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Structural Difference Between Walls from Ends and Sides of the Rod-Shaped Bacterium *Bacillus subtilis*

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Electron microscopy studies show that walls from ends of *Bacillus subtilis* cells are more resistant to autolytic degradation that walls from sides of the same cells. This observation suggests that these two parts of the cell are structurally different. A method is available for the isolation of walls from ends of cells.

Exponentially growing rod-shaped bacteria such as *Bacillus subtilis* have two morphologically distinct regions at the light microscopy level of resolution, ends and sides. Geometrically, the cell walls at the ends and sides obviously differ. The surface of the former is curved as a hemisphere, and in the latter the curvature is cylindrical. In order for the surface to bend differently at ends and sides, it might be proposed that walls at cell ends and cell sides are structurally different from each other. The data presented in an accompanying paper (5) support this proposal since morphological differences were found between the cell wall at ends and sides of whole cells examined in thin-section electron micrographs. The experiments below show that under certain circumstances walls from cell ends are more stable to degradation than walls from cell sides.

MATERIALS AND METHODS

Biochemical and bacteriological materials and methods were as previously described (1, 2, 3, 4) unless otherwise specified. Native cell walls were prepared from *B. subtilis* βAO cells harvested in the exponential growth phase (1). Substrate for autolysin was prepared by treating native walls with sodium dodecyl sulfate (SDS) (3). Native *B. subtilis* walls were centrifuged in a LiCl-sucrose gradient as described by Fan and Beckman (4) to isolate the autolytic amidase from the autolytic glycosidase. In incubations with isolated amidase, SDS-treated walls and enzyme solution were mixed. Then water was added to dilute the LiCl concentration to below one-tenth the concentration in the enzyme extract. The enzyme was bound to the walls upon the dilution, and the excess LiCl was discarded after centrifugation to pellet the walls with enzyme attached (4).

Thin-section electron microscopy. A modification of the electron microscopy methods described in the accompanying paper (5) was used. The only differences were as follows. In place of the Michaelis buffer, *p*H 6.12, 0.1 M sodium phosphate buffer, *p*H 7.0, was used. Walls were concentrated by centrifugation (1) instead of filtration. The post-staining step in 0.5% uranyl acetate was omitted.

Surface view electron microscopy. Formvar-coated grids which had been carbon-coated and surface-ionized in a glow discharge apparatus in a vacuum evaporator were used for the experiments. A small drop of sample was placed on the grid and an approximately equal volume of stain was added. The excess liquid was removed with filter paper; the grid was air-dried and examined. The stain solution was 0.5% uranyl acetate, *p*H 4.0. The sample was a portion of isolated cell walls in various stages of degradation suspended in the lysis buffer.

RESULTS

When isolated native *B. subtilis* βAO walls are allowed to autolyse in 0.3 M LiCl in TK buffer, there is preferential degradation of the walls from the sides of cells. The kinetics of autolysis (Fig. 1) are very rapid for the first 5 min. Then there is a decreased rate of lysis up to approximately 50 min, after which the lytic rate reaches a plateau region where degradation is very slow. The absorbancy at 540 nm (A_{540}) of this plateau region was 10 to 20% of that at the beginning of the experiment. Samples were taken at the beginning of the experiment and then during lysis as indicated in Fig. 1. Each sample was divided into two portions. Surface view specimens were made from one portion and thin-section specimens were prepared from the other. The electron micrographs (Fig. 2, 3, and 4) show good agreement

between the structures seen in parallel specimens at all stages of lysis.

In the first sample, four structures are evident from the thin-section micrographs (Fig. 2a): walls from cell sides, partial septa, complete septa, and cell ends. The vast majority of the material is clearly from the cell sides. At this same time in the surface view preparations, the cell ends and cell sides are easily seen (Fig. 2b). It is clear that the cell wall is seen in collapsed form on the specimen grid. Thus it is difficult to identify partial and complete sèpta because they tend to be surrounded by side material which obscures the septa. However, partial septa can be seen occasionally. In the surface view preparations, the texture of the ends can be seen to differ from that of sides. The sides seem to be more granular and uneven whereas the ends are smoother. This observation is in good agreement with the wall fringe measurements at the ends and sides of thin sections of whole cells (5) where it was also found that the surface of ends was more even than that of sides.

