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Ultrastructure of a Temperature-Sensitive Rod⁻ Mutant of *Bacillus subtilis*

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Mutant 168ts-200B, resulting from nitrosoguanidine treatment of *Bacillus subtilis* 168 (*trp*⁻ C2), exhibits a rod-to-sphere morphogenetic interconversion when the incubation temperature is 30 or 45 C, respectively. Ultrathin sections of rods grown at 30 C, after glutaraldehyde-osmium uranium-lead fixation and staining, show trilaminar cell walls with a well-developed underlying periplasm as in wild-type cells. However, the outer wall layer is irregular, and abnormal protrusions of wall material occur at the cross-walls. In contrast, cells growing at 45 C become rounded and are intersected randomly by irregular cross-walls which fail to split normally, resulting in large spherical masses. In these, the outer and inner wall layers and periplasm are lost, and the wall consists only of irregularly thickened and loosely organized middle layer. Wall ultrastructure is reversible in either direction as cell shape changes during temperature shifts. Mesosomes are rare and atypical at either temperature. It thus appears that cell wall ultrastructure is altered by the conditional (temperature-sensitive) mutation, and that loss of normal wall and submural organization is correlated with changes in cell size and shape as well as with inability to complete cell division. Preliminary studies after transformation of the mutant locus to another strain and growth at 45 C showed an increase in mucopeptide, loss of wall teichoic acid, failure of phage adsorption, and identical ultrastructural changes. The site of expression of the basic defect—be it in wall, submural region, or membrane—is undetermined.

Conditional morphological mutants of *Bacillus subtilis*, as well as of *Bacillus licheniformis*, were reported by Rogers et al. (22). These *rod*⁻ mutants grew as irregular spheres on an inorganic salts-glucose-tryptophan medium lacking added sodium chloride, but grew as rods when sodium chloride or several amino acids were added (21). A morphologically similar variation, for which the condition is temperature instead of medium constituents, was recently described by some of us (2) in a mutant of *B. subtilis* 168 (*trp*⁻ C2). In this report, we demonstrate changes in the ultrastructure of this mutant at the permissive and restrictive temperatures, and during transition from one temperature to the other.

MATERIALS AND METHODS

Organism. The temperature-sensitive rod⁻ mutant, designated 168ts-200B, was derived from *B. subtilis* 168 (*trp*⁻ C2), designated 168ts⁺, as previously described (2).

Culture. Media and growth conditions were described previously (2).

Electron microscopy. At temperatures and times of incubation indicated, 2 ml of 5% glutaraldehyde, buffered to *p*H 7.2 in 0.2 M sodium cacodylate (24), was added to 25 ml of bacterial culture. The mixture was immediately centrifuged, and the cells were suspended in 3 ml of the buffered 5% glutaraldehyde for 1 hr at room temperature. A 0.3-ml amount of 1% tryptone medium (23) was then added, and the suspension was maintained at the same temperature for 1.5 hr. After centrifugation, the cells were placed in 0.2 M sucrose buffered at *p*H 7.2 in 0.1 M phosphate and held for 2 days at 4 C.

Secondary fixation at room temperature in acetate-Veronal-buffered 1% osmium tetroxide, and subsequent washing, agar embedding, and treatment with uranyl acetate, were done according to Ryter and Kellenberger (23).

Dehydration was performed by passage of the agar cubes through a graded series of ethanol concentrations to propylene oxide, from which final embedment was made in Epon 812 (14).

Sections, silver-grey to gold, were cut with a diamond knife on an LK Ultratome I and were placed on carbon-Formvar-coated copper grids. All sections were stained for 3 min with alkaline lead citrate (20). Examination and photography were done with an Hitachi electron microscope, model HU-11C, operated at 75 kv.

Measurements of the dimensions of wall, submural

regions, and membrane were made by the use of a Bausch and Lomb measuring magnifier with a scale marked in 0.1-mm gradations. The dimensions given represent an average of at least 20 measurements of each component and were made on electron micrographs printed at a magnification of 105,000. Checks made on photographs at higher magnifications gave similar and consistent readings.

RESULTS

By electron microscopy, ultrathin sections of cells of *B. subtilis* 168ts-200B, grown from spores at the permissive temperature (30 C), are morphologically similar to those of normal or wild-type strains. At 6 hr, the cells are rod-shaped, frequently in pairs or short chains, and the centrally located cross-walls produce daughter cells of equal lengths (Fig. 1). Some late-germinating spores and emerging new vegetative cells are also seen. The appearance of the cell wall is roughly trilaminar (Fig. 2): there is a rather poorly defined outer layer (OL; Fig. 2, 3) of increased density of about 5 nm in thickness, followed next innermost by a less dense homogeneous layer (ML; Fig. 2, 3) which makes up the bulk of the wall. The combined thickness of these two layers varies from 14.5 to 28.2 nm, but is usually about 20 nm. Innermost is what appears at low magnification to be a wider dense layer; however, on close examination, this can be seen to consist of an inner dense layer of wall (IL) separated by an irregularly globular, granular, or stranded layer of decreased density (PP) from the dense outer leaflet of the cytoplasmic membrane (OM; Fig. 2c, 2d, 3). The total thickness of these combined layers (IL + PP + OM) is approximately 10 nm. The "globules" constitute the periplasm or "gap substance" of Murray (16), but are not always seen clearly nor well differentiated from the inner portion of wall. However, in cells which are lysing and in which cytoplasmic membrane is clearly separated from wall, the "globules" disappear and a dense inner layer of wall is clearly seen (Fig. 4a): it is occasionally also well seen in other cells (Fig. 4b). This layer, which is about 3 to 3.5 nm in thickness, is seen more clearly in some wild-type strains (Fig. 4c, 4d). The tripartite appearance of *B. subtillis* wall has also been described by Glauert et al. (8) and by Granboulan and Leduc (9), as well as being noted by others. The inner leaf of the cytoplasmic membrane (IM; Fig. 4b, 4c) is only rarely seen and is usually defined only in areas of right-angle sectioning by the separation of the middle electron-lucent membrane layer (MM; Fig. 2d, 3, 4d) from cytoplasm on one side and from the outer dense membrane leaflet on the outer (as in normal cells).

In other respects, however, the wall of 168ts-200B presents some peculiarities even at 30 C. The outer dense layer appears only partially present and is not sharply defined, and the outer wall surface thus appears correspondingly irregular and "fuzzy" to an extent greater than seen in most normal or wild-type cells (Fig. 2, 4). The cross-walls frequently show protrusions from the division plane (arrows, Fig. 1a–c, 2a), an effect which is seen occasionally in aging cultures of normal cells. Internally, the cytoplasm is often pale and relatively homogeneous, and ribosomes are not well defined (Fig. 1, 2), although fixation was carried out directly in the culture medium and tryptone was present during both primary and secondary (osmium) fixation. The nucleoid is usually represented by scattered electron-lucent areas, occasionally containing ill-defined coarse fibrils (Fig. 1, 2a, 2b). The variability of the nucleoid appearance after glutaraldehyde fixation has been reported by Granboulan and Leduc (9). Membrane invaginations or mesosomes are rarely seen and then appear atypical (Fig. 2a, 2b); they do, however, clearly contain the periplasmic-like layer (pp, Fig. 2b), as is characteristic and as was emphasized (though not in these terms) by others (6, 7, 9).

Cells of mutant 168ts-200B grown from spores at the restrictive temperature (45 C) are radically different from those germinated and grown at 30 C. Instead of rods, they appear (at 4 hr) as more or less rounded cells in irregularly spherical masses, separated within each mass by a profusion of abnormal and randomly situated cross-walls (Fig. 5). There is gross disturbance of cell wall architecture (Fig. 5, 6). The outer surface of the wall is very irregular and "fuzzy." The wall varies greatly in thickness (from 20 to 200 nm), and is, on the average, much thicker (50 ± nm) than in 30 C cells; and it is loosely organized, sometimes fibrillar, and homogeneous from the absence of the inner and outer dense layers. As a result of loss of the inner wall layer, the periplasm-containing layer (OM + PP, Fig. 3) is ill-defined, reduced in thickness (as best can be determined), or, in fact, seems to disappear (Fig. 6a, 6c). (In Fig. 6b some residual periplasm seems to be present; compare with Fig. 2 and 4.) The cross-walls are also fuzzy and irregular in outline and appear "moth-eaten" (Fig. 5b, arrow). Gross thickenings of wall appear, usually at or near the bases of the abnormal cross-walls (Fig. 5). The inner leaflet of the cytoplasmic membrane is poorly defined, as usual. The cytoplasm and scattered nucleoid areas appear as in cells grown at 30 C. However, occasional cells, closely associated with others in the spheroidal masses are extremely dense, seemingly because of a heavy concentration of densely-staining ribosomes (Fig.

