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Ultrastructural Studies on a Mutant of *Bacillus subtilis* Whose Growth is Inhibited Due to Insufficient Autolysin Production

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The growth of *Bacillus subtilis* mutant βA177 can be inhibited under special conditions in which not enough autolytic enzymes are produced for optimal growth. Electron microscopy studies show that during growth inhibition there is localized thickening of the cell wall at positions where cells bend. A model is proposed to explain this result. Rapid growth can be restored by adding lysozyme or a *B. subtilis* autolysin mixture to a growth-inhibited βA177 culture. Such addition reduces the localized wall thickening and causes other changes in surface morphology which are described and discussed. Septum formation seems to be relatively less inhibited than cell elongation when lytic enzyme levels are reduced. Measurements were made demonstrating that walls at ends of cells are morphologically different from walls at sides of cells in cultures of βA177 growing at 51 C.

It has often been suggested that growth of bacterial cell surface requires some controlled degradation of the cell wall to open gaps where new wall material can be added. Fan and Beckman (3) have reported the isolation of a mutant of *Bacillus subtilis*, βA177, whose growth under certain conditions was inhibited at the same time that autolytic activity was diminished. The growth rate could be enhanced by the addition to the culture medium of either egg white lysozyme or an autolysin preparation obtained from *B. subtilis*. The implication from these experiments was that in restrictive conditions the growth of this mutant was limited because the strain could not produce enough autolysins to allow for normal cell surface expansion. It was further found that, under the restrictive conditions, the cell morphology of this mutant as seen by light microscopy was quite different from that of the wild type. The mutant shape seemed to revert to wild type when a lytic enzyme was added. Thus the shortage of a lytic enzyme seemed not only to slow cell growth but also to alter cell morphology. Since the above observations were only made at the level of optical microscopy, it seemed desirable to study these morphological changes in more detail using electron microscopy techniques. This paper reports the results of such observations.

MATERIALS AND METHODS

Bacteriological and biochemical materials and methods have been described by Fan and Beckman (3). The growth rate of *B. subtilis* βA177 decreases if the bacteria are shifted from the exponential growth phase at 30 C to aeration at 51 C in Difco Penassay Broth. If this mutant is shifted from stationary phase at 30 C to aeration at 51 C in Penassay Broth, growth becomes exponential after a lag phase. The bacteria then continue to grow exponentially with a rapid doubling time and normal rod-shaped morphology upon serial dilution. Therefore, βA177 seems to have a control mutation in which autolysin synthesis is diminished only when an exponential culture growing at 30 C is shifted to 51 C.

Electron microscopy. Bacteria were fixed with osmium tetroxide and embedded in Epon following a modification of the procedure of Ryter and Kellenberger (7). For prefixation, acrolein was added to a flask of organisms in growth medium to yield a 3 to 5% solution (v/v). After 0.5 hr in this solution the cells were collected on a membrane filter (Millipore, 0.45 μm pore size). Cells collected by centrifugation had the same morphology as filtered cells. The filter was rinsed with Michaelis buffer, *p*H 6.12, and gently lowered into a shallow container of R-K fixative containing approximately 10% (w/v) tryptone (Difco). The tissue was fixed overnight at room temperature, rinsed with Michaelis buffer, and post-stained for 2 hr with 0.5% uranyl acetate in Michaelis buffer. Dehydration was accomplished by transfer through a graded series of acetone. The

tissue was then infiltrated with increasing concentrations of Epon in acetone over a 24-hr period. The plastic was polymerized at 60 C for 72 hr. Thin sections were picked up on coated grids and stained with uranyl acetate and Reynolds lead citrate. Electron micrographs were taken with a Hitachi HU-11C electron microscope. Negatives were taken at magnifications of ×3,500 and ×10,000 using Dupont Cronar COS-7 film.

Measurements from electron micrographs: minimum wall thickness. Thin-section micrographs were examined for median-sectioned cells as judged by looking for cells which had a sharply defined line where membrane touched wall and in which both ends were included in the section. In these cells a straightedge approximately one-tenth the length of a cell was laid down on a line that averaged the inside edge of the outside surface of a cell wall. An attempt was made to move this straightedge as close to the center of the cell as possible without placing it in a region of only cell wall and no wall surface. The distance was measured between this straightedge and the membrane-wall interface to give the minimum wall thickness (Fig. 1).

Wall fringe. On median-sectioned cells, a straightedge as above was laid down on a line that averaged the outside edge of a cell wall. An attempt was made to move this straightedge as far from the center of the cell as possible without getting into a region that had no cell wall at all. The distance was measured between this straightedge and the one laid down to average the inside edge of the outside surface of the wall (Fig. 1). This distance was defined as wall fringe.

Cell length measurements. Median-sectioned cells were measured from the inside surface of the wall at one end to the inside surface of the wall at the other end. Cells with and without partial septa were measured.

RESULTS

The phenomenon of growth enhancement of βA177 by added lysozyme or *B. subtilis* autolysins removed from isolated cell walls by 3 M LiCl (3) is shown in the experiment described in Fig. 2. It can be seen that without an added lytic enzyme the growth rate of βA177 decreases progressively. The cells were harvested for microscopy at the indicated time because their morphology had already reached the very twisted form typically observed with this strain (Fig. 3). Incubation was not continued in this culture because prolonged aeration under these growth-limiting conditions leads to extensive production of empty cells. In the culture with added lysozyme, there was an initial dip in the absorbancy reading at 540 nm (A_{540}), followed by a more rapid increase than found with no added lytic enzyme. Depending on how much enzyme is added, this dip reflects both digestion or already empty cells and lysis of living cells. A similar dip could have been found in cultures with added autolysin if more enzyme had been added. In the cultures with added enzyme, the incubation was continued until the twisted morphology had largely disappeared. Then the cultures were harvested for microscopy. From Fig. 2 it is clear that in the enzyme-added cultures the growth rate was exponential for at least five generations, indicating full physiological adaptation to the addition. The growth enhancement with added lysozyme was somewhat greater than with added autolysins in terms of steady-state growth rate. It is possible that if more autolysins were added the growth enhancement would equal that with lysozyme added. In fact, with more lysozyme added, the growth rate might have been even faster than in this experiment. It is difficult to judge exactly how much enzyme should be added because as the enzyme concentration increases the initial dip in the A_{540} curve (Fig. 2) becomes progressively greater. At very high enzyme levels

