation of strain 749 at 15 and 180 min after induction with 1 µg of cephalosporin C per ml.*

Fig. 19. *Stained with PTA 15 min after induction; intracellular vesicles (v), intracellular tubules (t).*

Fig. 20. *Showing many vesicles, 180 min after induction; infolding of peripheral membrane giving rise to small pockets (p) or large invagination (li).*

(Fig 29 and 30). Large numbers of tubules and some vesicles are found in the space between the cell wall and the plasma membrane.

After 30 min of lysozyme treatment, protoplasts have irregular convoluted profiles and large pseudopodia-like extensions from the surface which remain as long as 40 to 50 min after lysozyme treatment (Fig. 31). The convolutions contain intact and well-organized cytoplasmic material and have a well-defined unit membrane.

These structures are probably a consequence of increased membrane synthesis associated with penicillinase induction. Large convolutions appear to be a way of accommodating excess membrane. The convolutions are not very stable and usually become separated from the body of the protoplast by constriction at the cell surface (Fig. 34). The irregular profile indicates that the protoplasts are still in the process of transformation from rod to sphere. Figures 34 and 36 are low magnifications of protoplasts at 30 and 180 min, respectively, after

lysozyme treatment of cells that have been induced for 2 hr. Large numbers of slender tubules have separated from the protoplasts (Fig. 34). This is in marked contrast to the situation of uninduced 749, in which protoplasts produce a small number of similar tubules (Fig. 28). Formation of extra layers of membrane is evident in many protoplasts (Fig. 35).

After 180 min of lysozyme treatment, the protoplast profile is smooth and devoid of convolutions (Fig. 36). Tubule formation also is greatly reduced. A granular, highly electron-dense material frequently accumulates in close association with fibrillar nuclear material (Fig. 36, arrow). A feature of the 180-min specimen is the membranous projections (empty of cytoplasmic material) that extend and ultimately detach from the protoplast surface as empty tubules and small vesicles (Fig. 33 and 36). This again suggests that membrane synthesis is enhanced in the induced cells or protoplasts, resulting in the appearance of a variety of extracellular membranous structures.
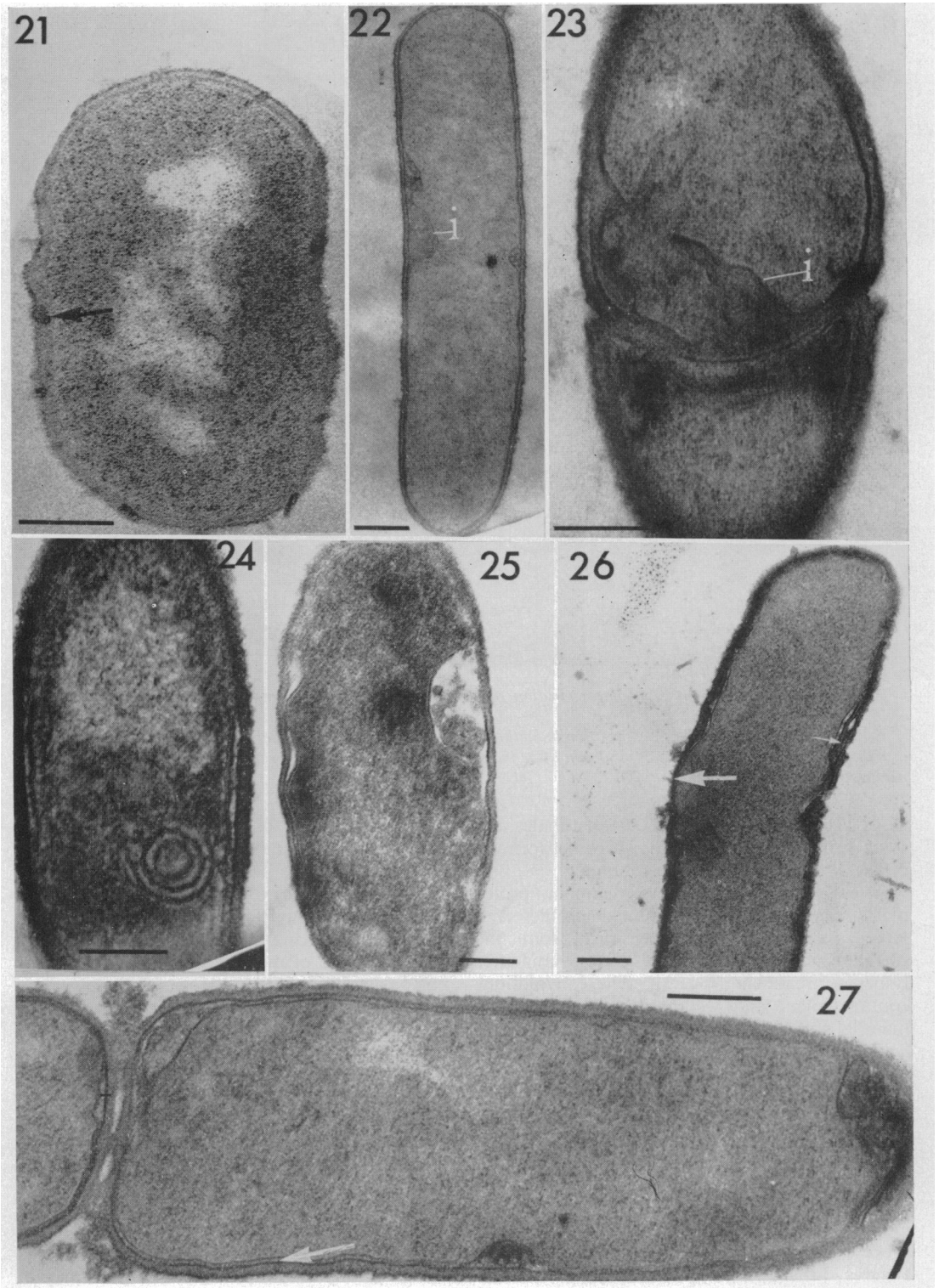
FIG. 21–27

