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Mode of Cell Wall Synthesis in Gram-Positive Bacilli

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Ultrastructural experiments on plasmolyzed cells suggested that the information for the position and orderly synthesis of septa is not determined by the attachment of cell membrane to previously formed wall. These experiments, in conjunction with others on cells disrupted by the freeze-fracture technique, are most consistent with wall growth over the entire surface of the rods, with wall material gradually moving from a position next to the cell membrane to a position at the outer surface of the cell.

The basic mechanisms of morphogenesis in bacteria remain unclear at the present time. For example, it is not even known how the walls of the septa in rod-shaped bacteria are placed at regular positions, all perpendicular to the long axis of the cells. In thinking about possible mechanisms of septum synthesis, it is essential to consider the cell membrane, because that is the location of wall synthetic enzymes.

In recent times, the fluid-mosaic model of the membrane (8) has come into wide acceptance. However, in this model, proteins and lipids are free to diffuse throughout the membrane. There is no mechanism for proteins involved in the synthesis of septal wall being localized in defined positions. But it is possible to imagine that wall synthetic enzymes are held in place by attachment to previously existing peptidoglycan. This wall material is relatively rigid, with the glycan strands held in a fixed array by covalent peptide cross-links. In this model, the order in the newly synthesized wall would be dictated in part by the spatial relation of material in previously formed wall. If this attachment is presumed to be the only force holding septal wall synthetic proteins in place, these enzymes would be free to flow in the fluid-mosaic membrane if the attachment of old wall to membrane were disrupted. One result would be that enzymes synthesizing septa would now become dispersed over the entire surface of the membrane and no coordinated septum synthesis would occur.

To test this model, *Bacillus subtilis* cells were plasmolyzed by the addition of 35% sucrose. After this disruption of attachment of wall to membrane, several aspects of wall synthesis were studied ultrastructurally, with particular emphasis on regions where septa were being formed.

MATERIALS AND METHODS

Strains. *B. subtilis* 168M carrying the *trpC2* mutation was obtained from B. Reilly (School of Dentistry, University of Minnesota). Strain βA205 was an *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine-mutagenized derivative of *B. subtilis* βA203 described earlier (1). βA205 was a strain tentatively characterized as having an autolytic amidase even more sensitive to high-temperature inactivation than the amidase in βA203 (unpublished observations). In studies of 35% sucrose-plasmolyzed cells grown at 51 C, both βA205 and 168M had precisely the same morphology at all stages of growth. Therefore, the ultrastructural results from these two strains were pooled. *B. megaterium* 899 has already been described (10).

Plasmolysis. Prewarmed 60% sucrose in Penassay broth (antibiotic medium no. 3, Difco) was added to *B. subtilis* cells growing exponentially at 51 C in Penassay broth to give a final sucrose concentration of 35%. Immediately the cells were centrifuged in a rotor prewarmed to 30 to 50 C. Then the cells were resuspended in Penassay broth at 51 C and aeration was continued.

Microscopy of whole cells. Whole cells were fixed and embedded according to the procedure of E. Hagen (unpublished data). The fixation involves glutaraldehyde followed by osmium tetroxide treatment.

Cell walls. *B. subtilis* walls were prepared and examined electron microscopically as described previously (3). *B. megaterium* cells were grown and disrupted by the freeze-fracturing technique according to published methods (7). The walls were prepared and examined ultrastructurally, using the procedures employed for the *B. subtilis* walls.

RESULTS

Growth of plasmolyzed cells. *B. subtilis* cells could grow exponentially at 51 C in Penassay broth. If sucrose was added to 35% and then quickly removed, the plasmolyzed cells continued to grow, but at a slower rate (Fig. 1). After a recovery period of 20 to 30 min, the cells

resumed their typical exponential increase (Fig. 1). Thus, the sucrose treatment only caused a temporary disruption of normal growth.

Ultrastructure of plasmolyzed cells. There was often very little difference between the morphology of the cells before plasmolysis and immediately after the sucrose addition and removal (Fig. 2). Unfortunately, the sucrose used in the plasmolysis interfered with fixation procedures, so plasmolyzed cells could not be examined ultrastructurally. In both types of cells at 51 C, the mesosomes appeared to be compressed against the wall. Mesosomes were found with high frequency at the septa and near the hemispherical caps of the cells, although mesosomes could also be seen anywhere along the length of the cells (Fig. 2).