As lysis proceeds, the sides are seen, in surface view preparations, both to shred and be covered with large holes. The degradation seems to occur preferentially in planes parallel to the ends. In contrast, the ends seem more numerous, stay relatively more intact, and retain their smooth morphology (Fig. 3b). Structures are seen which might be partial septa, but since ends are also somewhat degraded, it is difficult to ascertain whether a disc with a hole in the center came from a partial septum or a partially degraded end. In the thin sections (Fig. 3a), the preferential lysis of sides is also seen; there are many gaps in the sides whereas essentially none are visible in the ends. Partial septa were not often seen but they may have been difficult to identify with certainty because they may have lost rigidity, and hence distinctive shape, when significant amounts of side material were lost.

The relative resistance of ends to digestion is most clearly seen in the last sample examined (Fig. 4a and b). In the surface view samples, there are almost no identifiable sides. Instead, the predominant structures are discs corresponding to cell ends. These discs have sometimes undergone significant destruction since large holes are often apparent in them. Some of these holes are very symmetric and may have been present originally as partial septa. But as stated above, positive identification of these rings with symmetric holes as coming from partial septa is difficult. In the thin sections also, the crescent-shaped ends

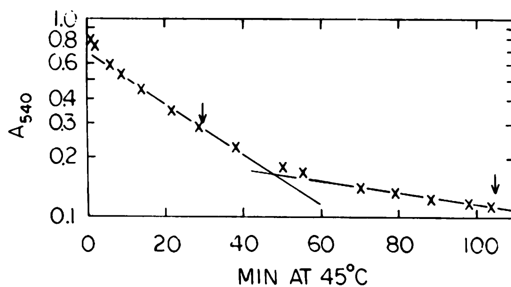

FIG. 1. *Kinetics of autolysis of Bacillus subtilis βAO walls in 0.3 M LiCl. Cell walls were prepared from exponential phase βAO cells (1) and suspended in 0.3 M LiCl in TK buffer. Loss of absorbancy at 540 nm (A_{540}) was followed as a function of time at 45 C. At 0 min, 30 min (arrow), and 105 min (arrow), samples were taken and divided into two portions. One portion was added to acrolein for preparation of thin sections. The other portion was frozen for examination by use of surface view techniques.*

are the main structures left at this stage of degradation.

In all three thin-section micrographs, the wall that remains after digestion does not become thinner, suggesting that the autolysins cut deep holes and then only expand the area of the holes. This interpretation is consistent with the results from the surface view specimens where the texture of undegraded cell wall does not change drastically during lysis.

In both the surface view and thin-section preparations, the amount of amorphous background material increases progressively with the extent of lysis, suggesting that the source of the amorphous material is fragments of degraded wall. This background material can be removed by extraction with SDS. Figure 5 shows a clean preparation of ends obtained by SDS treatment of walls harvested in the plateau region under the digestion conditions of Fig. 1. Since the surface view and thin-section procedures gave the same results, subsequent experiments were performed using the surface view technique only.

The preferential persistence of ends during the lysis incubation might be due to either of two causes. One possibility is that the structure of the end is somehow different from that of the sides so that the ends are more resistant to autolysin action. On the other hand, the preservation of ends might be a result of there being less autolysin at ends than at sides. In *Streptococcus faecalis*, for instance, it has been found that the autolysin is not distributed uniformly over the entire cell surface (6). To distinguish between these two hypotheses, an experiment was performed in which an auto-