Strain 749/C cells during protoplast formation. Thin sections of washed cells suspended in protoplasting medium show no plasmolysis or damage to the periplasmic structures in this hypertonic medium. After 5 min of lysozyme treatment, the changes are similar to those obtained with uninduced 749 cells. Although the cell wall has not lost the trilamellar structure, there is considerable separation of protoplast and cell wall, and the space between these two structures contains a large number of released tubules and vesicles (Fig. 37). Protoplasts, after 30 min of lysozyme treatment (Fig. 38), are roughly spherical and have attached tubules and vesicles. After 240 min of lysozyme treatment, highly electron-dense material is observed in the center of the protoplast (Fig. 39); fibrillar nuclear material occasionally surround these dense bodies. Slender tubules are still observed at this stage.

Unlike the protoplasts of induced 749, protoplasts of the constitutive strain 749/C do not exhibit a characteristic convoluted profile, nor does membrane growth, leading to empty tubule or vesicle formation, occur.

DISCUSSION

Cells of uninduced *B. licheniformis* (strain 749) in the logarithmic phase have no extensive intracytoplasmic membranes. After induction with cephalosporin C, characteristic tubules and vesicles with single-layered membranes appear in certain areas of the periplasm. The cell membrane usually is invaginated and closely surrounds the organelle. Strain 749/C, a magnoconstitutive penicillinase producer derived from strain 749, has structures similar to but generally more highly developed than those in induced 749.

The first observed reaction of the cell to cephalosporin C is the production of intracellular vesicles (Fig. 18B). Subsequently, the surface shows many invaginations and soon after vesicles appear in the periplasm, forming the organelles that characterize the fully induced cells. Unfortunately, we have been unable to show the relationship between these periplasmic structures and the small intracellular vesicles that are seen in the first 15 min. Once the periplasmic vesicles are formed, no structures which might, if present, indicate the mode of origin of the organelle can be seen on the inside of the bounding membrane.

We have also examined the behavior of cells during protoplasting. In uninduced strain 749, the cell wall and protoplast separate (3 to 5 min after addition of lysozyme); then the cell wall disappears (15 to 20 min), and, finally, the protoplast is gradually transformed from rod to sphere (20 to 30 min).

Lysozyme acted not only on the matrix of the cell wall but also at the interface of membrane and cell wall, indicating that a lysozyme-sensitive structural component may join the two under normal circumstances.