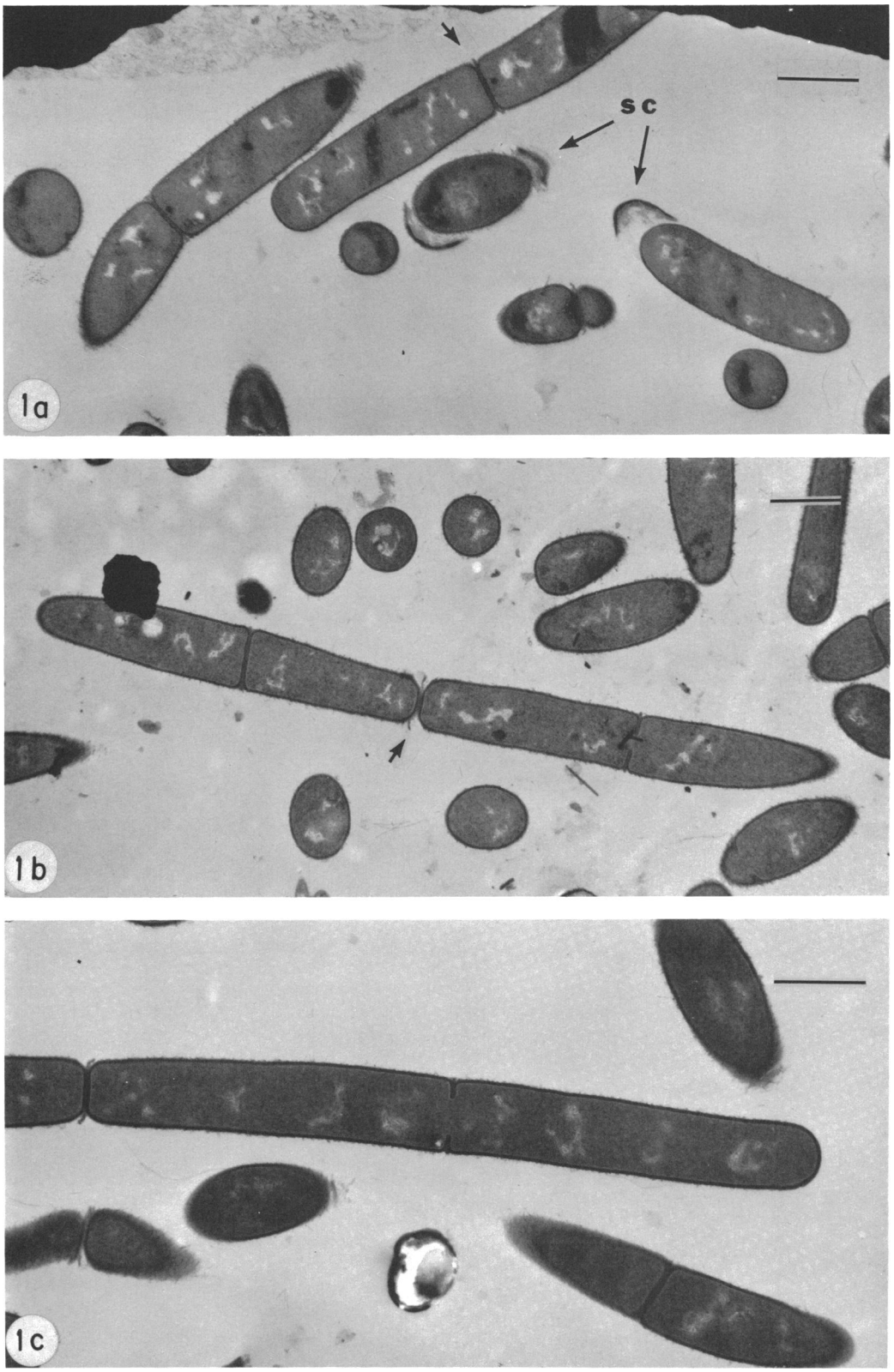
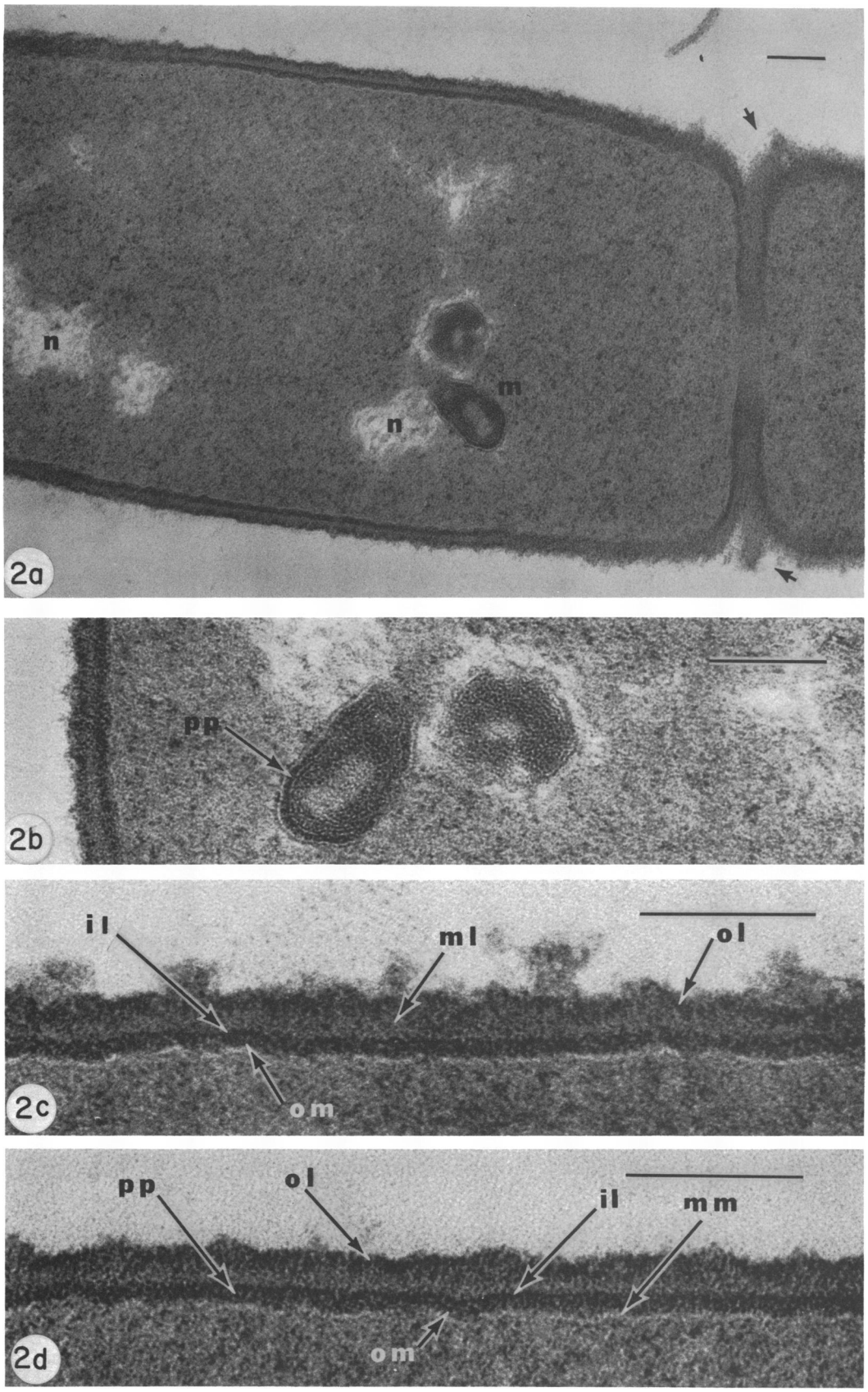


FIG. 1. *Examples of bacilliform growth, from spores germinated and incubated for 6 hr at 30 C. Note germinated cells emerging from spore coats (sc) in a and protrusions of cross-wall (arrows) in a and b. Magnification: a, ×12,250; b, ×10,500; c, ×13,600. Bars, 1.0 μm. All electron micrographs are of ultrathin sections of Bacillus subtilis 168ts-200B, except Fig. 4c and 4d.*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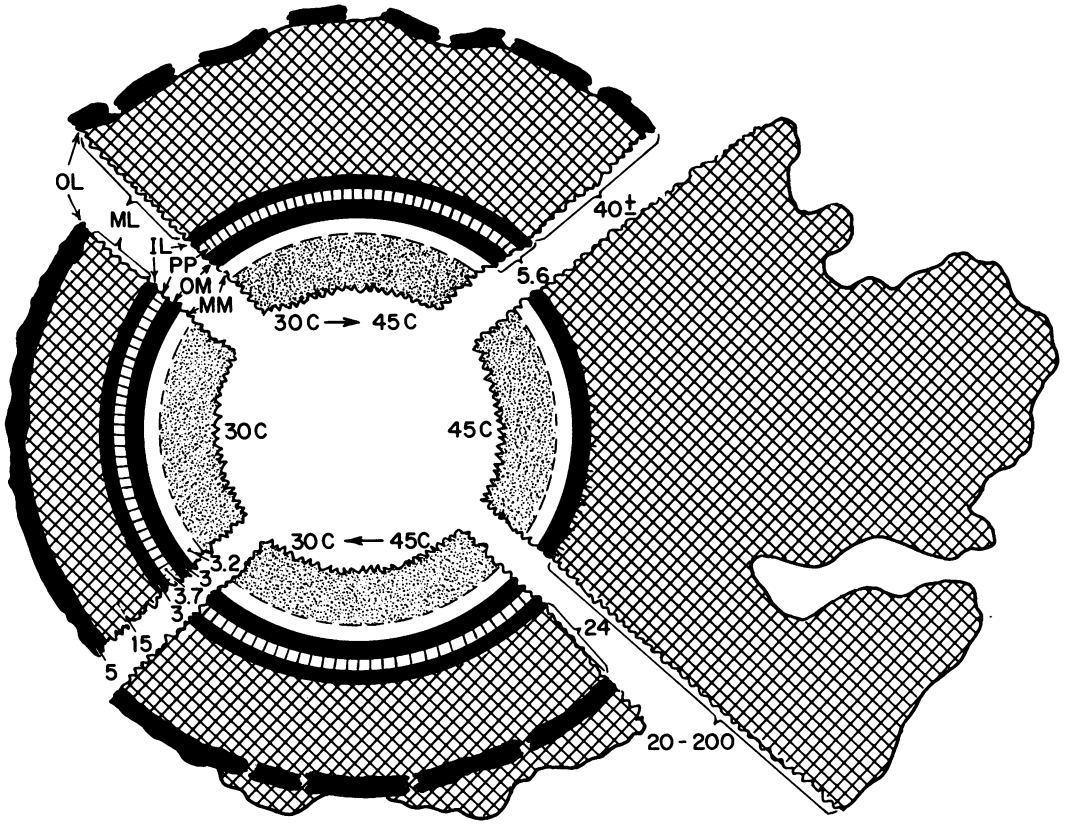
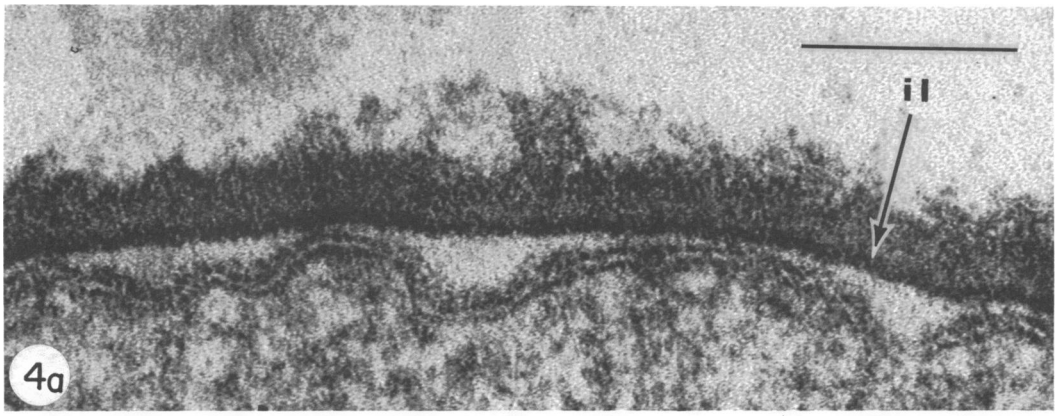


FIG. 3. *Schematic diagram of changes in cell wall and periplasm at the permissive and restrictive temperatures and during temperature shifts. Labels as in Fig. 2. Numbers represent approximate mean thickness, in nanometers, of the indicated layers.*

7). In addition, well-defined fibrils are seen in the nucleoids of these cells. Their walls are similar to those of the other cells. Mesosomes are rarely seen in cells grown at 45 C.