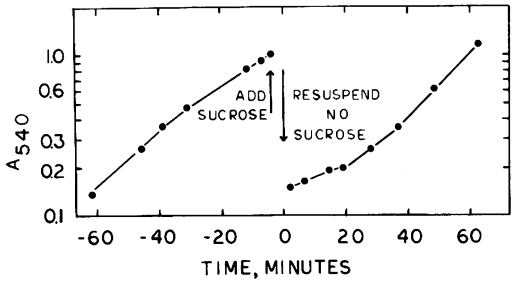

FIG. 1. *Plasmolysis of B. subtilis 168M cells. The bacteria were grown in Penassay broth at 51 C. At the indicated time, sucrose was added to a final concentration of 35%. The cells were immediately centrifuged and resuspended in Penassay broth at 0 min and aeration was continued at 51 C. The turbidity of the culture (absorbancy at 540 nm; A_{540}) was measured.*

However, during growth after the plasmolysis step, marked ultrastructural changes were seen (Fig. 3). Particularly at the early times of 5 and 10 min after the removal of the sucrose, wall was synthesized around pockets of cytoplasm. The positions and sizes of the pockets were very reminiscent of mesosomes, so a likely interpretation was the expulsion of the mesosomes from the cytoplasm during plasmolysis into the space between the cytoplasmic membrane and the wall. Then new wall synthesized on the cytoplasmic membrane was separated from the previously formed wall by the extruded mesosomes. A large proportion of the mesosomes was expelled at the septa so that pockets were often seen at these positions. In those regions where the new wall was separated from the old wall by a pocket of mesosomal material, there was obviously no connection between the membrane and the wall made before plasmolysis. Nevertheless, septa continued to be formed in the same positions as before. Therefore, the septal

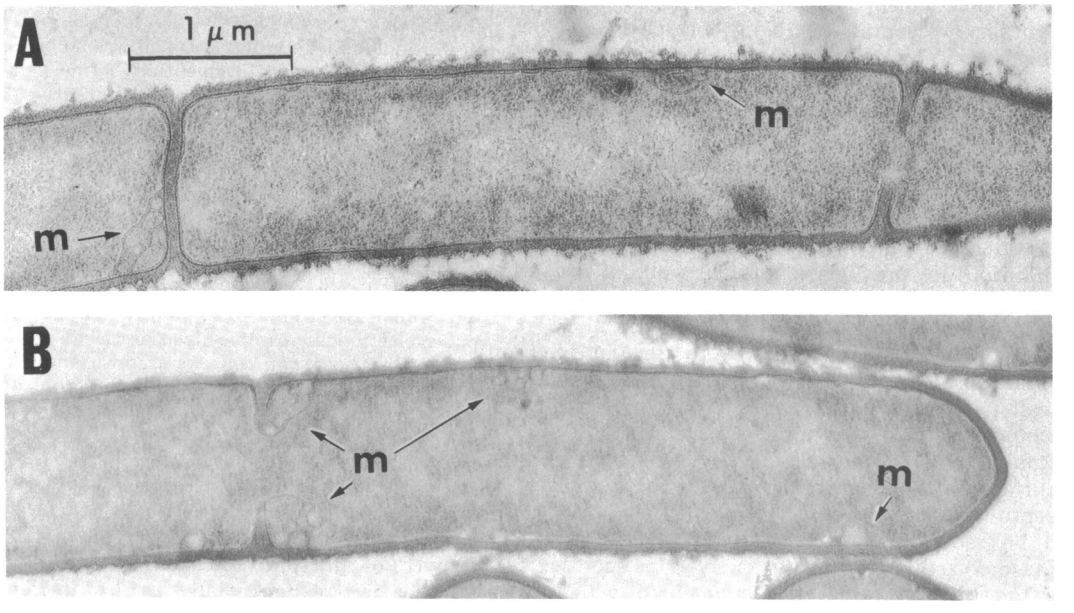

FIG. 2. *Ultrastructure of plasmolyzed B. subtilis 168M cells. The cells used in the experiment of Fig. 1 were examined in thin sections immediately before sucrose addition (A) and immediately after resuspension without sucrose (B). Mesosomes (m) were seen at the partial septa, along the length, and at the hemispherical caps of the cells.*