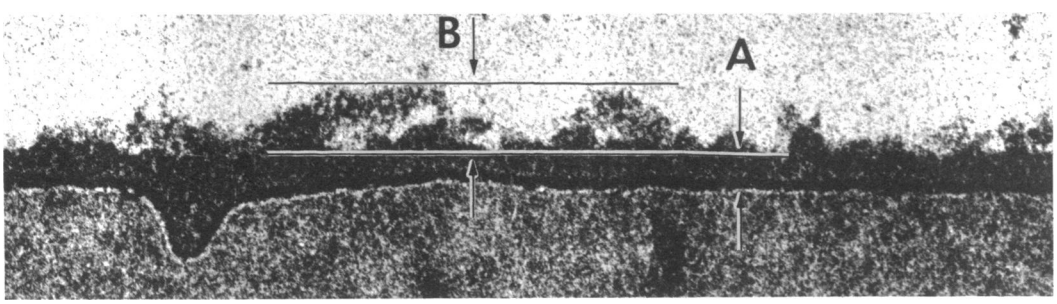

FIG. 1. *Minimum wall thickness and wall fringe measurements. In median-sectioned cells, a straightedge about one-tenth the length of a cell was laid down to average the minimum thickness of the cell wall. The distance between this straightedge and the membrane-wall interface was measured to give minimum wall thickness (arrows A). Another straightedge was laid down to average the maximum thickness of the cell wall, and the distance between this straightedge and the one averaging the minimum wall thickness was taken to give the wall fringe (arrows B). ×100,000.*

there is no recovery, and only lysis is seen. Furthermore, the optimal enzyme concentration depends on cell density since the added enzyme binds to the cells to which it has been added (3). Thus in both of these latter cultures the additions may have been suboptimal for maximum growth rate at the time of harvest. However, both cultures clearly show the growth enhancement and rescue of morphology characteristic of these additions.

For comparison, a phase-contrast micrograph of βA177 growing exponentially at 30 C is also presented in Fig. 3. The samples used for the micrographs of Fig. 3 were also sectioned for electron microscopy studies.

In studying morphologies, straightforward comparisons can be made between βA177 cultures growing at 51 C with or without added lytic enzymes. In these cultures the strain is always the same, and the only change in culture condition is added enzymes. Therefore, any differences can be directly attributed to the added enzymes. Upon inspection (Fig. 4 and 5) the most noticeable difference between the samples is that the walls of cells growing without added lytic enzyme at 51 C show localized thickening relative to the walls with lysozyme added. A micrograph of cells growing at 51 C with added autolysins is not shown since at this magnification the morphology is very similar to that of cells shown in Fig. 5 with lysozyme added. The thickening with no added enzyme at 51 C is most apparent at the insides of the curves of bent cells (Fig. 4). The fact that this phenomenon is general is seen in the measurements of Fig. 6 where the minimum wall thicknesses at the insides and outsides of cell curves are measured. The thicknesses at the outsides of the curves give a narrow distribution which shows almost no overlap with that of thicknesses at the insides of the curve. Furthermore, the same measurements made at the centers of the cells (Fig. 7) give a profile indistinguishable from that of the thicknesses at the outsides of curves. Since measurements at the center of cells should represent an unbiased random sampling of typical thicknesses along the sides of cells, it can be concluded that the principal sites at which the thickness is significantly greater are at the insides of curves. From the light micrographs of Fig. 3 it is evident that the major morphological difference between 51 C samples is in the great increase in formation of curves and bends when lytic enzymes are not added. Since these curves are the main sites of wall thickening, it is clear that lack of sufficient lytic enzyme during growth at 51 C leads to localized wall thickening as well as a curved cell morphology.

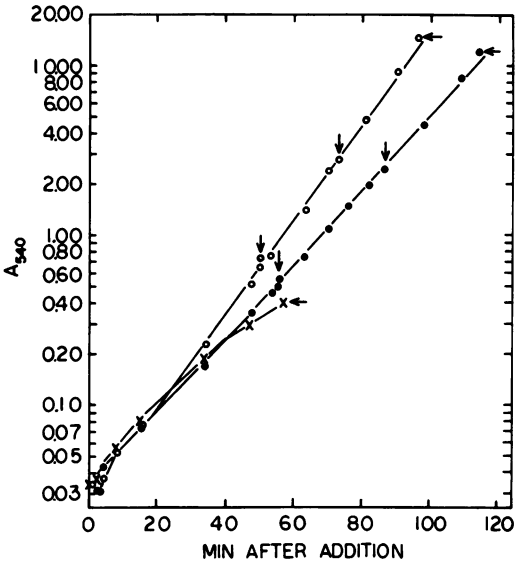

FIG. 2. *Growth of Bacillis subtilis βA177 at 51 C with added lysozyme, 3 M LiCl-extracted autolysins, or no added enzyme. A Penassay Broth culture of βA177 growing exponentially at 30 C was shifted to growth at 51 C. When the cells started to look twisted under the phase-contrast microscope, the culture was diluted to A_{540} = 0.03 and divided into three portions. To one 20-ml portion (●) was added 0.1 ml of 3 M LiCl-extracted autolysins (220 μg of protein/ml); to another 20-ml portion was added 0.1 ml of egg white lysozyme (60 μg/ml) (○) in the same buffer as that for the autolysins (3 M LiCl in TK buffer); to the last 20-ml portion (×) was added 0.1 ml of 3 M LiCl in TK buffer. A_{540} was followed as a function of time of aeration at 51 C. At the times indicated, the cultures were harvested by centrifugation for light and electron microscopy. At the times indicated by the vertical arrows the culture was diluted ½ into fresh medium containing the same additions as the original culture. The A_{540} readings are accordingly scaled five times in order to indicate what growth would have been like had the dilution not been necessary to prevent the cells from reaching stationary growth phase.*