Log-phase cells of 168ts-200B growing at either the permissive or restrictive temperature with the respective morphologies described, can transform to the alternate morphology when shifted to the alternate temperature. On shifting a culture of 45 C-grown spheres to 30 C, the first evidence of reversion to the bacillary form can be detected with the light microscope about 1.5 to 2 hr after transfer. By electron microscopy (Fig. 8), masses of bizarre cells are still present, although the masses are somewhat less spherical in shape, less compact, and some outgrowing cells appear semibacillary. There is both early and late lysis within many cells, thus accounting for the previously observed (2) failure of many spheres, as seen by phase microscopy, to revert to bacilli. The reason for this lysis is unknown. The most striking change on close inspection, however, is the gradual reappearance in the bacillary cells of inner and outer dense layers of cell wall, with a corresponding increased definition of the periplasm-containing layer and restoration of its thickness to about 10 nm (Fig. 9a, 9b). This does not occur in all cells simultaneously (Fig. 9c). Actually, the normal thickness and structure of wall and periplasm frequently appears to be restored beneath the thickened middle layer of the 45 C wall, the peripheral remnants of which then

FIG. 2. *Bacilliform growth at 30 C for 6 hr. In a, note nondense nucleoid (n), mesosome (m), and protrusions of cross-wall (arrows). ×90,000; bar, 0.1 μm. b, Detail from Fig. 2a; pp, periplasm in mesosome. ×188,000; bar, 0.1 μm. c and d, Two examples of wall detail: ol, outer dense layer of wall; ml, middle layer of wall; il, inner dense layer of wall; pp = periplasm; om, outer layer of cytoplasmic membrane; mm, middle, nondense layer of membrane. The inner layer of membrane (im, in Fig. 4b and 4c) cannot here be distinguished from the cytoplasm. ×288,000; bars, 0.1 μm.*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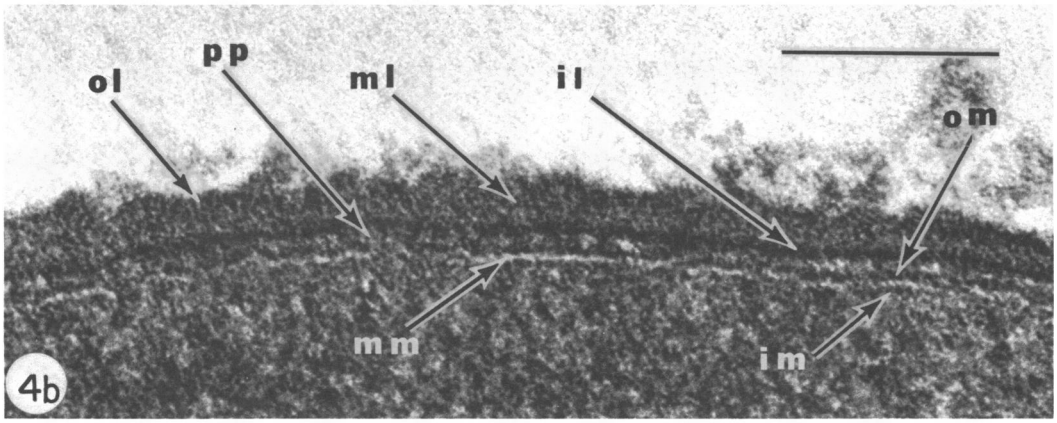
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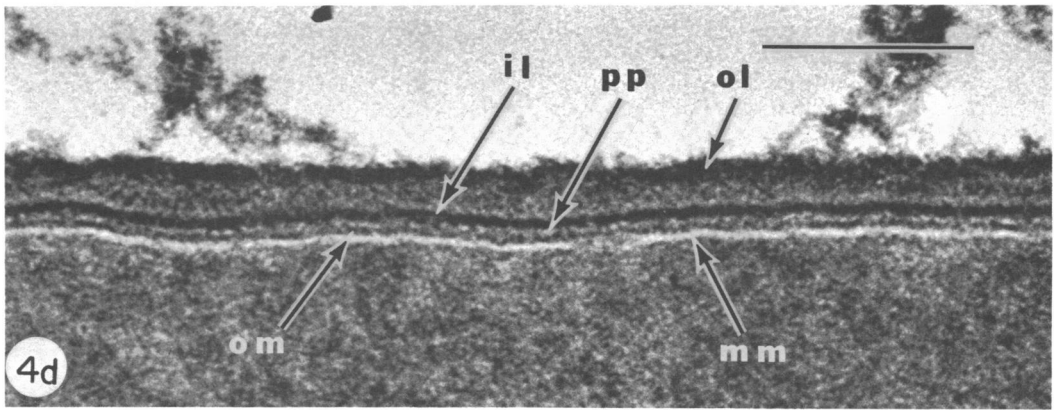
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seem to be gradually cast off (arrow, Fig. 8; Fig. 10). The thinner and more regular wall then surrounds more and more of the reverting cell, which gradually approaches the shape and appearance of cells grown from spores at 30 C. By 3 hr after transfer, many more semibacillary forms can be seen (Fig. 11). These occur as curved, irregular rods in irregular chains which appear, as sectioned in a single plane, as groups of cells of various size and shape. Cross-walls are formed at right angles to peripheral wall and often at sites more nearly approximating the center of the cell (Fig. 11b). Occasional but atypical mesosomes can be seen (*m*, Fig. 11a); and in cells undergoing what appear to be "early" lysis, collections of extruded vesicular elements resembling mesosomes are often seen (Fig. 12). Internally (cytoplasm, nucleoid, cytoplasmic membrane), the reverting bacilli resemble those grown at 30 C except that occasional dense cells are seen (arrow, Fig. 11a).

When spores germinated and grown to bacilli at 30 C are transferred to 45 C, the first change (noted at 30 min) is a slight rounding of the rods, which are nevertheless maintained in chains with cross-walls forming at right angles and at regular intervals (Fig. 13). There is a "patchy" early loss of the outer dense layer of wall, and a corresponding more or less regular fraying of the outer surface which gives a "scalloped" appearance (Fig. 13a; arrows, Fig. 14a, 14b). The inner layer of wall also becomes ill-defined. After 2 hr, the cells are still in chains. The periplasm-containing layer is indistinct and narrowed from apparent increasing loss of the inner layer of wall (Fig. 14d). The remainder of the wall becomes gradually thicker (about 40 nm), frayed, and irregular (Fig. 14c, d), and cross-walls of irregular shape, thickness, and origin begin to appear (Fig. 15). The appearance continues to approach that of cells germinated and grown at 45 C. An occasional dense cell is seen in the developing spheroidal masses. In the other cells, the cytoplasm and nucleoid are unchanged, and mesosomes continue to be rare or absent.

Throughout these changes, in either direction, the width of the cytoplasmic membrane components appear constant. The outer dense leaflet (OM, Fig. 3) is 3.0 to 3.5 nm wide, and the middle nondense layer (MM, Fig. 3) is 3.0 to 3.3 nm in width. The inner leaflet (IM, Fig. 3), when it can be seen as in the "lytic" profile (Fig. 12), has the same dimensions as the outer; in nonlysing cells, as mentioned above, it cannot be delineated from cytoplasm and therefore cannot be measured.

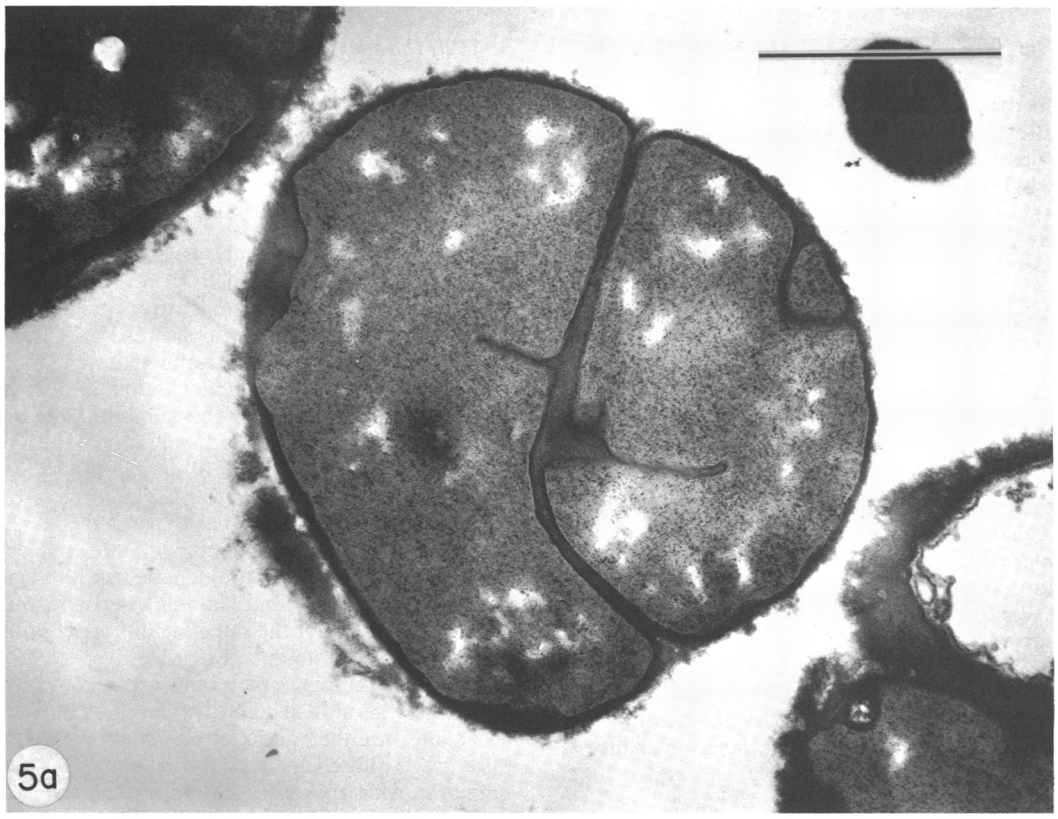
DISCUSSION

This ultrastructural study verifies the original report (2) of the growth of *B. subtilis* 168ts-200B as rods at the permissive temperature and as large spheroidal cells in masses at the restrictive temperature. Furthermore, it shows that the latter condition is clearly associated with loss of normal ultrastructure of wall and the submural region. This suggests that the trilaminar appearance of wall often seen in sections of *B. subtilis* (8, 9), other bacilli (6, 17, 18), and other gram-positive rods (7) may not be artifactual as claimed (17, 18), but may in fact represent components that are demonstrated by the glutaraldehyde-osmium-lead sequence of fixation and staining used and that are lost, as seen after the same conditions of preparation, when the temperature-sensitive defect is expressed at the restrictive temperature.