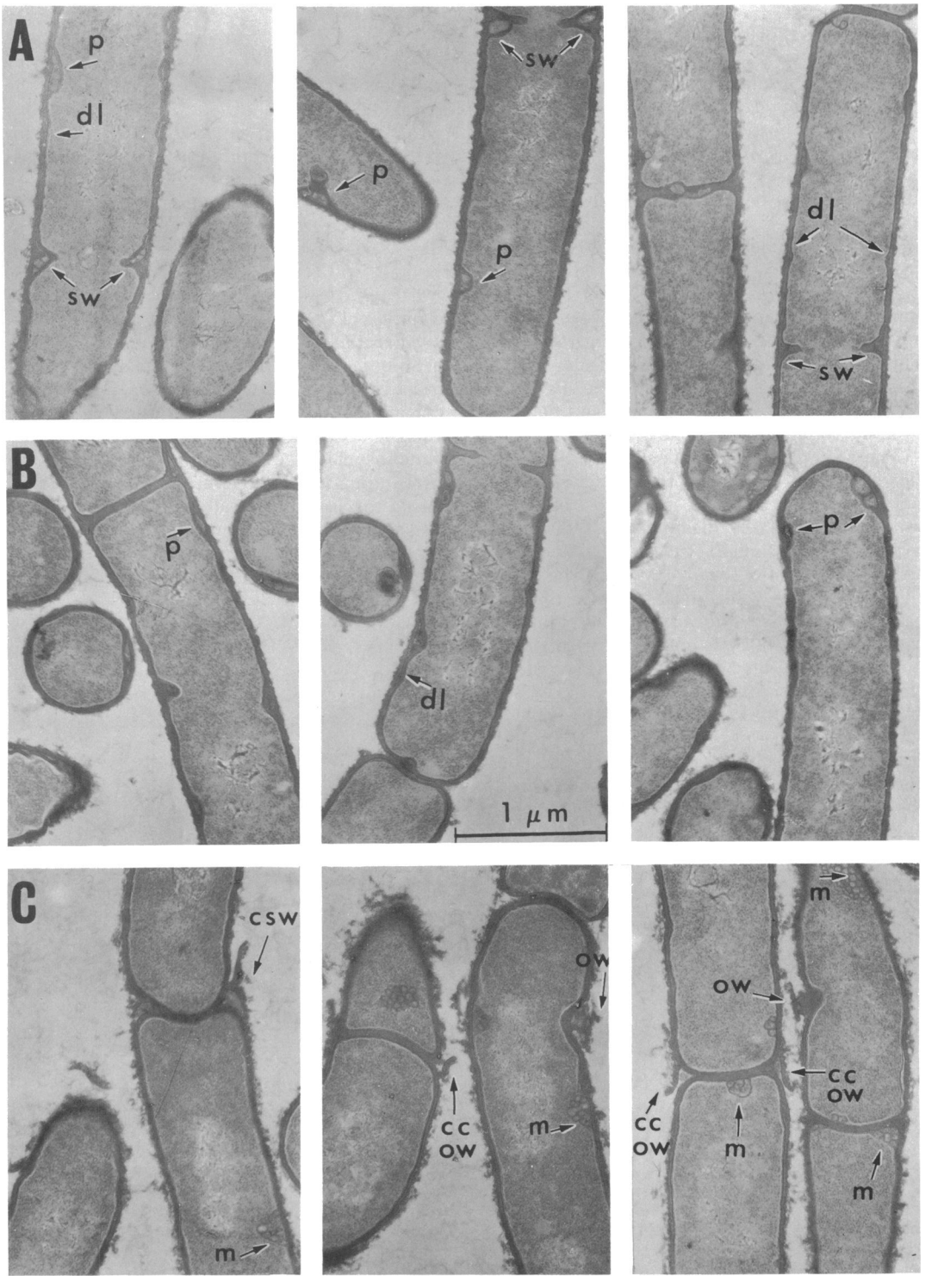

FIG. 3. *Ultrastructure of plasmolyzed B. subtilis 168M cells after growth at 51 C. Cells from the experiment in Fig. 1 were examined in thin sections at 5 (A), 10 (B), and 30 (C) min after resuspension without sucrose. Newly synthesized cell wall could be seen to form pockets (p) separating mesosomal material from the cytoplasm. Much of the new wall was also seen to be separated by a dark line (dl) from wall made before plasmolysis. Based on the morphology around the pockets and the dark lines, septal wall could be seen to be synthesized after plasmolysis in the same positions as before plasmolysis (sw). After 30 min of incubation, some of the septa had become completed in the region of septa started before plasmolysis (csw). At this late time, only fragments of old wall formed before plasmolysis (ow) were visible, especially around what formerly were mesosomal pockets. Also, mesosomes (m) were again apparent inside the cells. The fragments of walls often had the shape of convex curves (cc).*

wall made after plasmolysis was synthesized by membrane not attached to previously formed wall. Often, a portion of old septal wall could be seen in positions where new septal wall was also laid down (Fig. 3). Thus, the model postulated above could not be correct. The organization of proteins synthesizing septa could not be due solely to attachment to old wall.

Since pockets were seen along the length of the cell as well as in the regions of the hemispherical caps and the septa, the cells must have been making wall over their entire surface. This fact was more clearly seen in isolated walls (Fig. 4). There was often a dark line that could be followed between the old wall and new wall over the total surface of the cell.