The wall surfaces of βA177 cells growing at 51 C under different conditions also vary. Qualitatively it can be seen in the micrographs of Fig. 7 that the surface of cells growing without added enzyme is very irregular, often with large pieces in the process of dissociating from the cell. If autolysins are added, the surface becomes much smoother and more uniform. On the other hand, if lysozyme is present, the surface is also more uniform, but there is a rather frayed appearance. These qualitative differences are large enough so that the growth condition can be immediately identi-

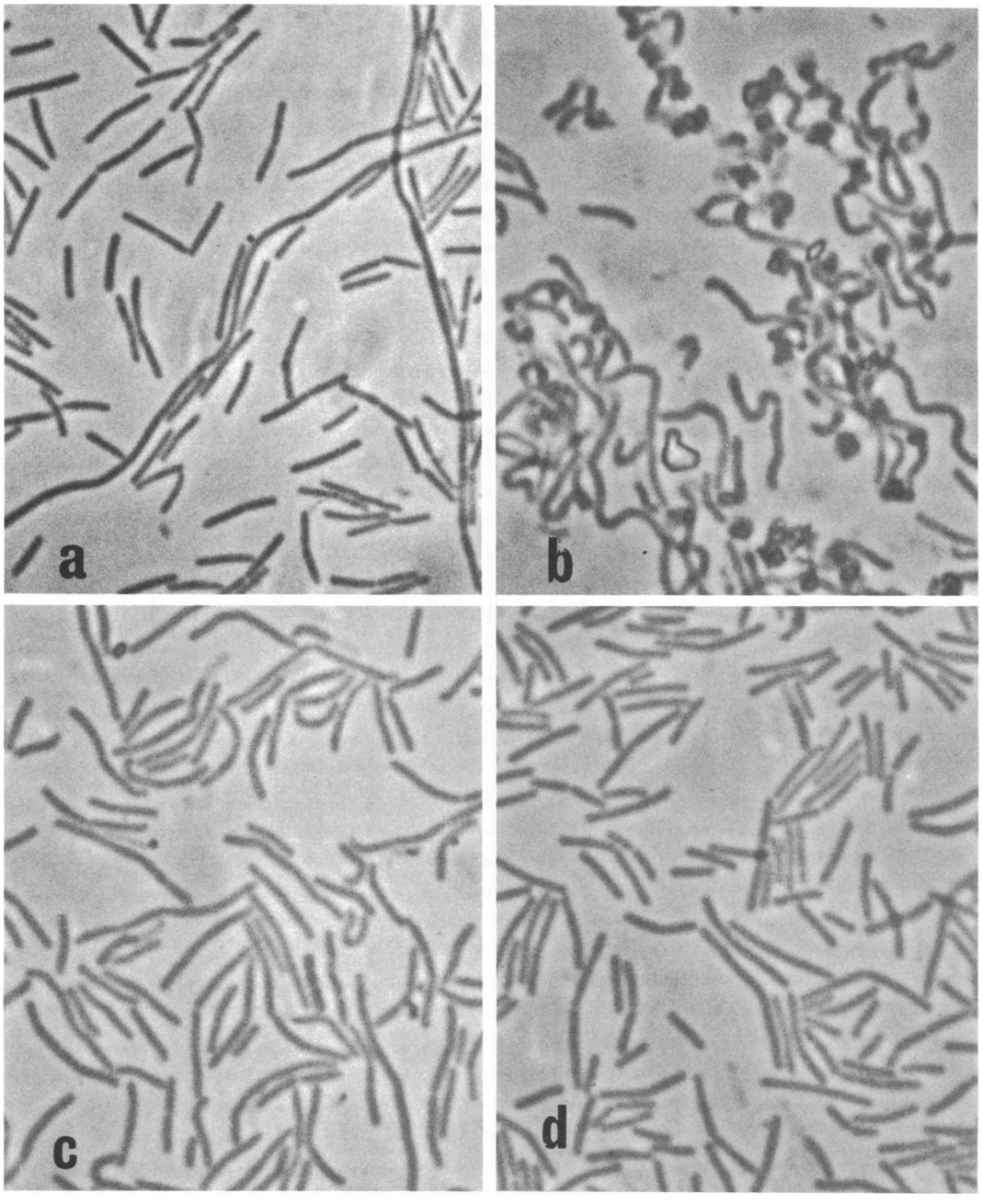

FIG. 3. *Phase-contrast micrographs of βA177 growing under various conditions; ×1500. (a) Exponential growth at 30 C. (b, c, d) Cells harvested in the experiment of Fig. 2: (b) growth at 51 C with no enzyme addition, from curve (×); (c) growth at 51 C with autolysins added, from curve (●); (d) growth at 51 C with lysozyme added, from curve (○).*

fied upon examination of micrographs on which the sample source is not marked. In addition, quantitative measurements can be made to substantiate the differences. Figure 8 shows that the fringe on the wall surface differs in thickness quite appreciably among the three samples. However, the distribution of minimum wall thickness does not change ap-