Because the ultrastructural changes of wall are reversible in either direction as incubation temperature is shifted, it is clear that they are concomitant with the gross alterations of cell size and shape; it is likely that the ultrastructural changes are responsible for such alterations. However, until studies are made of the chemistry of the layers of normal *B. subtilis* wall and of mutant wall at both temperatures and correlated with examination of ultrathin sections prepared by glutaraldehyde-osmium-lead, the molecular or biochemical nature of the losses expressed conditionally in the mutant cannot be determined.

At 45 C, the appearance of *B. subtilis* 168ts-200B is similar to that shown by Rogers et al. (22) in *B. subtilis* and *B. licheniformis* mutants grown in the absence of additional salt or certain amino acids. Whether there is loss of wall layers and periplasm under these conditions cannot be determined from the published electron micrograph. However, it was stated (22) that these forms did not lyse in water: neither do the 45 C-grown spheres of 168ts-200B. Furthermore, the change in shape of 168ts-200B at 45 C is not prevented by making the medium hypertonic (2), suggesting that internal osmotic pressure against a nonrigid wall is not a primary factor in determining the shape. Consequently, we presume that there is some residual wall rigidity present in both types

FIG. 4. *Detail of cell walls. a and b, B. subtilis 168ts-200B at 30 C for 6 hr. c and d, B. subtilis Marburg (wild type) at 37 C for 6 hr. Note distinct inner layer (il) of wall in plasmolysing cell in a and "fuzziness" of outer layer of wall in a and b. The wall is more regular in thickness, and the various layers more clearly defined, in cells of the wild-type strain (c, d). Occasionally, in either mutant (b) or wild type (c), the inner layer of cytoplasmic membrane (im) can be seen in intact cells. ×280,000; bars, 0.1 μm.*



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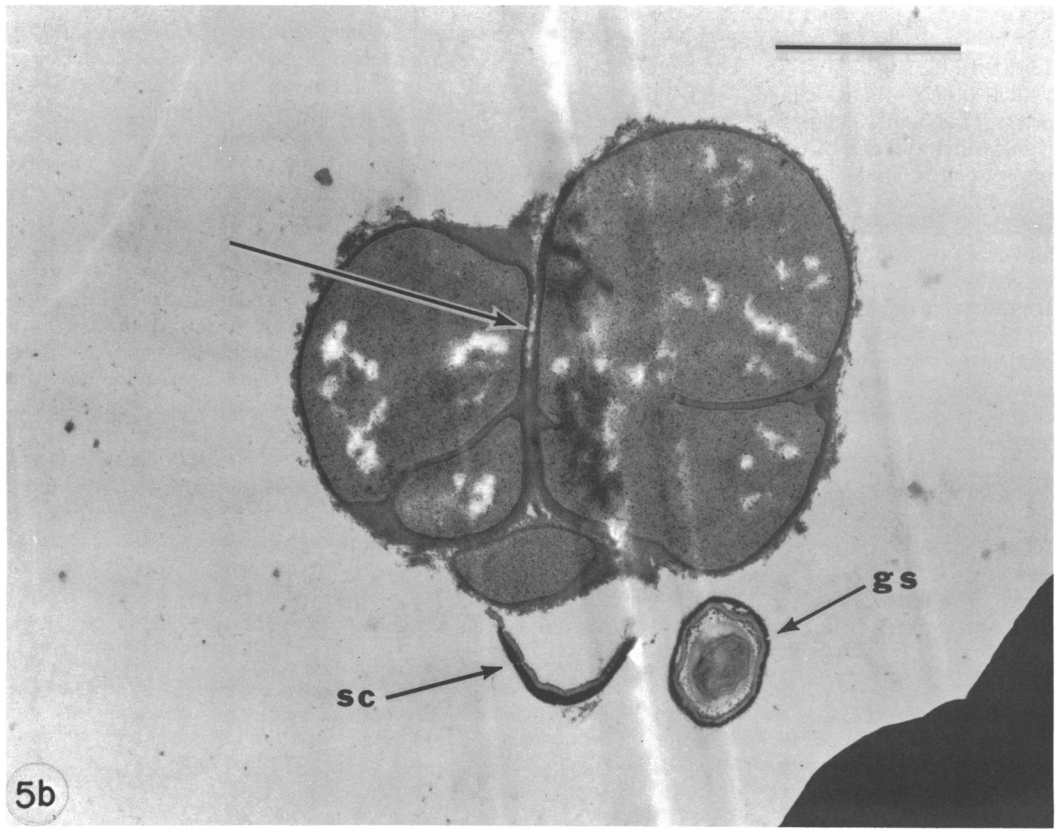

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of mutants under the respective restrictive conditions. Whether this is due, in 168ts-200B, to the continued presence of mucopeptide, or to what the state of the mucopeptide, if present, may be, remains to be demonstrated. At least one of the mutants of Rogers is said to possess mucopeptide in the restrictive condition, and this mucopeptide shows a lower degree of cross-linking than that isolated from the same mutant growing under permissive conditions (21).

A somewhat similar sphere-rod transition, which is also controllable nutritionally, has been investigated by Krulwich et al. (12, 13) in *Arthrobacter crystallopoietes*. Although great differences in the gross chemical compositions of walls and spheres were not shown, detailed analyses of the peptidoglycans demonstrated that, comparatively, the spheres contained shorter glycan chains of greater heterogeneity in length. Some longer peptide cross-bridges, due to glycine, were also present, although the extent of cross-linking was slightly less than in the rod form. [Ultrastructural studies of another species of *Arthrobacter* (26) do not show a thickened wall of the coccoid forms, but the electromicrographs presented do not permit a detailed analysis of the cell wall ultrastructure.] Krulwich et al. (13) conclude that the sphere wall of *A. crystallopoietes* is more loosely organized than that of the rod; they later showed that increased activity of an *N*-acetyl-muramidase is positively correlated with the transition to the sphere form (11). Studies of some other bacteria have also indicated that gross morphological changes may be associated with changes in the extent of peptide cross-linking of the peptidoglycan, although other alterations in surface structure may also occur (19, 30). In view of our ultrastructural studies of the spherical form of 168ts-200B, a chemical alteration in wall structure appears a reasonable although as yet unproven possibility. Whether the temperature-sensitive step affected by mutation involves autolysin production or activity, production or activity of wall-synthesizing enzymes, or other processes leading to unbalanced replication of cell wall, will not be known until completion of further studies.

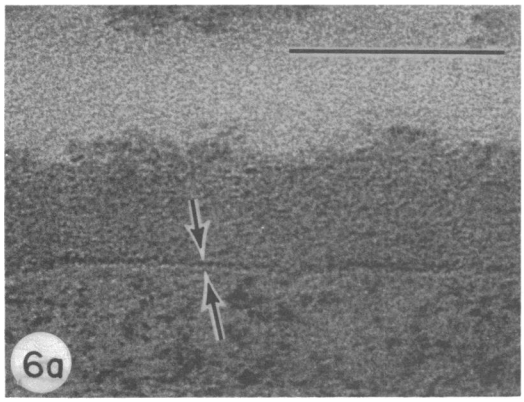
An interesting aspect of the cell wall mutants relates to the last stage of cell division, that is, the splitting of the cross-wall to separate daughter cells. This stage appears to be defective under the restrictive conditions in both the mutants of Rogers (22) and our temperature-sensitive mutant. In the latter at 30 C, protrusions from the division plane were noted (Fig. 1, 2a); these seem to represent excess middle wall layer which is cut off but not degraded while ingrowth of wall of normal thickness and trilaminar appearance is taking place at the future new poles of each daughter cell. A similar appearance, although of lesser degree, can be seen in wild-type cells. However, at 45 C the bases of the cross-walls become broad and irregular (Fig. 5, 7), and the entire cross-wall, like the peripheral wall, seems composed entirely of middle layer.

In addition, there is seemingly gross misorientation of cross-wall formation. Cross-walls appear to originate at sites on pre-existing cross-wall (Fig. 5); and several walls can often be seen radiating in different planes from the above-mentioned basal accumulations of thick middle wall material. However, the factor(s) which regulate cross-wall location during normal bacterial growth are not fully understood, and loss of normal topography in 45 C-grown cells of 168ts-200B makes it impossible to determine whether cross walls are truly originating from other cross-walls, or rather from peripheral wall which is mislocated because of unbalanced growth and shape distortion. This problem is further compounded by the paucity of visible mesosomes, which seem to be essential extensions of the membranous septum which is the initial determinant of subsequent cross-wall site in normal division. Most importantly, however, in 168ts-200B at 45 C, cell separation fails to occur in the absence of trilaminar wall of normal thickness. Only an inefficient and occasional fraying of the loosely organized cross-wall is seen (arrow, Fig. 5b; Fig. 7), and the cells remain in spheroidal masses.

Similar failure of daughter cell separation in cell wall mutants with large, thick- and rough-walled cells has been reported not only by Rogers et al. (22) in *B. subtilis* and *B. licheniformis*, but also by Chatterjee et al. (5) in a *Staphylococcus aureus* mutant lacking polymeric teichoic acid. This mutant contained more murein than the parent strain, more nucleotide-bound *N*-acetylated amino sugar murein precursors, and more wall-bound autolysin, and the walls were twice as thick as those of the parent cells. No studies of the nature of the murein have been reported. The *rod*⁻ mutants of Rogers et al. (21, 22) contain more wall per unit weight than the wild type; in the round form under the restrictive conditions, mucopeptide is present, but as mentioned above, is less cross-linked than in the rod-shaped cells or in the parent (21).