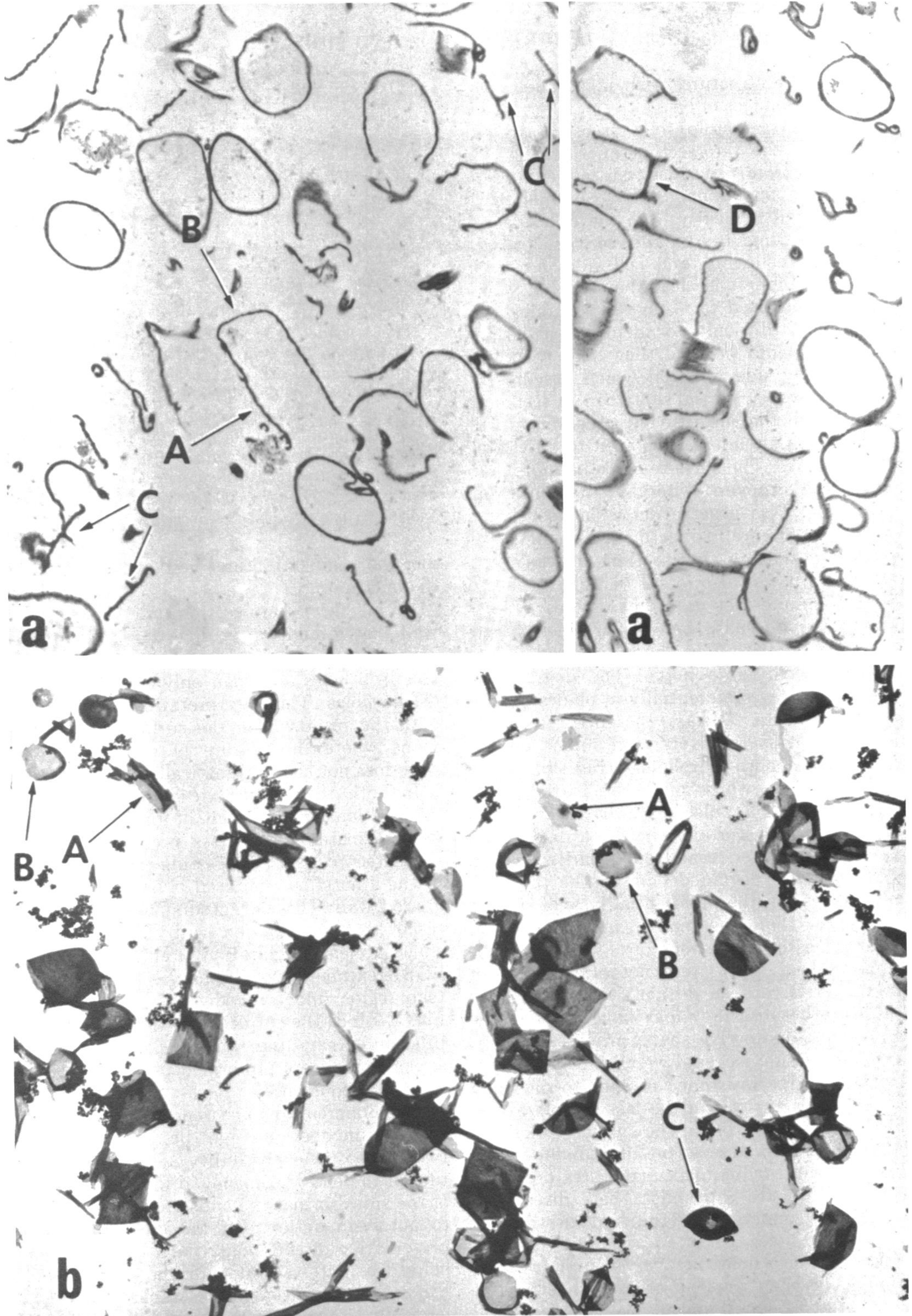

FIG. 2. *Morphology of freshly prepared Bacillus subtilis walls. Cell walls were sampled at 0 min (Fig. 1). (a) Thin-section electron micrographs; ×15,000. Arrows: A, cell side; B, cell end; C, partial septum; D, full septum. (b) Surface view electron micrograph; ×6,000. Arrows: A, cell side, note rough texture; B, cell end, note smooth texture; C, possible partial septum with wall from cell sides collapsed on top.*

