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Sites of Cellular Autolysis in *Lactobacillus acidophilus*

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Ultrastructural changes which occur during cellular autolysis of *Lactobacillus acidophilus* strain 63AM Gasser in 0.05 M citrate buffer, pH 5.0, were examined. Early in the process, randomly distributed electron-dense patches were seen on the wall surface, along with an accompanying eversion of mesosomes. Later, after a loss of about 20% of the initial cellular turbidity, dissolution from the outside of nascent cross walls was seen. This observation was related to the normal process of cell separation. After this stage, short lengths of the cylindrical portion of the wall appeared to be completely removed in a random manner over the entire surface. This dissolution produced gaps in the wall which allowed the extrusion of membrane and cytoplasm. Although membrane was usually extruded through one major, polar, subpolar, or septal site, other secondary points of membrane extrusion were also frequently seen in the same cell section.

Roles for autolytic enzymes in bacterial cell surface enlargement and cell division have been proposed by a number of investigators (summarized in recent reviews; 12, 15). These roles frequently rely on the hydrolysis of selected bonds in peptidoglycan at highly localized sites in the bacterial cell wall. Direct, ultrastructural evidence of such localization of the very early stages of cellular autolysis has been obtained in only a very few bacterial species (12). Less direct evidence suggestive of localized autolytic activity has also been obtained with a limited number of species. This type of evidence usually relies on the localization of penicillin-sensitive cell surface sites (12).

Here we examined the early stages of cellular autolysis in *Lactobacillus acidophilus* strain 63AM Gasser. This species contains a single detectable autolytic enzyme activity. The autolytic enzyme system of *L. acidophilus* resembles that described in *Streptococcus faecalis* (3) in that it is (i) a β-*N*-acetylmuramideglycan hydrolase, and (ii) strongly bound to and closely associated with the cell wall. The *L. acidophilus* and *S. faecalis* systems differ in that (i) *L. acidophilus* enzyme activity was inhibited by reagents which bind to sulfhydryl groups, (ii) a proteinase-activatable latent form was not detected, and (iii) a significant portion of the autolytic activity was not wall bound and can be found in the soluble fraction of the cell (2, 3). The two organisms also differ in that the peptidoglycan fraction of *L. acidophilus* showed extensive turnover, whereas turnover of the peptidoglycan of *S. faecalis* was below detectable levels (1).

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MATERIALS AND METHODS

Growth of the organism. *L. acidophilus* strain 63AM Gasser was grown at 37 C in previously described organic medium (2). Exponential growth, at a doubling time of about 45 min, was stopped at an optical density (adjusted to agree with Beer's law) of 0.6 to 0.8 at 675 nm by rapid chilling of the cultures.

Cellular autolysis. The exponential-phase cells were harvested and washed rapidly on a membrane filter (0.45-μm pore size). The cells were then resuspended in 0.05 M sodium citrate, pH 5.0, to an optical density of 0.4 to 0.5. Cellular autolysis at 37 C was monitored by measuring turbidities at 675 nm (3).

Cell counting and sizing. Cells undergoing autolytic wall hydrolysis in citrate buffer, pH 5.0, containing 5 mM $MgCl_2$ and 15% polyethylene glycol 4000 as an osmotic stabilizer were removed at zero time and after 200 min and fixed in 6.5% Formalin. Suitable dilutions were counted and placed in size classifications with the Coulter particle counter (18) by using a 30-μm-diameter orifice tube. Amplification and current setting of 1 and 0.7, respectively, were used.

Electron microscopy. Glutaraldehyde was added to control and autolyzing cultures to a final concentration of 3%. After 2 h of fixation at room temperature (25 C), the cells were washed, postfixed in

osmium tetroxide, stained with uranyl acetate, dehydrated, and infiltrated with Epon 812 as described previously (8, 11). Silver-gray and gray sections, stained with uranyl acetate and lead citrate, were examined with a Siemens Elmiskop 1A at instrumental magnifications ranging from 30,000 to 50,000.

Because of the poor staining characteristics of the cell wall of *L. acidophilus* in thin sections, the photographic prints of the electron micrographic plates used in this study generally required much more exposure than is normally used. This in part accounts for the high density of the cytoplasm in most of the illustrations presented in this paper.

Glutaraldehyde-fixed cells were also freeze-fractured with a Balzer freeze-fracture microtome model BA360M, following the procedures outlined in the Balzer instruction manual no. A11-3992e. All preparations were freeze-etched 3 min before being replicated.