Other instances of failure of daughter cell sepa-

FIG. 5. *Cells germinated from spores and grown for 4 hr at 45 C. Note spheroidal shapes; irregular cross-walls; thickening, irregularity, and "fuzziness" of peripheral walls; and "moth-eaten" appearance in cross-wall (arrow in b). Cytoplasm is pale and nucleoids appear as scattered electron-lucent areas. gs, Germinating spore. Magnification: a, ×31,500; b, ×24,050. Bars, 1.0 μm.*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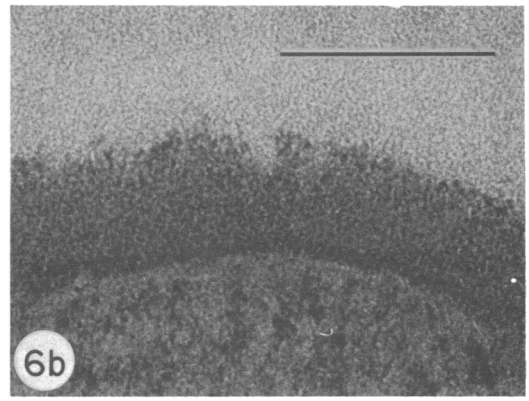
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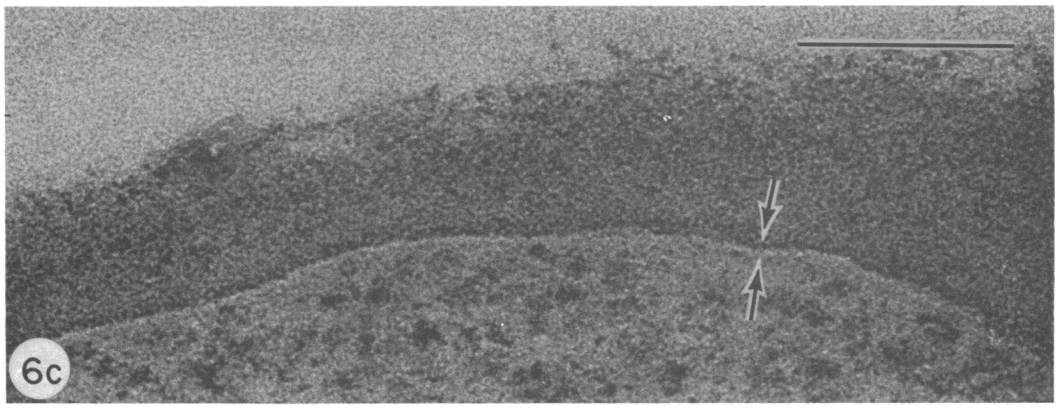
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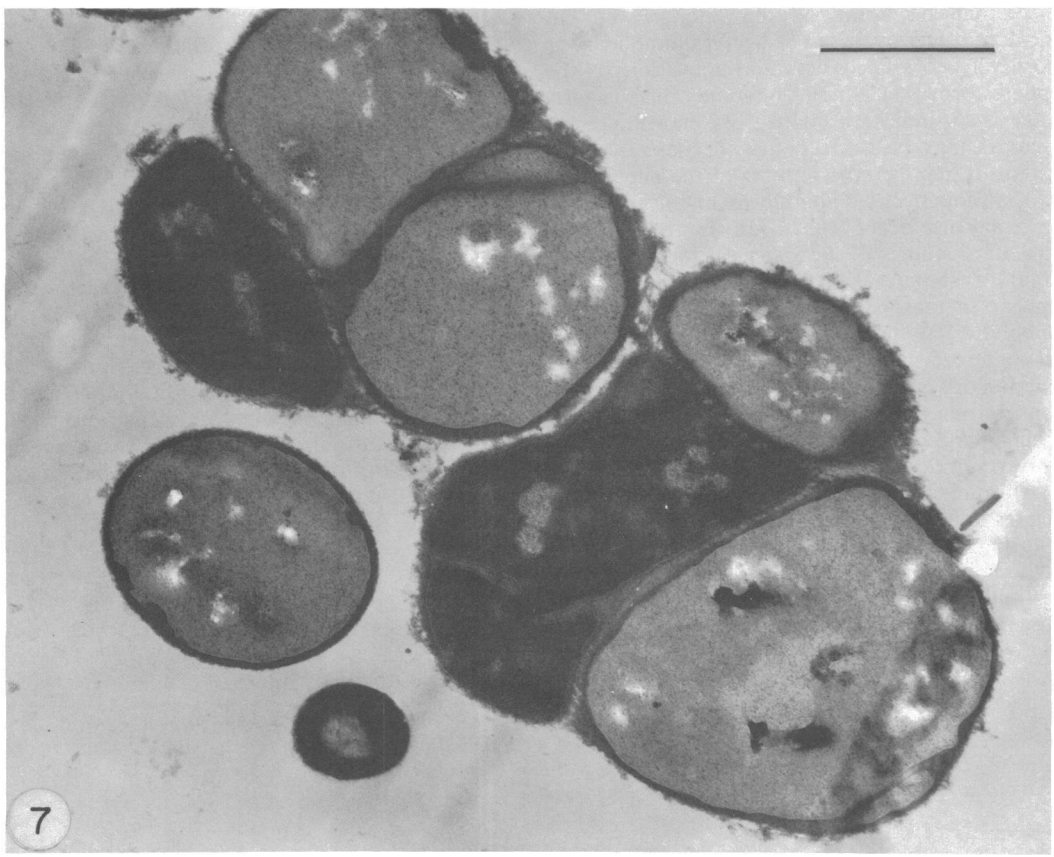
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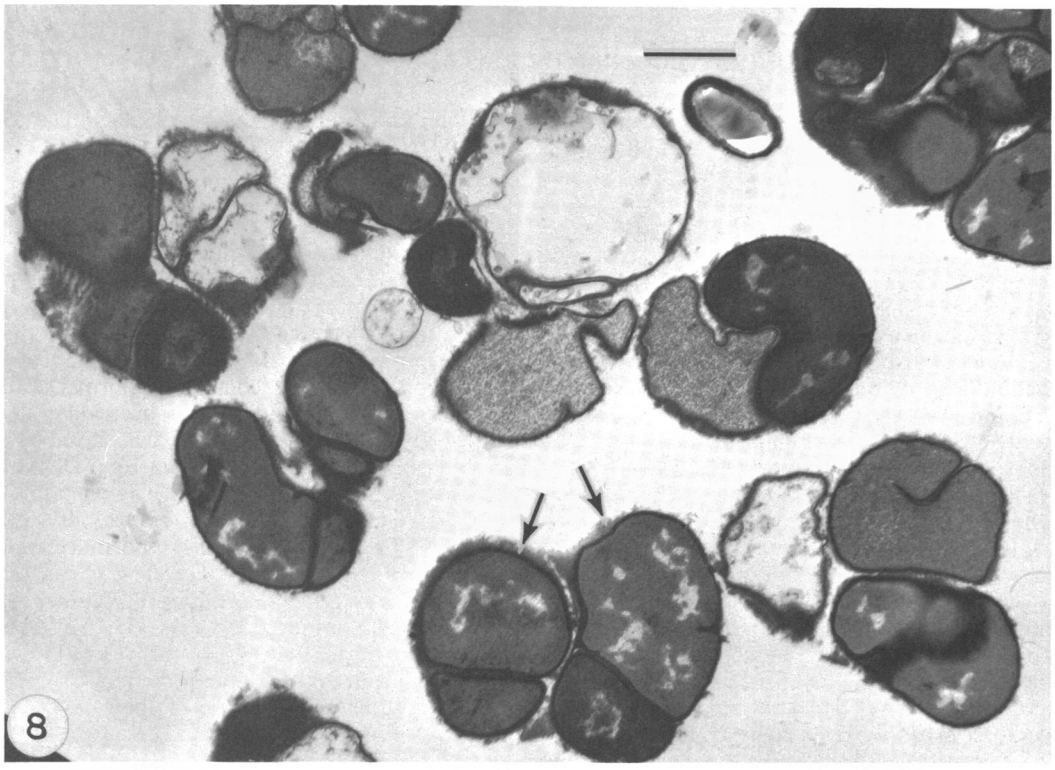
ration, resulting in strongly chaining cells that appear to have normal peripheral wall structure, have been reported in opaque colony variants of group A streptococci (15, 27) and in a filamentous mutant of *B. cereus* T (29). Chemical analyses of these showed no gross differences in cell wall composition, as compared with wild-type or parent walls, but no chemical structural studies were made. In *Pneumococcus*, however, the substitution of ethanolamine for choline in the ribitol teichoic acid of the cell wall (4) also results in chaining (28). These cells fail to autolyse, cannot be lysed by added autolysin or deoxycholate, and are genetically incompetent.

The significance of these scattered observations cannot be assessed at present. They suggest that the presence of ultrastructurally and chemically defective cell wall can result in failure of the last stage of cell division, as well as in changes in cell shape, and that such failure may also occur as a separate event apparently limited to the cross-wall region itself. However, ultrastructural and chemical comparisons of normal and mutant walls in more detail are required to assure the normality of wall structure in the latter instances. In addition, although a few studies (5, 11, 28) report either increased or absent autolytic activity in cells which fail to separate or which show changes in shape, the relationships of autolytic activity, ultrastructure and chemical structure of walls, changes in cell shape, and separation of daughter cells, are not clear. Some possibilities, in relation to teichoic acids, have been recently mentioned by Heptinstall et al. (10).

The most interesting and obvious question raised by our ultrastructural findings in 168ts-200B is that of control of normal thickness of cell walls of *B. subtilis*. It is a common electron microscopic observation that cell walls become thickened and irregular in old cultures of gram-positive bacteria, and that supernumerary and irregular cross-walls may appear. Unbalanced growth resulting from amino acid deprivation or from chloramphenicol treatment can also lead to overall wall thickening in *Streptococcus faecalis* (25). An unpublished report (E. K. Neale, and G. B. Chapman, Bacteriol. Proc., p. 39, 1969) indicates that a streptomycin-resistant tryptophan$^-$ mutant of *B. subtilis* 168, grown at high (as well as at low) temperatures, shows irregular and thickened cross-walls and peripheral wall with protrusions at division planes similar to those seen in our 30 C-grown cells. The bacteria continue to grow as rods. (Unfortunately, fixation in that study did not include glutaraldehyde; thus, no direct comparison of wall ultrastructure can be made with that seen in our studies.) Nevertheless, the parent strain of our mutant also does not form spheres at 45 C. We have not yet examined the ultrastructure of wall of the parent strain under these conditions. It appears, however, that genetic control of normal wall thickness may be conditionally overridden by altered environmental conditions; however, the gross changes leading to spheres in 168ts-200B at 45 C suggest that, additionally, the temperature-sensitive mutation is affecting control of wall synthesis. The absence of inner and outer wall layers in spheres of our mutant at 45 C suggest that control of normal wall thickness is somehow related to the presence of these layers; their appearance during sphere to rod transition is compatible with this concept, although not proof thereof. The chemical nature of these layers is unknown. However, teichoic acid comprises 60% of the weight of *B. subtilis* 168 (1, 31), and is intimately bound to the autolytic amidase (3). It also appears somehow related to wall thickness, autolytic activity, and to failure of cell separation as mentioned above (5, 11, 28). It is therefore difficult to avoid speculation on the role of this wall polymer in the morphogenetic changes of 168ts-200B. In this respect, it is important to note that glucosylation of wall teichoic acid in *B. subtilis* is regulated by at least three genes (*gta* A, *gta* B, and *gta* C) clustered to the right of the *hisA* locus in the middle of the chromsome (32), and that preliminary mapping of 168ts-200B by PBS-1 transduction indicates that the temperature-sensitive mutation is also in this region of the chromosome (R. J. Boylan, N. H. Mendelson, and F. E. Young, *in preparation*). These collaborators have also transferred the mutant locus, by deoxyribonucleic acid-mediated transformation, or "congression" (32), into *B. subtilis* BR-19 (*trp*$^-$ *hisA1*). The transformants exhibit the same gross morphological changes, with temperature shifts or after germination of spores at either temperature, as does 168ts-200B; electron microscopy shows exactly the same ultrastructural changes. At 45 C, the walls of transformants show an increase in total mucopeptide (as per