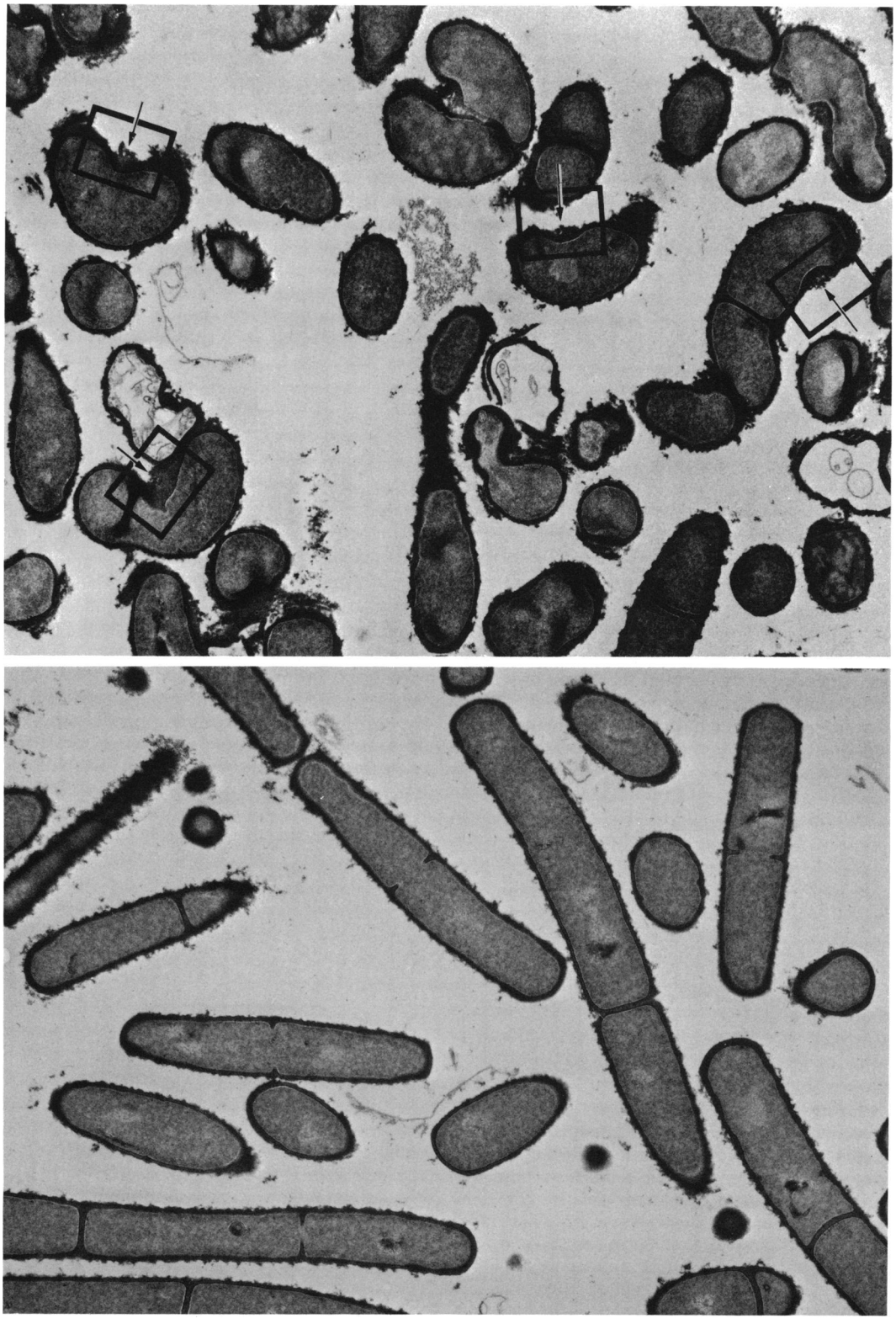

FIG. 4. *Electron micrograph of βA177 growing at 51 C without added enzyme, sample pictured in Fig. 3b. Curved cells with wall thickening at insides of the curves is shown by arrows. ×12,500.*

FIG. 5. *Electron micrograph of βA177 growing at 51 C with added lysozyme, sample pictured in Fig. 3d. ×12,500.*

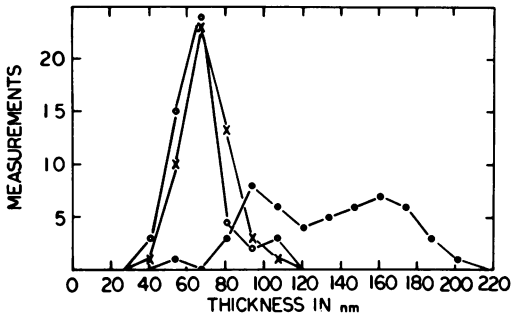

FIG. 6. *Minimum wall thickness measurements on βA177 growing at 51 C with no added enzyme (cells of Fig. 3b). Measurements were made midway from both ends of relatively straight cells (×). If the cell had a partial septum, the measurement was made at a position equidistant between the partial septum and one cell end. Measurements were also made on median-sectioned curved cells at the position shown by arrows in Fig. 4, to give the minimum thickness on the inside of the curves (●). The region of wall directly opposite the portion indicated by the arrows was measured to give the minimum thickness on the outside of curves (○).*

preciably even when an external lytic enzyme is added. The mean minimum wall thickness values are 62 nm for no enzyme added, 63 nm for autolysins added, and 70 nm for lysozyme added. For comparison, the mean value for minimum wall thickness of cells growing at 30 C with no added enzyme is 44 nm. Thus it seems that walls of βA177 growing at 51 C are on the average thicker than the same cells growing exponentially at a lower temperature.

Another set of differences among cells growing at 51 C under various conditions is the fraction of cells that show layers of wall beginning to peel and separate from other layers (Fig. 9, arrows). Distinct layers are most clearly seen in samples grown with added autolysins. However, layering can be seen in all samples. In the micrographs, any cell which showed at least one region of peeling wall as shown in Fig. 9 was counted as a peeling cell regardless of the plane of secretion. Also, the number of cells which were considered for the above enumeration was also counted. It was necessary to count all sectioned cells and not only median-sectioned cells because the density of peeling cells was very low. The morphology of the peel differed in the three growth conditions, so an attempt was made to count only cells showing unambiguous peeling. The results were found as follows: 26 peeling cells per 1,158 total cells examined when no enzyme was added; 26 peeling cells per 2,900 total cells when autolysins were added; and 3 peeling cells per 2,626 total cells when lysozyme was added. Using the χ^2 test of significance, all three measurements are different from one another at the 99% confidence level. Thus there is the most peeling when no enzyme is added, less peeling when autolysins are added, and the least peeling when lysozyme is added.