During conversion of strains 749 (2 hr after induction) and 749/C to protoplasts, large numbers of tubules and some vesicles were liberated from the periplasm. In induced 749, there was very extensive reorganization of the peripheral membrane; also (on a smaller scale) in 749/C. In induced 749, large pseudopodia-like structures and long, empty tubules that originated from the plasma membrane were seen, and extra layers of membrane were present beneath the normal surface membrane.

Five aspects of these observations should be noted. (i) When protoplasts are formed, the components of the organelle are liberated within a few minutes of lysozyme treatment, indicating that they are in fact tubules and vesicles. (ii) Certain free tubules show constrictions along their entire length. This may indicate the origin of the vesicles. (iii) The number of tubules in a suspension of growing protoplasts of induced 749 is much greater than that in uninduced 749, suggesting they probably are produced in response to the inducer. That some are present in the uninduced protoplast, however, could conceivably indicate a role independent of the penicillinase secretion process. (iv) The massive convolutions of the surface of protoplasts of induced 749 imply that membrane synthesis is greatly en-

FIG. 21–27. *Thin sections of strain 749 cells after induction with cephalosporin C (1 μg/ml).*

FIG. 21. *Electron-dense pockets in the cell wall (arrows), 10 min after induction.*

FIG. 22. *Invagination of membrane and undifferentiated material in the periplasm (i), 10 min after induction.*

FIG. 23. *Note invagination of membrane (i), 30 min after induction.*

FIG. 24 and 25. *Whorl type of membrane material in the cytoplasm, 60 min after induction. Unit membrane character of this membrane can be seen (Fig. 24). Invagination of peripheral membrane to form periplasmic structure, vesicles, and some undifferentiated material (Fig. 25).*

FIG. 26. *Damaged cell wall 1 hr after treatment with 50 μg of cephalosporin C per ml. Note abnormal layering of cell wall material (small arrow) and partial removal of cell wall material resulting in pushing out of protoplast at the weakened point of cell wall (thick arrow).*

FIG. 27. *Further development of periplasmic structure 120 min after induction. Note clear definition of spike-like structure between cell wall and plasma membrane (arrow).*
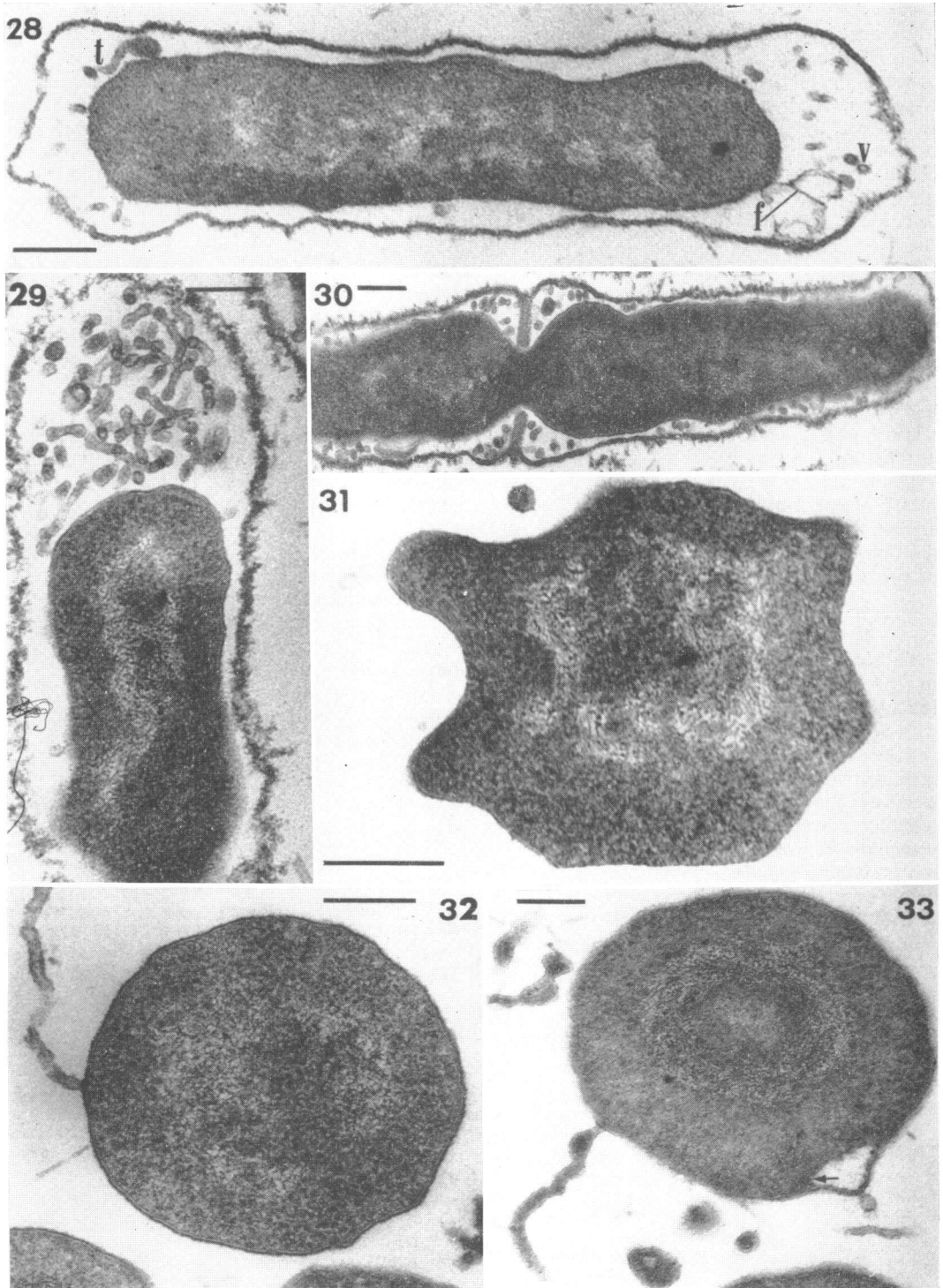
FIG. 28–33