FIG. 6. *Examples of wall detail of 45 C-grown cells. Note increase in wall thickness (all of middle layer) and "loose" organization; compare with Fig. 2 and 4. The cytoplasmic membrane can be defined (arrows in a and c), but the usual layers of wall and periplasm are not seen. Occasional cells (b) retain some periplasm that is poorly delimited.* ×*280,000; bars, 0.1 μm.*

FIG. 7. *Example of spheroidal masses of 45 C-grown cells containing dense cells with fibrillar nucleoids.* ×*22,750, bar, 1.0 μm.*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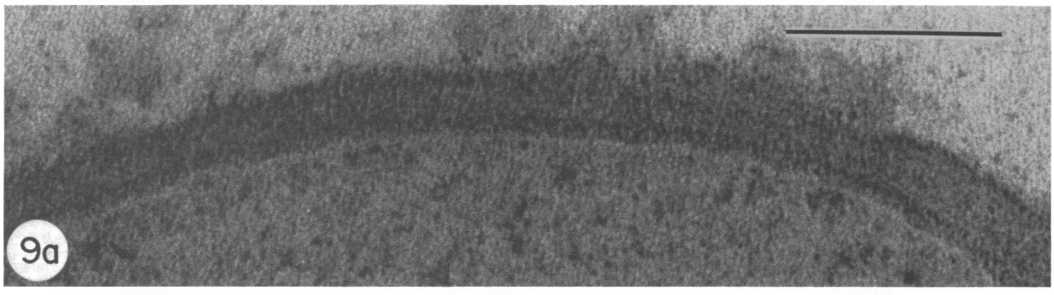
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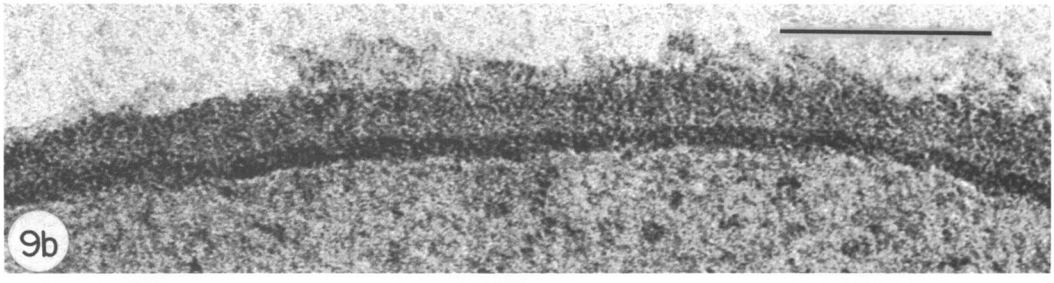
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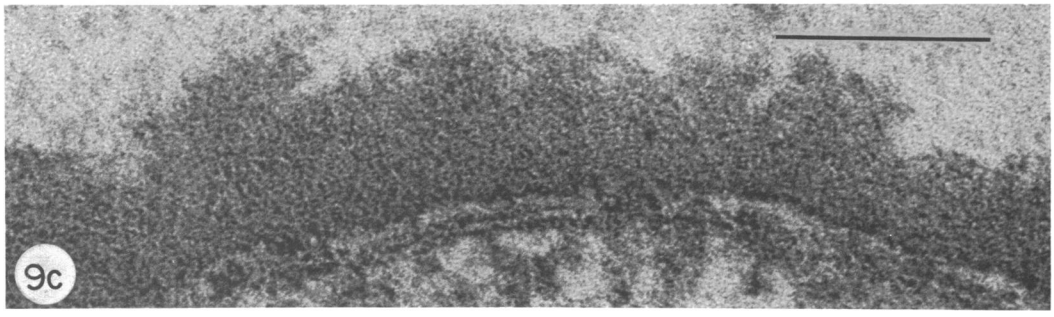
9c

cent of wall weight), and wall teichoic acid is reduced by at least 80%. Correspondingly, phage ϕ25 (32) no longer adsorbs to these walls as it does to walls of 30 C-grown cells.

It seems important to emphasize also the disappearance of ultrastructurally recognizable periplasm in 168ts-200B at the restrictive temperatures. This submural region, which is seen only as a space when glutaraldehyde fixation is omitted (6, 8, 9), is a presumptive site of many enzymes and precursor materials, although few such have actually been demonstrated. As noted by several investigators (6–9), as well as in this study, it extends into the membranous septa and mesosomes. It is, therefore, fully formed at normal cross-wall sites (future poles) before the final separation process is initiated. It is quite possible that the site of the intitial defect in cell wall mutants is in the periplasm, and that this is the reason for its disappearance, in 168ts-200B, at the restrictive temperature. The disappearance is coincident with loss of normal wall structure which is, in turn associated with loss of ability to complete cell division in normal fashion. [However, the periplasm does not seem absent or abnormal in the streptococcal variants (27) nor the *B. cereus* mutant (29) that fail to complete division.] Whether the periplasm, as seen ultrastructurally, is really only a reflection of the state of the cytoplasmic membrane or an extension thereof [with the real defect in the membrane proper, as suggested by Rogers et al. (22)] is somewhat of a semantic question which cannot be answered now.

ACKNOWLEDGMENTS

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FIG. 8. *Cells grown at 45 C after shift to 30 C for 1.5 hr; ×12,250; bar, 1.0 μm.*

FIG. 9. *Examples of cell wall detail from cells in Fig. 8, showing the gradual reappearance of layers of cell wall and periplasm in some (a, b) but not all (c) cells. ×280,000; bars, 0.1 μm.*

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FIG. 10. *Examples of 45 C-grown cells after shift to 30 C for 1.5 hr, showing reappearance of normal wall thickness beneath excess residual middle layer. (a) ×39,000; bar, 1.0 μm. (b) ×105,000; bar, 0.1 μm. (c) ×120,000; bar, 0.1 μm.*

(Fig. 10 on page 807)

FIG. 11. *Cells grown at 45 C after shift to 30 C for 3 hr. Note appearance of more regular semibacillary forms with rare mesosomes (m) and cross-walls forming at right angles to peripheral wall (arrow in b). The plain arrow in a indicates a dense cell. Magnification: a, ×12,250; b, ×17,500. Bars, 1.0 μm.*

(Fig. 11 on page 808)

FIG. 12. *Detail of lysing cell from 45 C-grown culture shifted to 30 C for 3 hr. Note extruded mesosome-like vesicles (m). Many walls are still thick and irregular, but those in the bacillary forms are approaching a normal thickness and structure (arrow, left). ×96,000; bar, 0.1 μm.*

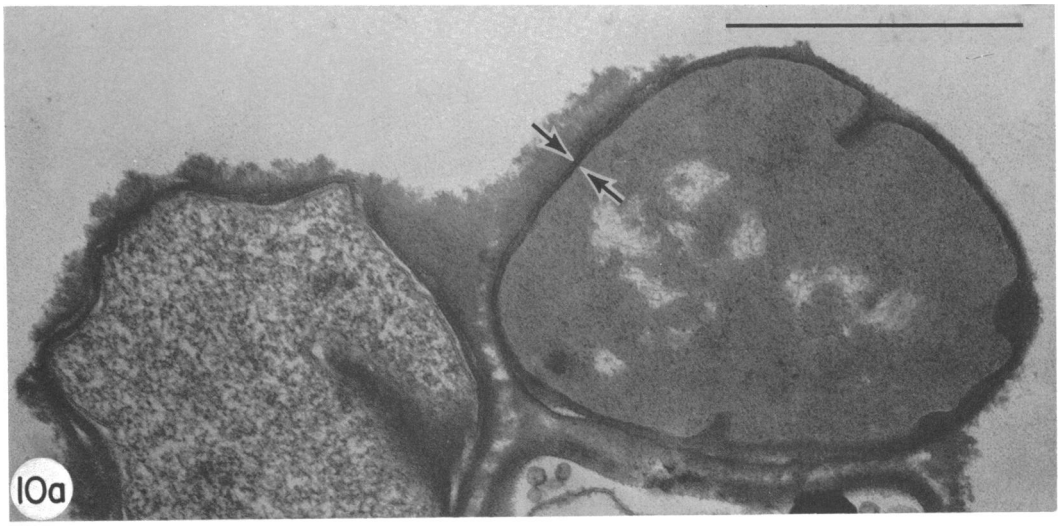
FIG. 13. *Examples of cell grown at 30 C after transfer to 45 C for 1 hr. Note slight rounding and "patchy" or scalloped appearance of peripheral wall surface. Magnifications: a, × 22,750; b, ×12,250. Bars, 1.0 μm.*