The walls between the pockets and the interior of the cell continued to thicken as growth proceeded (Fig. 3). At 30 min, most of the old wall had completely disappeared. Only in the regions around some of the pockets were there still remnants of old wall. However, the overall thickness of the wall did not change dramatically. These observations were consistent with the model that wall was made exclusively at the membrane. As new wall was synthesized, the old wall would have moved toward the exterior of the cell. Autolytic enzymes could have acted continuously on the cell wall. The wall at the exterior of the cell would have been the oldest and would have been exposed to autolysin for the longest time. Therefore, this wall would have been the most digested and the loss of cell wall would take place preferentially at the exterior surface of the wall.

Curves in broken cell walls. This model for wall growth at the membrane followed by digestion at the exterior surface also explained a common observation made on the walls of gram-positive rods. An example was found in Fig. 3 and 4. Often, where there were breaks in the cell wall, the wall around the breaks assumed a convex curve. The most dramatic examples of these curves (Fig. 5) were seen in *B. megaterium* cells broken by the freeze-fracture technique (7). In this method, cells were frozen in water and then the ice was crushed with a mortar and pestle. In the process, cells were cracked open like fossils in a rock, with the plane of cleavage being determined by the surrounding ice. Therefore, the cleavage planes passed through cells at all angles. This result is unlike that obtained by most other mechanical techniques for breaking cells. There the structure of the cell is such that breakage is usually

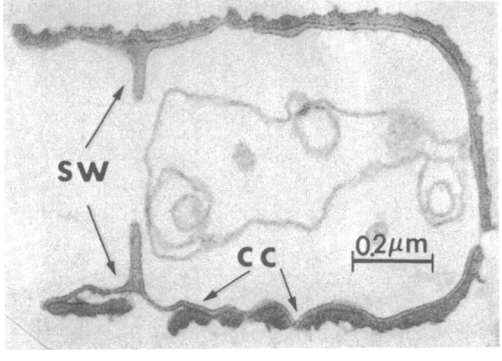

FIG. 4. *Walls of B. subtilis BA205 cells grown after plasmolysis. βA205 cells were treated as described in Fig. 1. At 20 min after the resuspension, the walls were isolated and examined in thin section. Newly made septal wall (sw) could be seen in the region of a mesosomal pocket that had ruptured. A dark line separated the newly synthesized wall from the wall made before the plasmolysis. In regions where the previously formed wall was broken, the walls often assumed a convex curvature (cc).*