When the project was begun to look for mutants such as βA177 which could not expand their cell surface due to lack of a lytic enzyme, it was hoped that such mutants might be able to continue to form septa even when size increase was blocked (3). This continued septum formation would result in very short cells. However, mutants like βA177 would be predicted to grow as longer cells if a lytic enzyme were added so that cell elongation as well as septum formation were permitted. Such a result is suggested in the cell length measurements of Fig. 10. The average cell length is 2.6 μm for no enzyme added, 2.8 μm for autolysins added, and 3.3 μm for lysozyme added. As can be seen by comparing these results with the data from Fig. 2, the average cell length increases as the growth rate increases. The increase is unfortunately not dramatic but merely represents a shift in the distribution toward the longer cells more typically observed at lower temperature. It is possible that if more enzyme had been added the shift to longer lengths would have been even greater. It is also possible that in a cleaner mutant in which autolysin levels can be more rapidly decreased, the cell lengths would be even shorter with no added enzyme. An attempt is now being made to isolate such a mutant.

Although differences were seen between the 51 C cultures with and without added enzymes, there was one feature of wall growth that was common to all three samples. This feature was the manner in which cell septa and cell ends were formed. In all cases the septum seemed to thicken as it grew. Simultaneously there was a thickening of the cylindrical portion of wall adjacent to the septum. The wall material in these thickened regions was smoother than the major portion of the wall in the region of the sides of the cell. Since these results were qualitatively the same in all three 51 C cultures, the quantitative data will be presented in detail only for the culture with autolysins added, since under these growth conditions the effects were most clearly seen. The positions of various measurements made are given in Fig. 11, and the results of these measurements are presented in Fig. 12. In cell

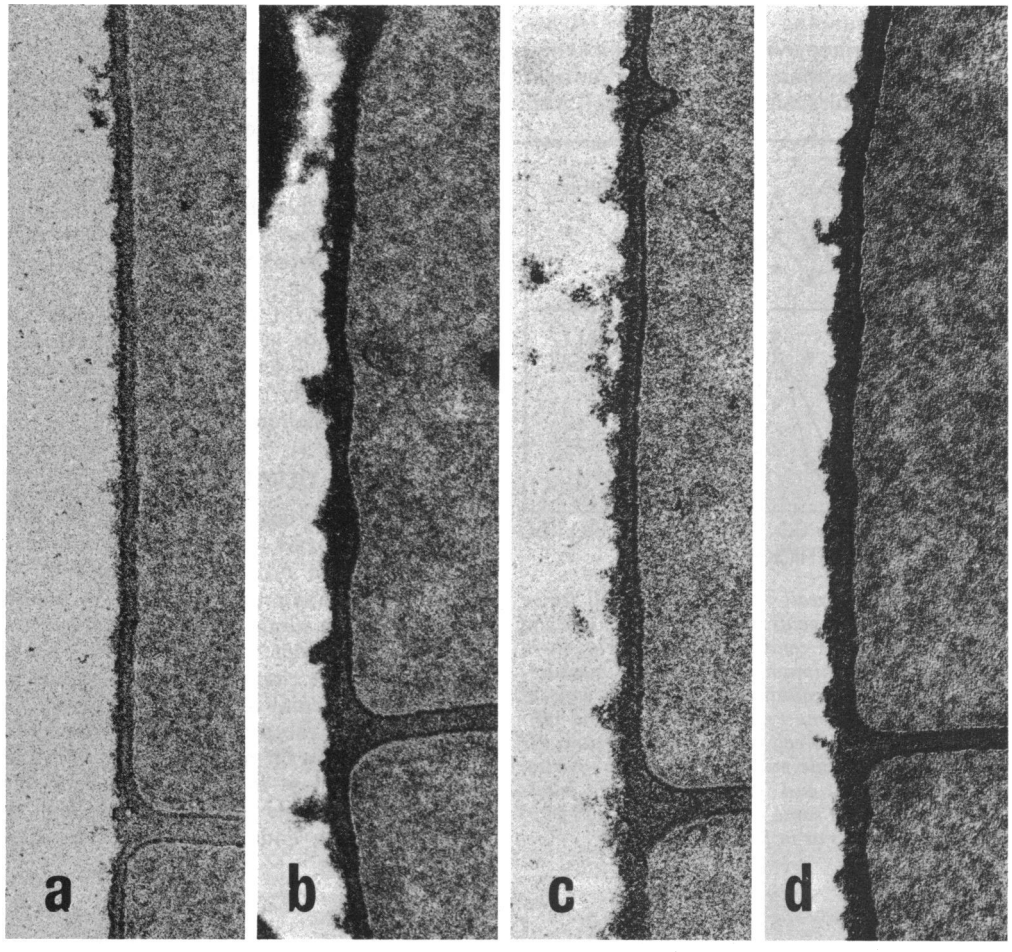

FIG. 7. *Electron micrograph of cell surface structure of βA177 growing under different conditions; ×50,000. (a) 30 C with no added enzyme, sample pictured in Fig. 3a; (b) 51 C with no added enzyme, sample pictured in Fig. 3b; (c) 51 C with lysozyme added, sample pictured in Fig. 3d; (d) 51 C with autolysins added, sample pictured in Fig. 3c.*

division, a partial septum is formed which grows to become a full septum, which in turn is cleaved in two by lytic activity leaving a thickness of wall from half of the septum only at the end of each of the two daughter cells. Thus in the case of cell ends, the wall thickness equivalent to that of a septum is actually twice the thickness of the end. Therefore in Fig. 12 (lower panel) septum thickness at the end of cells is taken to be double the cell end thickness. It is clear from the data that the septum thickness increases greatly as the septum ages and becomes a cell end. At the same time, the cylindrical portion of the wall adjacent to the end almost doubles in thickness (Fig. 12, upper panel). Thus the cell wall thickening occurs not only at the septum position but also a small distance into the wall at the sides of the cells. The wall in the entire thickened region is smoother than that in the cylindrical region. This fact is seen from Fig. 12 (inset) where wall fringe measurements are plotted. The fringe in either the middle of cells or in the region of the wall just outside the thickened region is identical. Also visual scanning of many thin sections shows that the entire surface in the thin wall regions has the same rough texture. However, measurements made at either the edge or the center of the thickened region show that the wall fringe there is approximately half as thick on the average as that at the center of the cells.