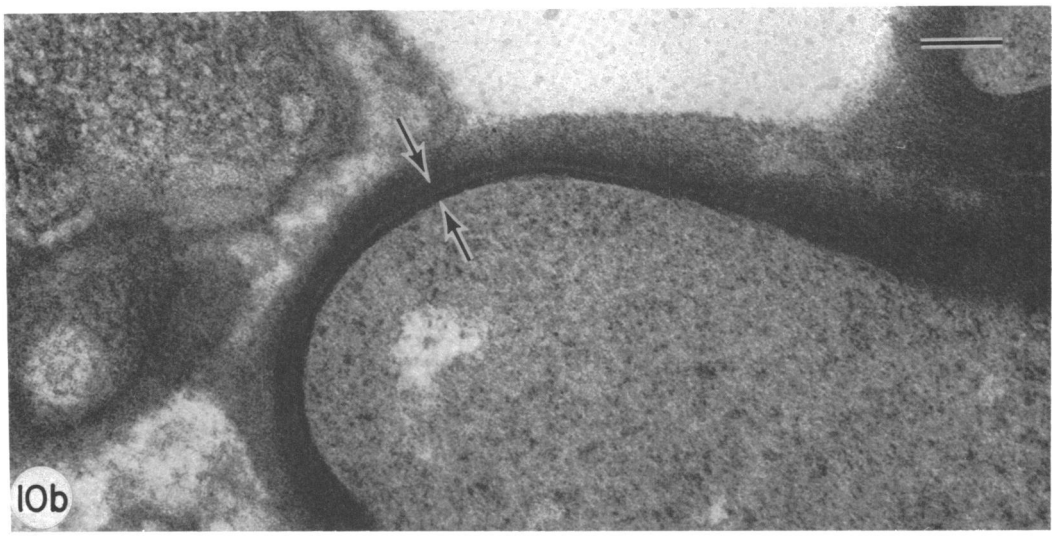
(Fig. 12 and 13 on page 809)

FIG. 14. *Examples of cell wall detail of cells after shift from 30 to 45 C for 1 hr (a, b) or 2 hr (c, d). In a and b, note patchy loss of outer wall layer (arrows) and some loss also of inner wall layer, resulting in narrowing and poor definition of the periplasm. In c and d, the wall has become thicker and more irregular, and definition of the periplasm may become entirely lost (d). ×280,000; bars, 0.1 μm.*

FIG. 15. *Cell grown at 30 C after transfer to 45 C for 2 hr. Note increasing irregularity of cell shape and of cross-walls and wall thickening and irregularity of surface. ×22,750; bar, 1.0 μm.*

(Fig. 14 and 15 on page 810)





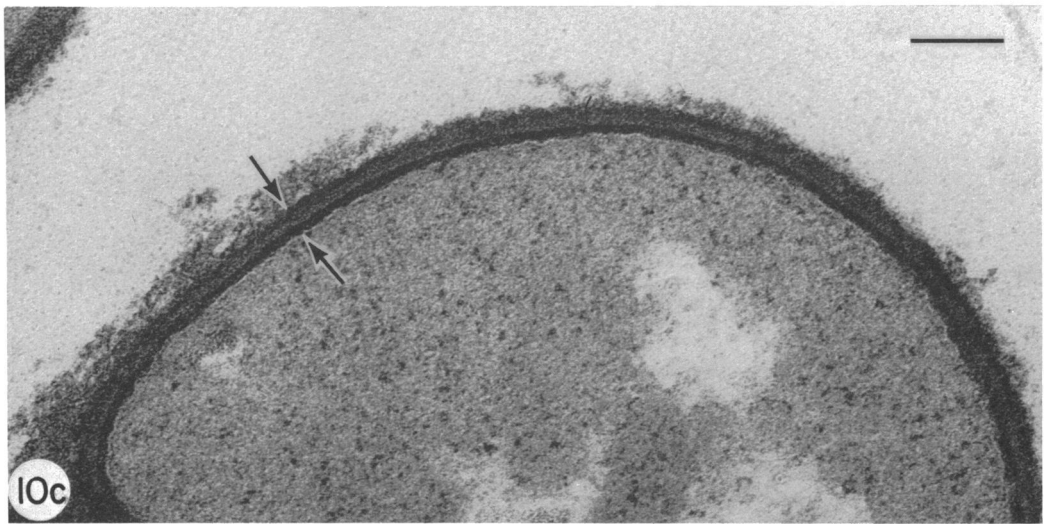
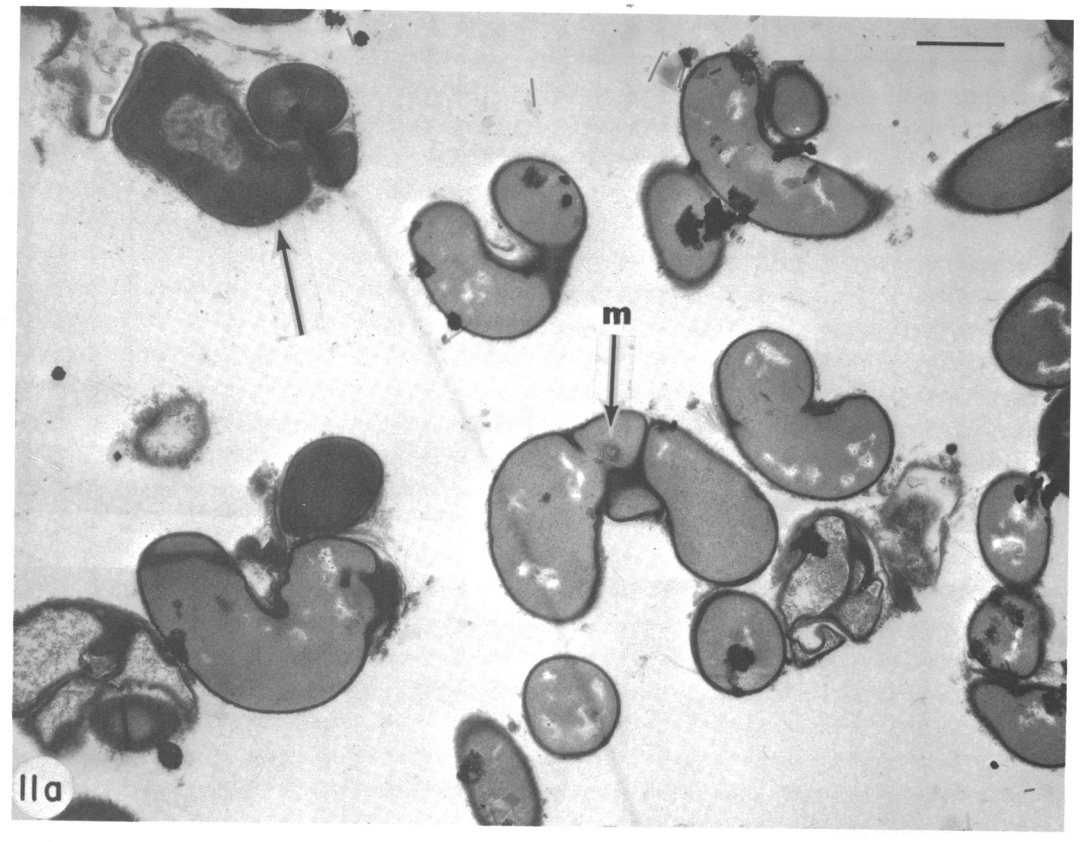


FIG. 10



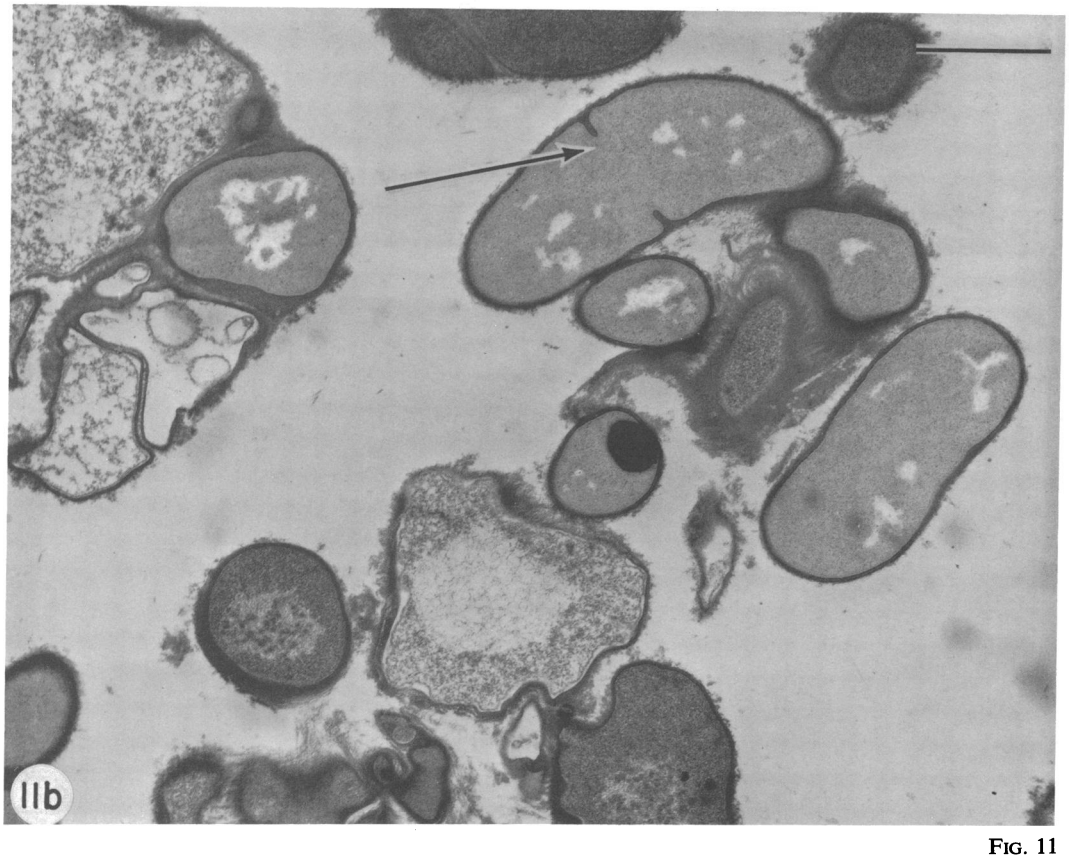
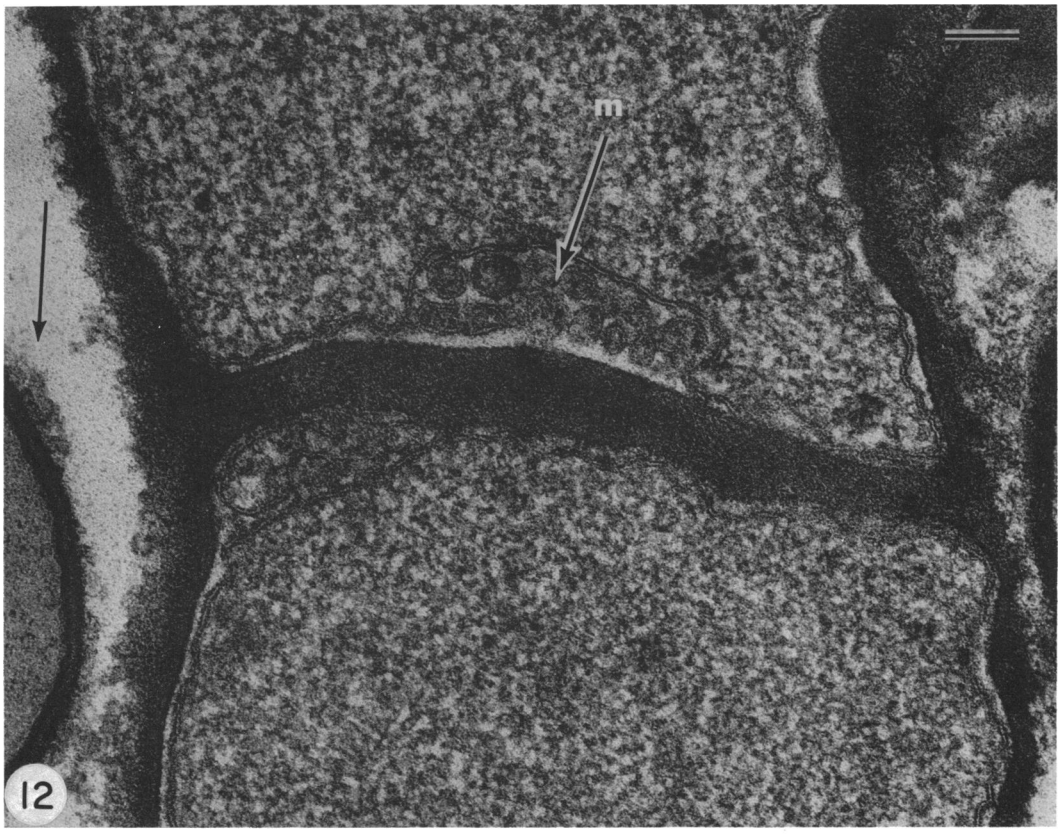
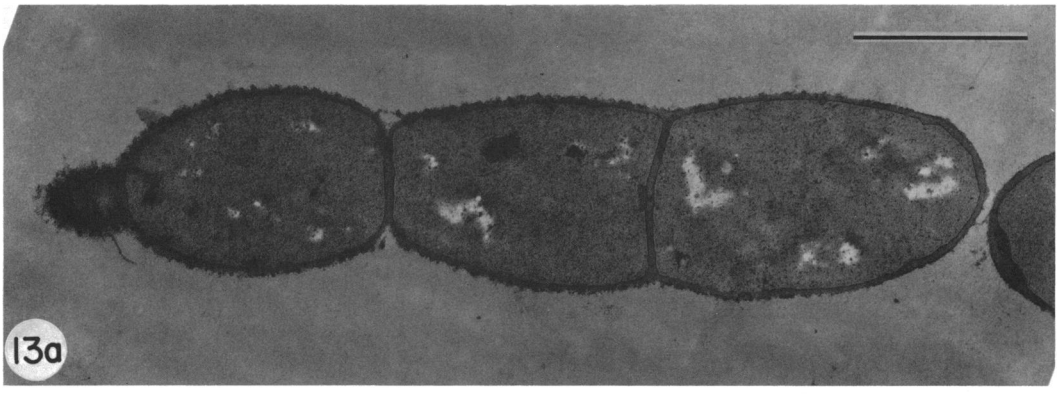