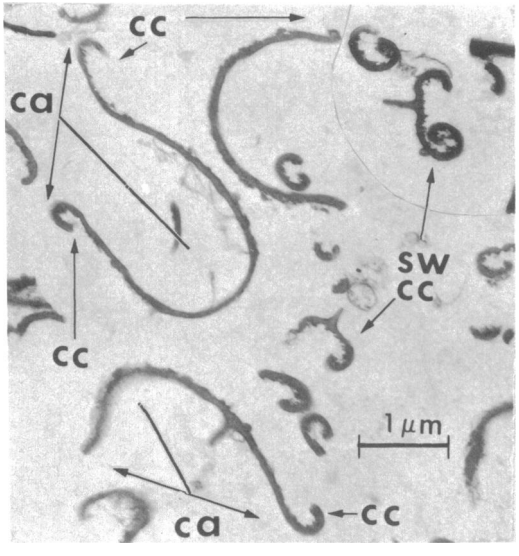

FIG. 5. *Walls of freeze-fractured B. megaterium 899 cells. Cells growing exponentially at 30 C in Penassay broth were disrupted by the freeze-fracturing technique. The walls were isolated and examined in thin sections. Convex curves (cc), some so extended as to form spirals, were seen in the walls in which the cells had been cleaved along the diagonal or along the length of the cells. The inner part of the curves had a rough surface typical of the outside of the cell, whereas the outer part of the curves had the smooth appearance characteristic of wall next to the cytoplasmic membrane. Some of the curves were seen in the regions of septa (sw). Cleavage angles (ca) are marked with double-headed arrows connected to a line indicating the long axis of the broken cell.*

perpendicular to the axis of the cells. The experiment in Fig. 5 was performed with *B. megaterium* instead of *B. subtilis* because the former cells were larger, so a greater percentage of cells were broken by the freeze-fracture method. This technique is very inefficient because most fracture planes do not pass through cells.

Our explanation for the presence of the curves takes note of the fact that the cells were constantly growing in length. Therefore, the wall at the exterior of the cell was made while the cell was shorter, whereas the wall at the interior was made after the cell had grown in length. Consequently, the wall at the exterior of the cell was constantly being stretched to be longer than the size at synthesis. If the wall was cut, then the wall at the exterior could contract whereas the wall at the interior would not change in size. As a result, the wall would curl as described.

The fact that the spirals and curves (Fig. 3–5) were seen all along the length of the cells suggested that the wall material along the total length of the cells was under tension. This suggestion was consistent with the interpretation that wall was synthesized over the entire length of the cell.

DISCUSSION

Our experiments with plasmolyzed cells showed that the organization of protein for septum synthesis could not be explained exclusively by the binding of otherwise free-flowing proteins to the old cell wall. Instead, the positioning of septum-synthesizing enzymes and the organized formation of septum must have been dictated by using some other information. It should be mentioned that wall synthesis around expelled mesosomes has already been reported in the literature (4, 9).

The model most consistent with the observed growth of new wall after plasmolysis is that wall is synthesized over the entire surface of the cell membrane, accompanied by the simultaneous degradation of cell wall by autolytic enzymes. The oldest and most digested material would be at the cell surface, so the outside surface of the wall would be the site of loss of wall material. The convex curves seen in broken cell walls support this model. Furthermore, this loss of wall material at the cell surface only after passage from the membrane surface is consistent with the cell wall turnover data of Mauck et al. (5), who showed a long lag before newly made *B. subtilis* wall was lost. This model of wall growth for rod-shaped gram-positive bacteria requires cell wall turnover so that the wall can be kept at a constant thickness. In fact, the visible disappearance of old cell wall after plasmolysis is one way to measure wall turnover. Also, wall turnover has also been measured biochemically in the two organisms we have studied, *B. subtilis* and *B. megaterium* (5). Pitel and Gilvarg reported no wall turnover in *B. megaterium*, but they considered any acid-precipitable breakdown products to be native wall (6). This consideration overestimates the amount of wall not turning over in light of the experiments of Mauck et al. to the effect that many of the degradation products of *B. megaterium* walls can have very large molecular weights (5). Thus, there may actually have been wall turnover in the experiments of Pitel and Gilvarg.

Dark lines in the walls of cells with unusually thick walls have been described from time to time in published experiments. An example is seen in the walls of the autolysin-deficient mutant of *B. subtilis* reported previously (2). These lines are often very much like the lines seen between the old walls and new walls synthesized after plasmolysis. In this latter case, the line resulted from a separation of membrane and wall. Perhaps some membrane material was retained with the old wall after plasmolysis. Thus the lines in unusually thick walls might also mark the time when membrane shifted relative to the wall for one reason or another.

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