hanced. It is probable that similar stimulation of membrane synthesis occurs in intact cells of 749 after induction, because in thin sections the plasma membrane is extended to surround the new organelle. (v) The extra layers of membrane present beneath the normal protoplast membrane (Fig. 35) usually are not seen in thin sections of whole cells, and might represent a repair phenomenon necessitated by small lesions incurred during protoplasting. However, these extra layers can be seen after 3 to 5 min of protoplasting. It is unlikely, therefore, that they were synthesized during this period.

A variety of experimental approaches will be required to elucidate the entire development of the organelle; nevertheless, it appears that a new structure develops in response to penicillinase induction. It may be argued, however, that this structure is concerned not with penicillinase secretion, but with repair of cell wall damage caused by the antibiotic. Under the conditions of induction employed here (1 μg of cephalosporin C per ml), the inhibition of growth is very small. At higher concentrations of cephalosporin C, the structure of the damaged wall is very characteristic (Fig. 26), and similar effects cannot be detected in cells that were treated with the lower concentration of cephalosporin C used in the induction experiment. Moreover, the constitutive mutant (749/C) produces a similar organelle in the absence of antibiotic. This, too, could be interpreted as a consequence of wall damage due to a genetic defect [a peculiarly attractive point of view, in view of recent claims that penicillinase may have been derived from a cell wall hydrolase during evolution (H. J. Ozer et al., Bacteriol. Proc., p. 124, 1967)]. However, it is very clear that penicillinase is located predominantly in vesicles corresponding to those in the electron micrographs described above, and their specific activity is at least six times that of the general plasma membrane (29). We conclude, therefore, that in response to penicillinase induction a new organelle appears which is concerned with penicillinase secretion (penicillinase secretory apparatus).

Recently, Beaton (1) implicated the mesosome of Staphylococcus aureus in penicillinase secretion. Grown on "Tris plus CY" medium, the organism had three different types of mesosome, and 98% of the penicillinase synthesized was cell bound. After being transferred to a "glucose plus phosphate" medium, the mesosomes disappeared and pockets that contained vesicles appeared in the periplasm. Simultaneously, 30% of the cell-bound penicillinase was liberated. However, other treatments which liberated some penicillinase (dextran sulfate) did not alter the mesosome structure. Beaton concluded, therefore, that mesosome eversion must be an early step in penicillinase secretion.