FIG. 11





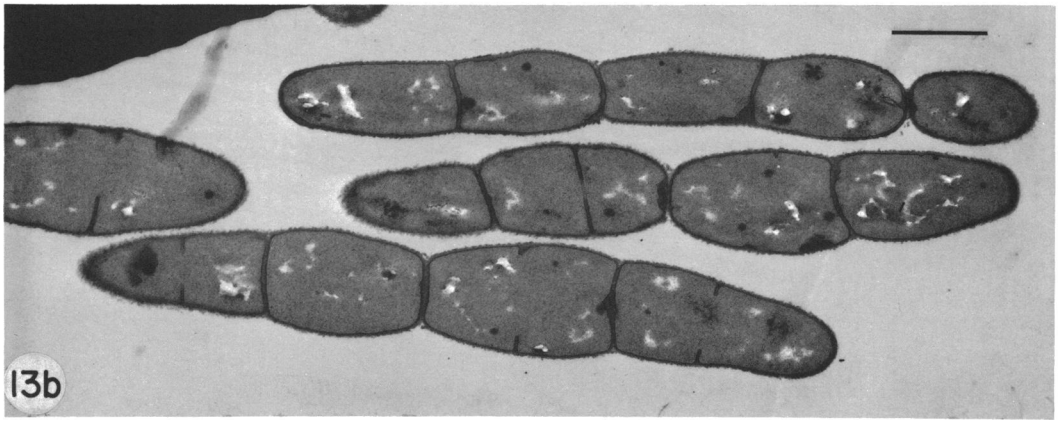
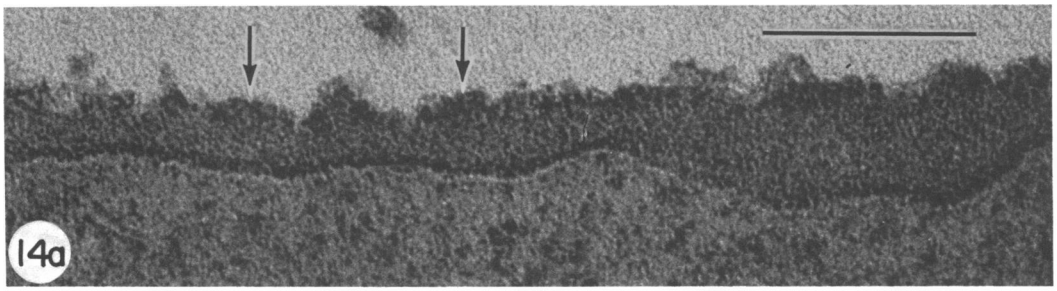
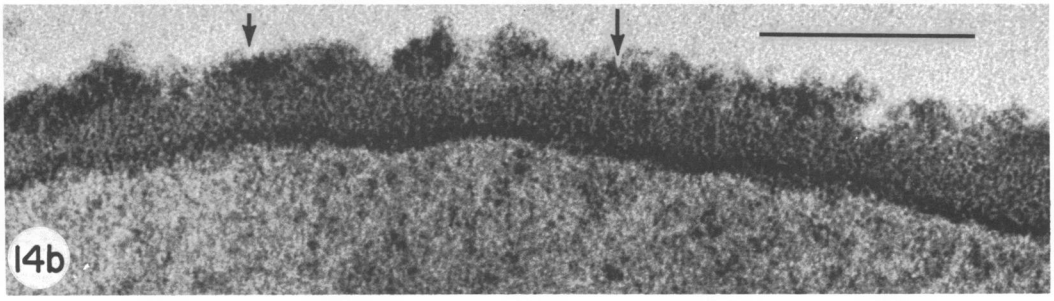
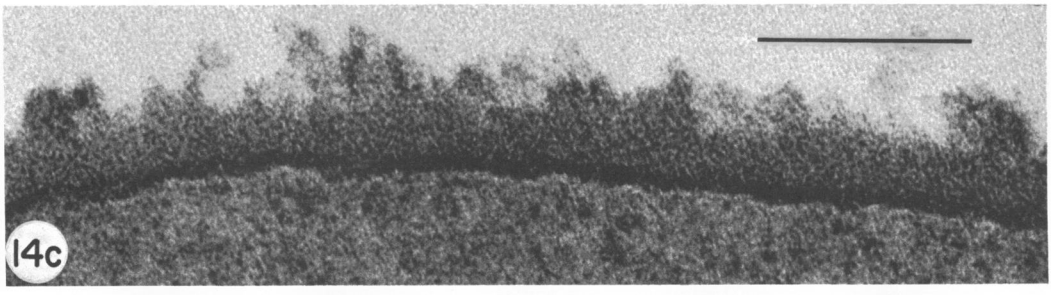
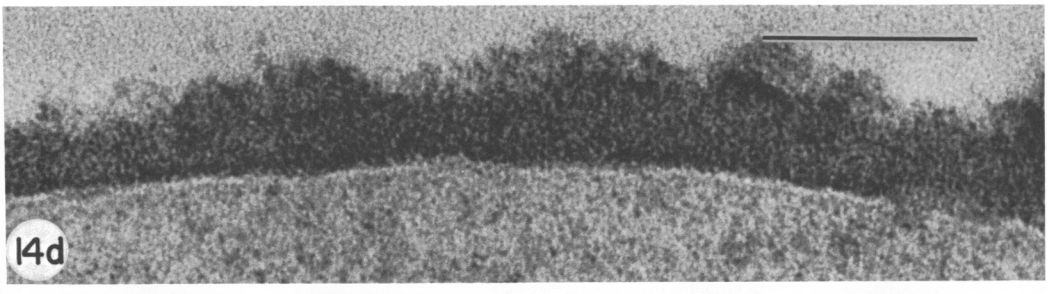


FIG. 12 and 13









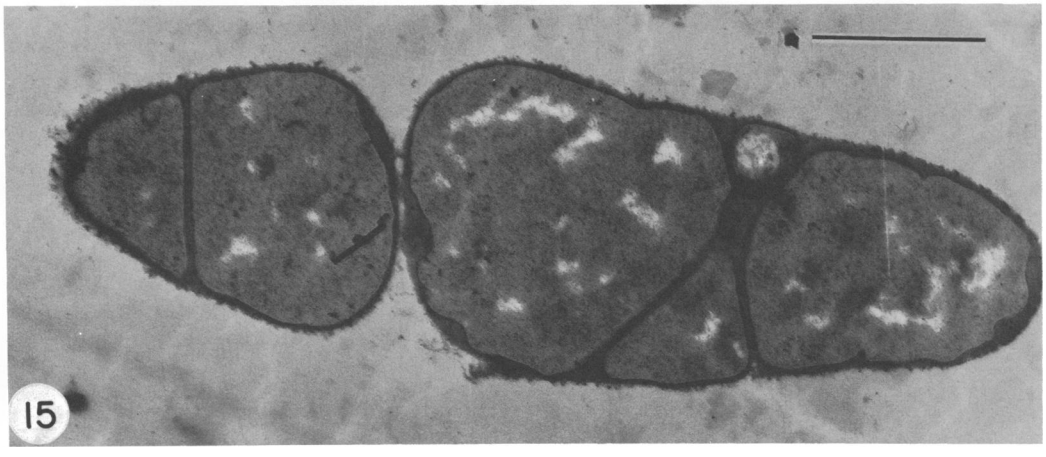


FIG. 14 and 15