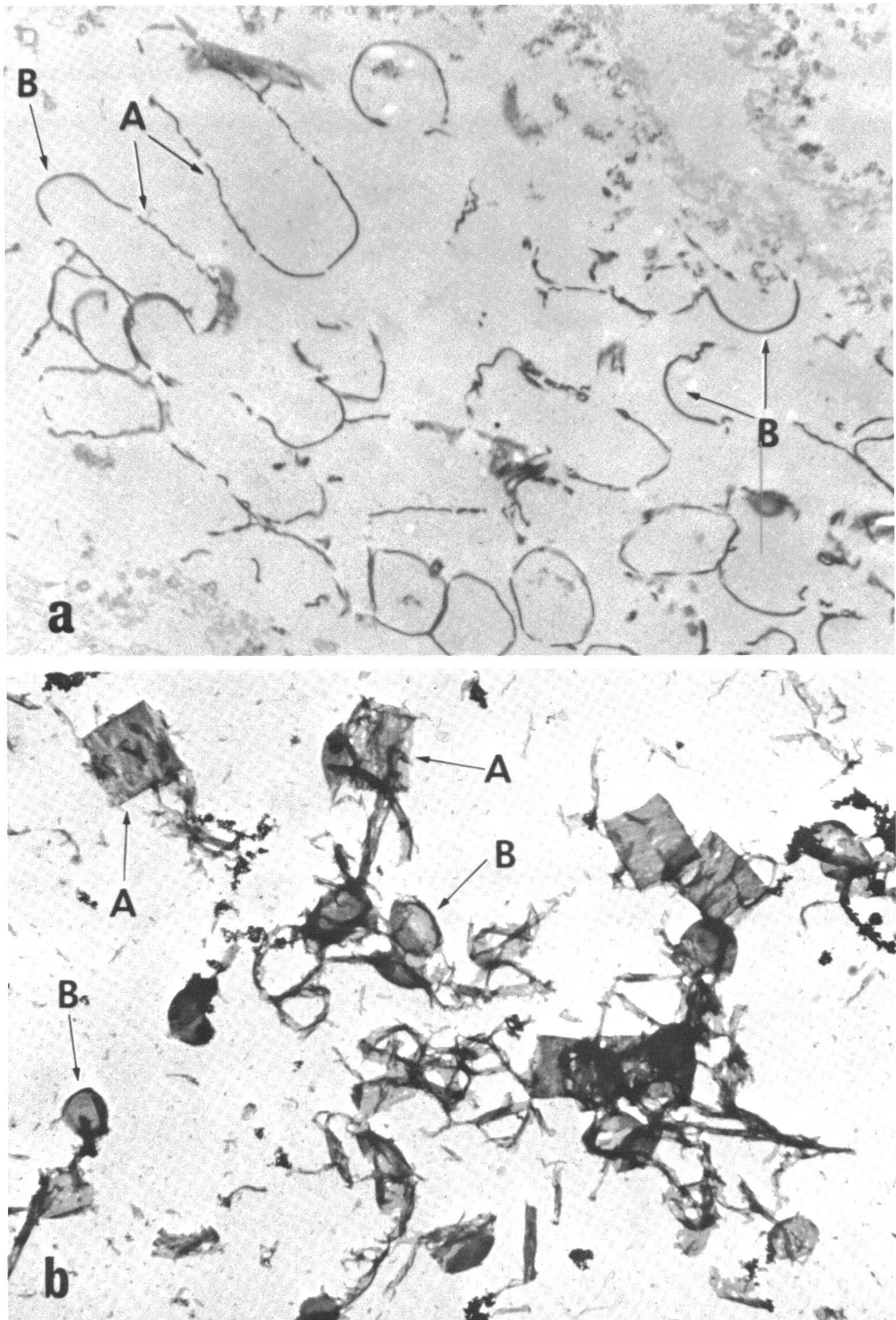

FIG. 3. *Morphology of partially autolyzed Bacillus subtilis walls. Cell walls sampled at 30 min (Fig. 1). (a) Thin-section electron micrograph; ×20,000. Arrows: A, partially degraded cell side, note gaps; B, relatively undegraded end, note absence of gaps. (b) Surface view electron micrograph; ×7,000. Arrows: A, partially degraded cell side, note degradation in direction parallel to cell end; B, relatively undegraded cell end.*