Again, visual scanning of micrographs shows that the wall surface over the entire thickened region is smoother than that in the cylindrical region. These results suggest that the cell wall is composed of two different components. The one along the sides of the cell is rough and relatively thin whereas that at the ends is smooth and capable of being preferentially thickened. The results in the accompanying communication (4), showing that cell wall ends are structurally different from cell wall sides, is in good agreement with this observation.

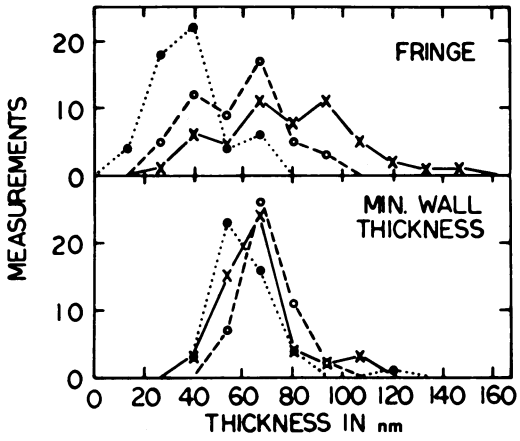

FIG. 8. *Minimum wall thickness and wall fringe measurements at the centers of βA177 cells growing at 51 C under various conditions (cells of Fig. 8b, c, d). Lower panel: minimum wall thickness measurements at a position equidistant from both ends of cells: no added enzyme (×); autolysins added (●); lysozyme added (○). If cell had a partial septum, the measurement was made midway between the partial septum and one cell end. Upper panel: wall fringe measurements at the same positions on the cell as in lower panel; symbols as in lower panel.*

As shown in Fig. 11, the smooth and thickened wall associated with the ends extends a small distance into the cylindrical region of the cell so that in the median sections the shape of the ends is in the form of the letter "U". The average distance between the tips of the arms of the "U" and the trough is 0.5 μm, based on 50 measurements.

DISCUSSION

The principal observations made in these experiments can be summarized as follows. (i) When autolytic enzyme levels decrease so much as to impair growth, there is localized wall thickening along the cylindrical portions of cells at positions where cells bend; (ii) cell surface structure changes when the growth rate of βA177 growing at 51 C is increased by addition of either lysozyme or *B. subtilis* autolysins; (iii) septum formation seems to be inhibited relatively less than cell elongation when lytic enzyme levels are decreased; (iv) wall material at the ends of cells can be distin-

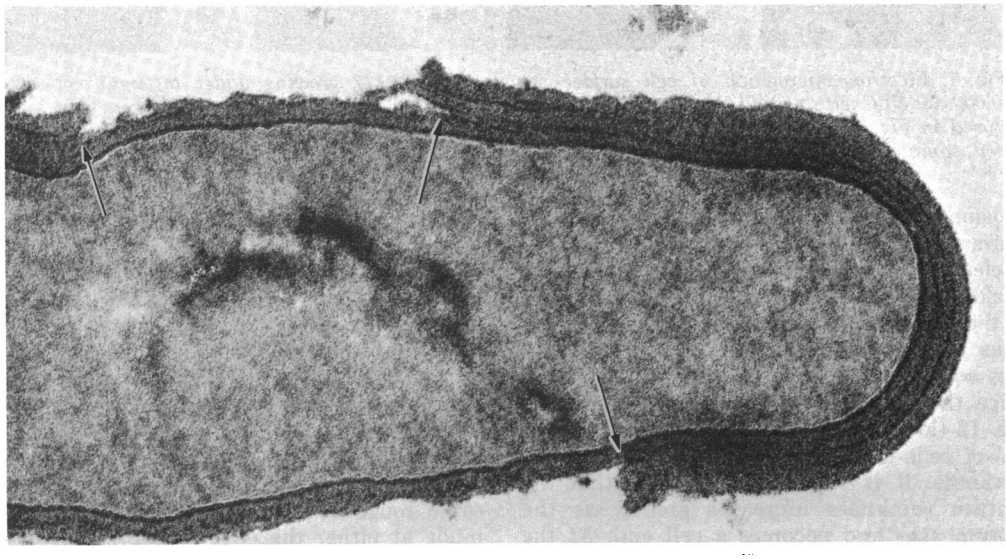

FIG. 9. *Electron micrograph of βA177 growing at 51 C with added autolysins. One of the few cells from the sample picture in Fig. 3c that shows many wall layers and extensive wall peeling. Cells were scored as having peeling wall if they had surface morphology as shown by the arrows.* ×50,000.

guished morphologically from wall material at the sides of cells.

The fact that localized wall thickening and cell bends occur at the same sites can be explained by the model shown in Fig. 13. Here it is assumed that normal wall expansion will take place in some plane perpendicular to the axis of the bacilli to produce cell elongation. It is immaterial how many such planes there might be. There could be many such planes very close together if wall growth were by uniform intercalation along the length of the cell or there might be very few planes spaced far apart leading to wall growth in discrete zones. The first possibility is perhaps more likely in light of the report of Mauck, Chan, and Glaser (5) that the cell wall of *B. subtilis* turns over almost completely during cell growth. This fact suggests that turnover must take place over almost the entire cell surface. Therefore, wall synthesis would have to occur over almost the whole cell surface with the net result being uniform intercalation of wall material over almost all of the cell length. It is proposed that normally the action of the lytic enzyme is to make breaks in the cell wall in a plane of growth allowing for uniform surface expansion. However, if lytic enzyme levels drop, as in the case of βA177 growing at 51 C, the loss will eventually reach a point where a decrease of one more enzyme molecule from the cell will lead to some part of the surface in the plane of growth not having a needed lytic enzyme, so growth will stop at that point. Other wall portions in the plane will still be able to expand, so the cell will bend. However, the synthetic machinery in the cell is assumed to be undisturbed and will continue to add material uniformly in the entire growth plane. Thus, in those regions where expansion is possible, synthesis will add wall material to make a wall of normal thickness but larger surface whereas in those regions where no surface expansion is possible, wall material will be added inside the cell to cause localized wall thickening. With this model it is easy to see how the simple addition of a lytic enzyme will replace the autolysins no longer on the mutant cell wall and thus allow for uniform surface expansion in the entire plane of growth and, hence, the appearance of a straight cell with walls of regular thickness. It would further be predicted that instantaneous and complete removal of lytic activity from a cell would not lead to curved cells with localized wall thickening but rather straight cells with uniform thickening.