Structures that resemble the vesicles and tubules liberated during protoplasting of B. licheniformis (749/C) have been observed in other organisms during protoplasting or plasmolysis (4, 5, 19, 27, 28, 32). These have been termed mesosomes, and they have not been implicated in secretion phenomena. The mesosome is widely believed to be concerned with partition of the genome and cell septation (27, 33). The structures associated with penicillinase secretion in B. licheniformis have a superficial morphological resemblance to mesosomes, but in this case they are found in greater numbers in cells that synthesize penicillinase; therefore they probably are not identical to mesosomes. Remsen detected the presence of two types of mesosomes in B. subtilis (24) by the freeze-etching technique; one is a vesicular structure, or a cluster of vesicular structures, occasionally surrounded by a single membrane and the other is a lamellar structure consisting of concentrically arranged membrane. The vesicular structures were smooth and devoid of particles, whereas the membrane surface of the lamellar structure was covered with particles (about 0.8 pm). Remsen suggested that the vesicles may be associated with the transport of cell wall material. Most interesting in the context of our observations is that the vesicles were occasionally found in the periplasmic space. Similar structures, also of undetermined significance, have been reported in Listeria

FIG. 28. Thin section of uninduced strain 749 cells 5 min after the addition of lysozyme. Note the fibrillar connection (f) between cell wall and plasma membrane; few vesicles (v) and tubules (t).

FIG. 29 and 30. Thin sections of induced (2-hr) cells of strain 749, 5 min after addition of lysozyme. Note tubules and vesicles released into the space between cell wall and protoplast. Considerable digestion and destruction of trilamellar structure of cell wall can be seen.

FIG. 31. Thin section of a protoplast of induced (2-hr) strain 749, 30 min after lysozyme treatment. Note the pseudopodia-like projections.

FIG. 32. Thin section of protoplast of uninduced strain 749, 180 min after the addition of lysozyme. Note the presence of an attached tubule containing agranular material. Membrane is unit membrane type, but outer layer is thicker than inner layer.

FIG. 33. Protoplast of induced 749 cell 180 min after lysozyme treatment. The peripheral membrane has extended and become separated from the cytoplasm (arrow). Structure may elongate to form empty tubule.
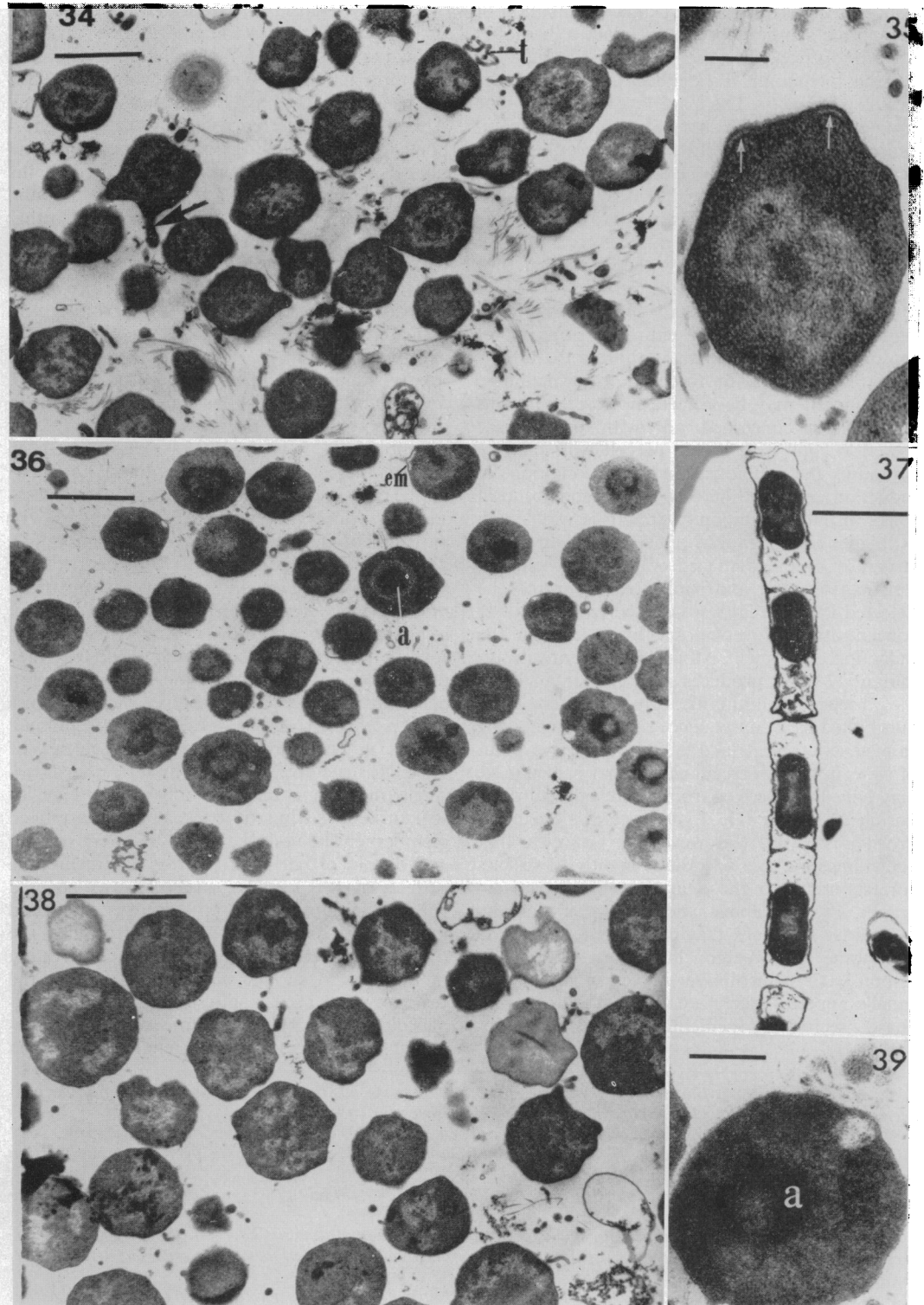
FIG. 34–35