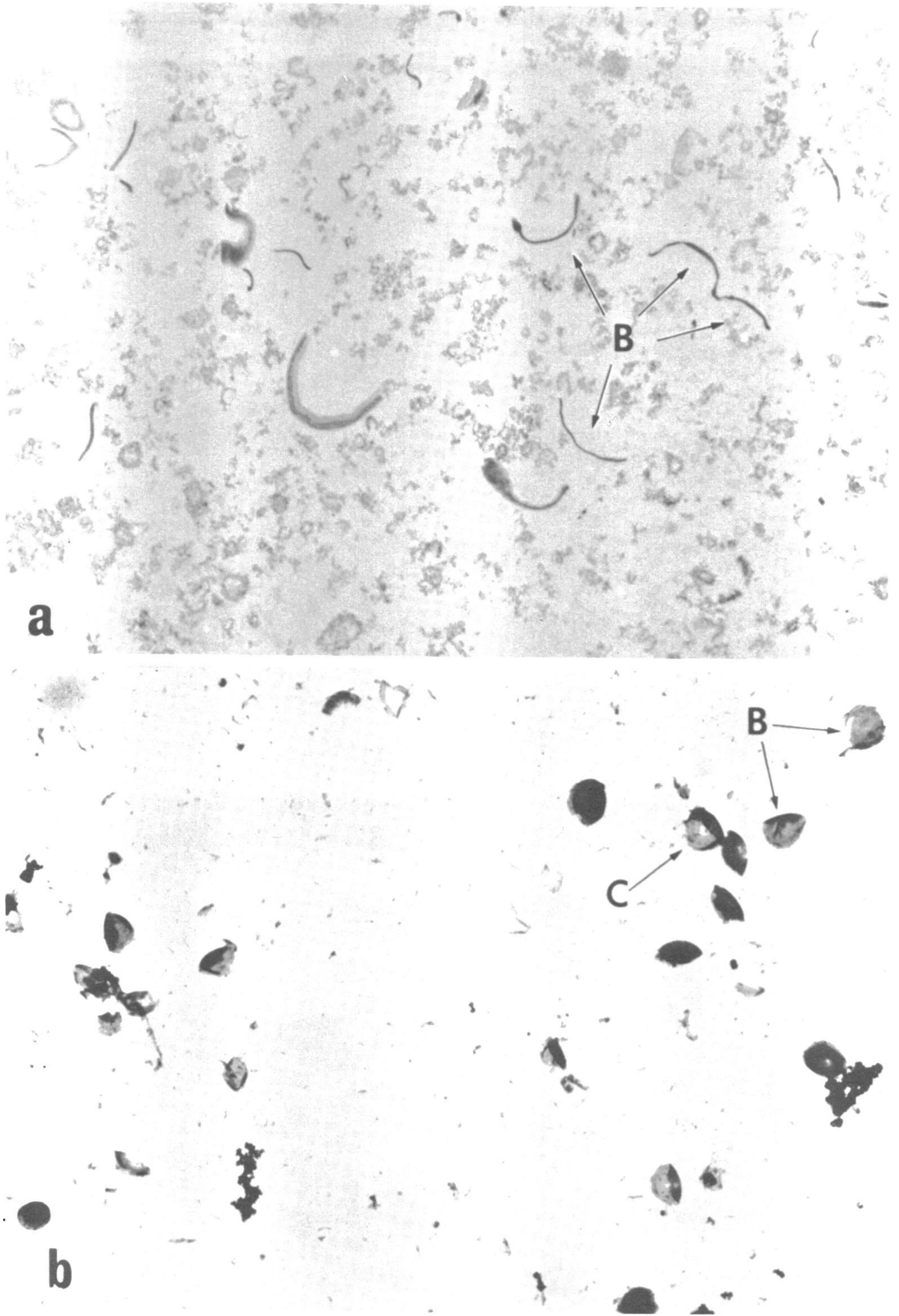

FIG. 4. *Morphology of cell ends remaining after full autolytic degradation of cell sides. Cell walls sampled at 105 min (Fig. 1). (a) Thin-section electron micrograph; ×20,000. Arrows: B, relatively undegraded cell end. (b) Surface view electron micrograph; ×6,000. Arrows: B, cell end; C, possible partial septum.*