The changes in cell surface structure during

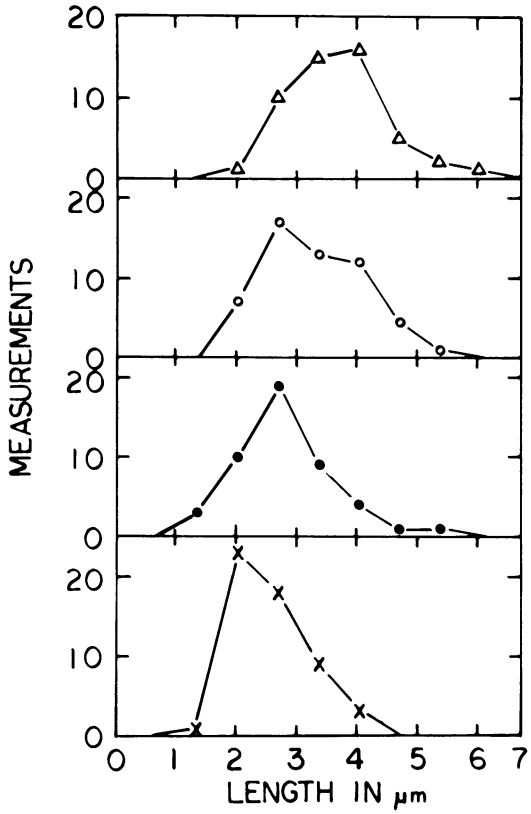

FIG. 10. *Cell length measurements on βA177 growing under various conditions. Measurements were made on electron micrographs from the same samples as in Fig. 3. Growth at 30 C (△); 51 C with no added enzyme (×); 51 C with autolysins added (●); 51 C with lysozyme added (○).*

growth with a lytic enzyme might be explained by the possibility that autolysin molecules, which are known to attach tightly to cell walls (3), might move over the cell surface digesting cleanly everything in their paths. This possibility could explain why the surface is very smooth when autolysins are added. Lysozyme, on the other hand, may bind walls much less firmly. So it might digest a portion of the wall and then dissociate from the wall to attach elsewhere on the wall before digesting further. This action could explain why lysozyme addition leads to a very rough cell surface.

Layers in the wall are seen most prominently in the culture with autolysins added. The nature of these layers is still obscure, although such layers have also been seen by other investigators under other circumstances (6). It is possible that adjacent layers are very loosely attached one to another so that enzyme

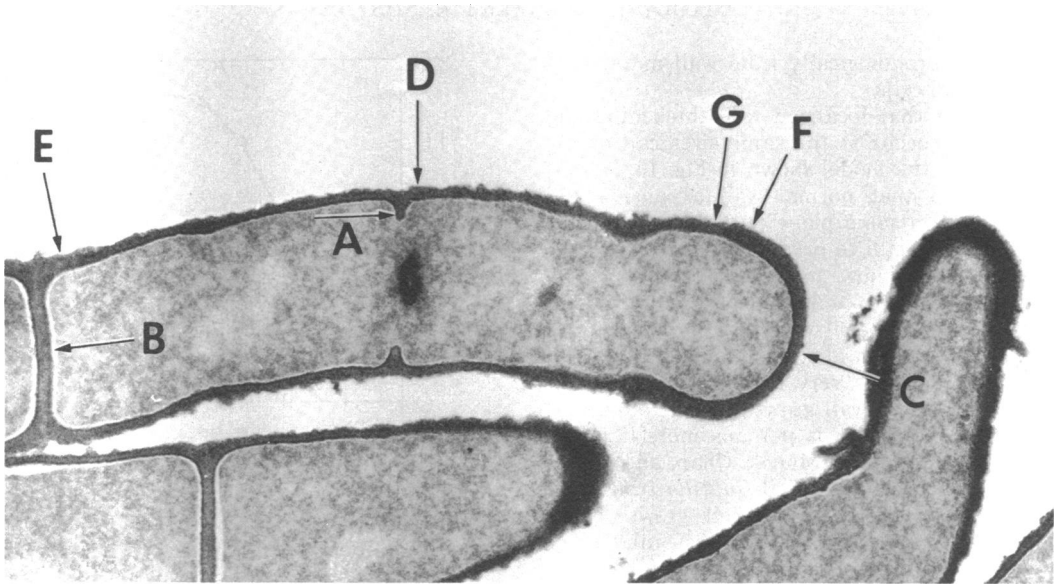

FIG. 11. *Cell illustrating the measurements made on βA177 cell growing at 51 C with added autolysins to demonstrate septum wall and cell end wall growth. Median section electron micrograph of a cell from the same sample as pictured in Fig. 3c; ×25,000. Arrows indicate positions at which measurements were made. (A) Thickness of partial septum, measured as the distance from the membrane-wall interface on one side of the partial septum to that on the other. (B) Thickness of complete septum, measured as above. (C) Thickness of cell ends, measured as the minimum wall thickness. Wall fringe measurements were also made at this position. (D) Thickness of the cylindrical portion of wall adjacent to partial septum, measured as minimum wall thickness. (E) Thickness of the cylindrical portion of wall adjacent to complete septum, measured as minimum wall thickness. (F) Thickness of cylindrical portion of wall adjacent to cell end, measured as minimum wall thickness. Wall fringe measurements were also made at this position. (G) Wall fringe measurements on the cylindrical portion of the wall where the wall abruptly becomes thinner.*