monocytogenes (6), in fungi (2, 18), and in higher plants (14). The structures in fungi have been designated lomasomes (border bodies) and a secretory function has been ascribed to them (although there is no evidence for this).

Matile (15) provided convincing evidence that protease secretion in *Neurospora crassa* occurs by reverse pinocytosis. Because this was demonstrated by use of the freeze-etching technique, it is difficult to make a direct comparison with our findings.

Recently, in freeze-etched preparations of *Saccharomyces cerevisiae*, it was demonstrated that vesicle formation from intermediate endoplasmic reticulum acts as an initiator of bud formation (17). It has been proposed that small vesicles (10 pm) that contain protein disulfide reductase traverse the plasmalemma to the periplasmic area for highly localized action on the cell wall. It would seem, therefore, that such transfer vesicles may have an important role in the secretion of macromolecules in micro-organisms.

In recent years, intensive investigations have been made of macromolecule transport in the specialized secretory cells of mammals (20, 31). There is a superficial morphological resemblance between the tubules and the vesicles of the secretory apparatus and the zymogen granules of mammalian secretory systems. Both are bounded by a single layer of membrane, and neither has any visible substructure. However, penicillinase is tightly bound to the secretory apparatus of *B. licheniformis*. In contrast, enzymes in the zymogen granules are free and have originated from the cisternae of the endoplasmic reticulum. There is, therefore, no strict parallel between mammalian secretory systems and penicillinase secretion.

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FIG. 34–39. *Thin sections to show general nature of protoplasts of induced (2-hr) strain 749, after 30 min (Fig. 34 and 35) and 4 hr (Fig. 36) of lysozyme treatment. Note the presence of large numbers of tubules released (t), pinched off convolutions of protoplast (Fig. 34, thick arrow), extra layer of membrane beneath normal peripheral membrane (Fig. 35, arrows), empty membranous projections (Fig. 36em), and electron dense agranular material (Fig. 36a) in association with the fibrillar nuclear material.*

FIG. 37. *Thin section of cells of strain 749/C, 5 min after addition of lysozyme. Note the large number of released tubules and vesicles in the space between cell wall and protoplast.*

FIG. 38. *Thin section of protoplasts of strain 749/C 30 min after addition of lysozyme. Note the presence of attached tubules.*

FIG. 39. *Protoplast 240 min after lysozyme treatment of 749/C cells. Pockets (arrow) can be seen occasionally at the base of tubule. These disappear after the release of tubules. Note the dense agranular material at the center of the protoplast (a). Markers in Fig. 34 and 36–38 indicate 1 μm.*

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