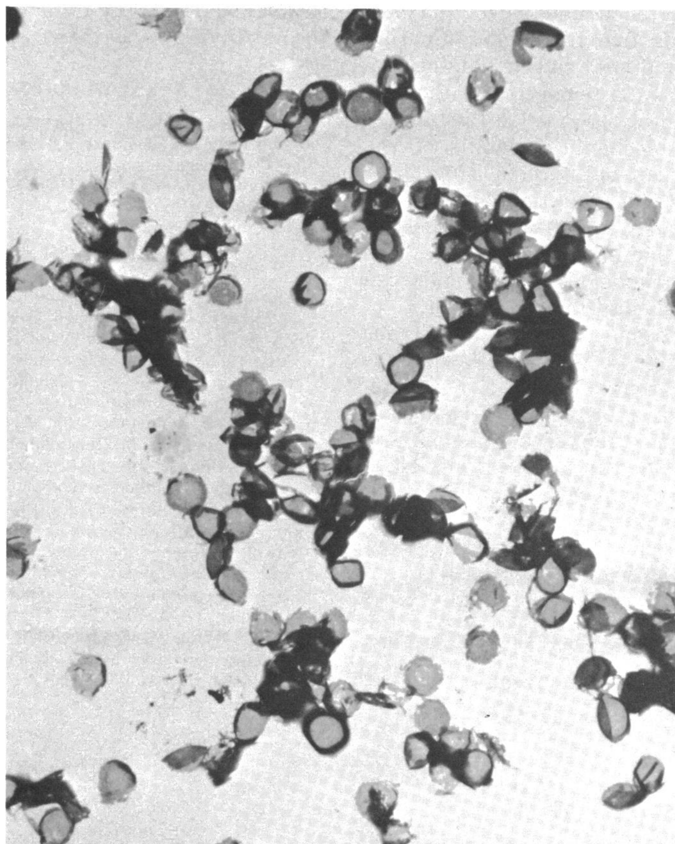

FIG. 5. *Morphology of isolated cell ends. Native cell walls were allowed to autolyse under the conditions given in Fig. 1. After the kinetics of autolysis had reached the plateau region, the sample was treated in sodium dodecyl sulfate as described previously (3). Then the sample was examined using the surface view technique.* ×*6,000.*

lysin prepared by soaking cell walls with LiCl was added back to SDS-treated walls from which all endogenous autolysins had been removed. Any preferential preservation of ends should now be due only to structural differences.

We have recently found that *B. subtilis* native walls have attached an alanine amidase and a glycosidase. When assayed in TK buffer, the amount of amidase activity present is approximately ten times greater than glycosidase activity. These enzymes can be separated from each other because the glycosidase can be eluted from native walls with 0.5 M LiCl, whereas the amidase is selectively removed by 1.5 M LiCl (4). Since the predominant autolytic activity in 0.3 M LiCl in TK buffer is due to the amidase (4), isolated amidase was tested on SDS-treated walls. When the amidase was added, the kinetics of lysis and the morphology of the samples at various stages of degradation were identical to those shown in Fig. 2b, 3b, and 4b, so no detailed data are given. This result indicates that ends are structurally different from sides. The fact that lysis with the amidase alone will give the same result as autolysis where some glycosidase activity is also present is not surprising because the glycosidase is present in very small amounts and is inhibited 80% by the LiCl present in the lysis buffer.

DISCUSSION

Both thin-section and stain techniques show that autolysis in 0.3 M LiCl will preferentially leave walls from cell ends and perhaps also cell septa. Experiments using *B. subtilis* amidase acting on SDS-walls indicate that the resistance of ends to digestion is due to a structural

difference between ends and sides. Furthermore, purified ends free from contaminating fragments of side material can be obtained by SDS treatment of walls remaining in the plateau region after autolysis in 0.3 M LiCl in TK buffer. Thus the biochemical details of the structure of ends can be studied. Since over 80% of the wall is side material (Fig. 1), any bulk biochemical analyses of whole walls will reflect essentially side material. Therefore, the biochemical properties of sides and ends can be studied separately and then compared.

In experiments from which 0.3 M LiCl was omitted, the relative resistance of cell ends was diminished so that the plateau region reflecting cell ends (Fig. 1) was largely absent, and complete destruction of both sides and ends was accomplished in a short time. However, electron microscopy examination in experiments with no LiCl also showed that primarily ends were seen after approximately 90% lysis. Addition of 0.3 M LiCl might well preferentially reduce the binding of autolysin to ends and hence cause ends to be more autolysin-resistant than sides.

The implications of structural differences between cell sides and ends is discussed in an accompanying paper (5).

ACKNOWLEDGMENTS

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