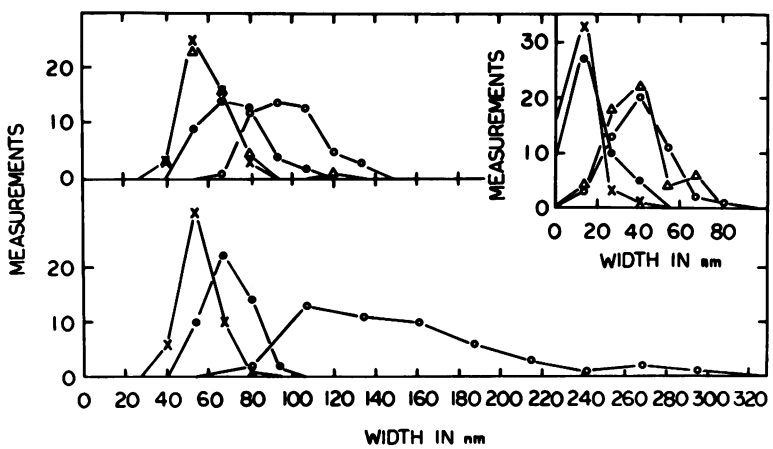

FIG. 12. *Wall thickness and wall fringe measurements of βA177 growing at 51 C with added autolysin. Sample pictured in Fig. 3c; measurements described in Fig. 11. Lower panel depicts measurements of septum thickness. Symbols: (×) thickness of partial septa (Fig. 11A); (●) thickness of complete septa (Fig. 11B); (○) thickness that septum would have had if lytic activity had not cleaved the septum in two to give the cell end. The plotted value was twice the thickness of a cell end (Fig. 11C) (see text). Upper panel depicts measurements of minimum wall thickness of the cylindrical region immediately adjacent to septum or cell end region. Symbols: (×) thickness around partial septum (Fig. 11D); (●) thickness around complete septum (Fig. 11E); (○) thickness around cell ends (Fig. 11F); (△) minimum wall thickness measured at the centers of cell (as in Fig. 8, ●, lower panel). Inset depicts wall fringe measurements of cylindrical and end regions of cells. Symbols: (△) fringe at centers of cells (as measured in Fig. 8, ●, upper panel); (○) fringe in cylindrical region adjacent to thickened region (Fig. 11G); (●) fringe in thickened region around cell end (Fig. 11F); (×) fringe at cell ends (Fig. 11C).*

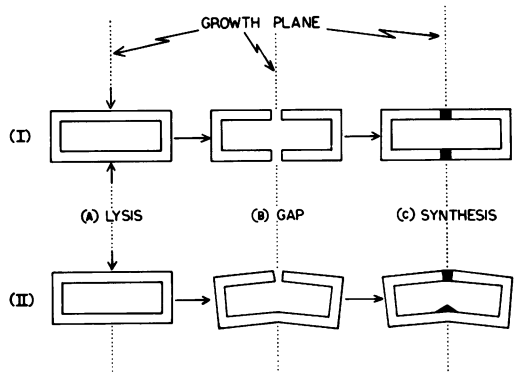

FIG. 13. *Model for curved cell formation in Bacillus subtilis when not enough wall lytic enzyme is present to permit balanced growth. Diagrams represent the cell walls of bacteria in median thin sections. Each cell is assumed to have one plane of growth perpendicular to the cell axis. This plane is given by the dotted vertical lines. If in fact many growth planes are present, then events portrayed can happen at any of these planes. (I) In balanced cell growth there is enough lytic enzyme present to cover the cell surface in the entire plane of growth. Therefore, lysis can occur at opposite sides of the cell in the plane of growth in the median section shown. Lysis positions are indicated by vertical arrows (A). The lytic enzyme cleaves a tiny opening diagramatically exaggerated by the gaps (B). Wall synthetic machinery fills in the gaps to give a longer cell with new material indicated by shaded region (C). (II) When lytic enzyme levels are reduced in a cell so that there is not enough enzyme to coat the entire surface in the plane of growth, it is assumed that there will be some part of the cell surface which will not have enzyme while the opposite region will. The median section showing this inhomogeneity of enzyme is given in (A). Lysis at the position of the vertical arrow will give a gap (B), but only on one side of the cell. The presence of a gap on one side only will lead to a bent cell morphology when the gap is opened. Wall synthesis in the plane of growth will fill in the gap normally to give an expanded surface at that position (C). Opposite the gap will be no opening, but synthesis will still proceed and wall material will be added to thicken the wall since surface expansion cannot take place. The end result is localized wall thickening at the insides of bends.*

cleavage through a layer would allow them to separate and give a peeled morphology. It is possible that the layers are tightly bonded together with autolysins being able specifically to cleave between layers while lysozyme cannot and must work perpendicularly to the wall surface.

Since the ends and sides are structurally (4) and morphologically different, it is reasonable to assume that their mechanisms of synthesis are not the same. It might well be possible that the synthesis of ends proceeds in a manner similar to that for the growth of *Streptococcus* bacteria, in light of the fact that one half of a Streptococcus cell looks like a *B. subtilis* end and in view of the fact that *Streptococcus* like *Bacillus* grows with only one plane of division. Because *Streptococcus* cells grow with no wall turnover (2), *B. subtilis* cell wall ends may also be conserved. On the other hand, since the sides form 80 to 90% of the total material of *B. subtilis* walls, and since Mauck, Chan, and Glaser (5) have found that almost all of the *B. subtilis* wall material turns over, the synthesis of cell wall sides might involve very extensive turnover. *B. subtilis* makes both an autolytic amidase and an autolytic glycosidase (1) and also makes both cell wall ends and sides, whereas *Streptococcus* synthesizes only one autolysin, a glycosidase (8), and makes only the equivalent of *B. subtilis* ends. Thus the *B. subtilis* glycosidase may play the same role in end synthesis as the *Streptococcus* autolysin plays in *Streptococcus* wall growth, whereas the *B. subtilis* amidase might be predominantly used for cell side growth. This model would be quite consistent with the finding that cell wall ends are more resistant to the autolytic amidase than the sides (4).

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