RESULTS

In freeze fractures, the cell wall appears to be a homogenous layer with a fairly smooth outer surface (Fig. 1). This is in contrast to the picture obtained in thin sections where the wall does not appear to be the typical tribanded structure observed in thin sections of many gram-positive organisms (7). In sections, the wall of *L. acidophilus* was seen as a largely diffuse structure which appears to be electron dense only in the zone immediately adjacent to the outer layer of the cell membrane (Fig. 2A–E).

As in the case of many other gram-positive organisms, the cross wall appears to be laid down between the two layers of septal membrane. Most of the cross wall appears to be made of the same "spongy" low-density cell wall material observed in the outer zone of the peripheral wall. It seems that the cross wall does not begin to split into two cell poles until after the cross wall has completed, or is in the last stages, of cell septation (Fig. 2). At this point the cross wall is in the form of a thick disk (Fig. 2D). This disk is converted into two rounded cell poles by the concentric severing of the

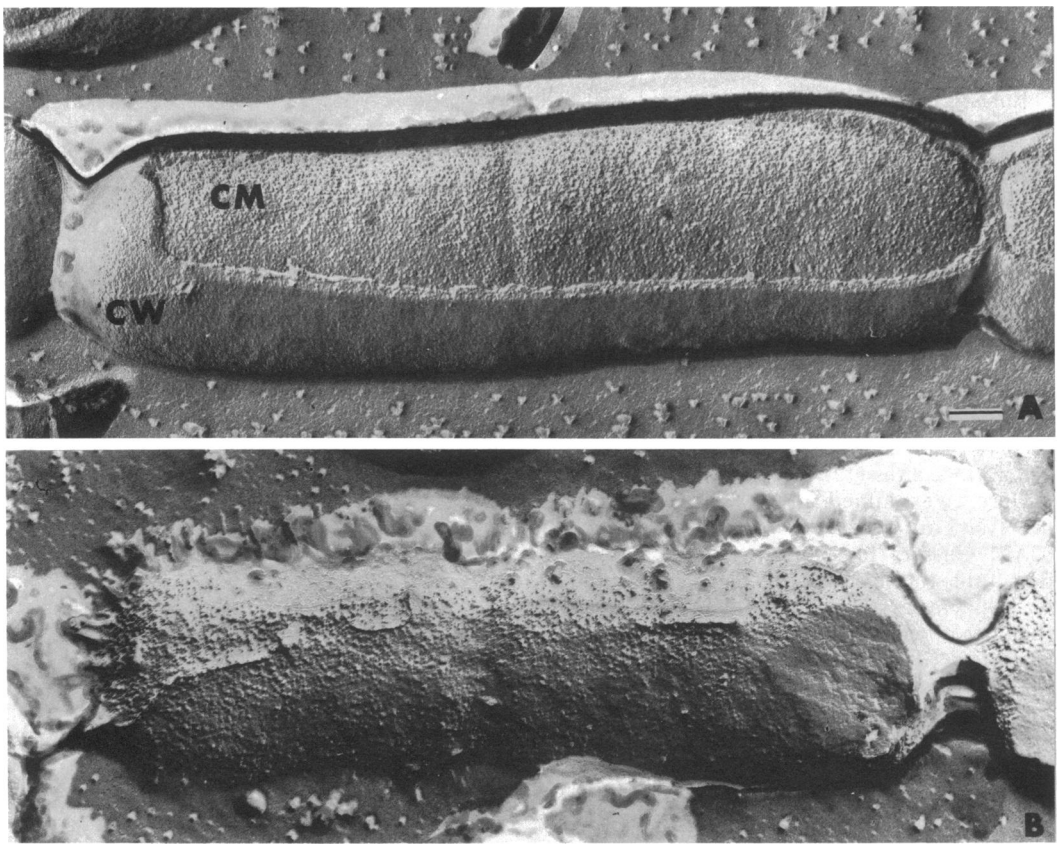

FIG. 1. *Freeze-fracture preparations of an exponential-phase (A) and autolyzing cell (B). In A, a portion of the cell wall (CW) has been removed, thus exposing the cell membrane (CM). In B, a cell taken from an autolyzing cell suspension after a 36% decrease in initial turbidity is shown. The cell wall surface has a "pebbled" texture. Both cells were fixed for 30 min in 3% glutaraldehyde before being frozen. Etch time was 3 min. Bar equals 100 nm.*

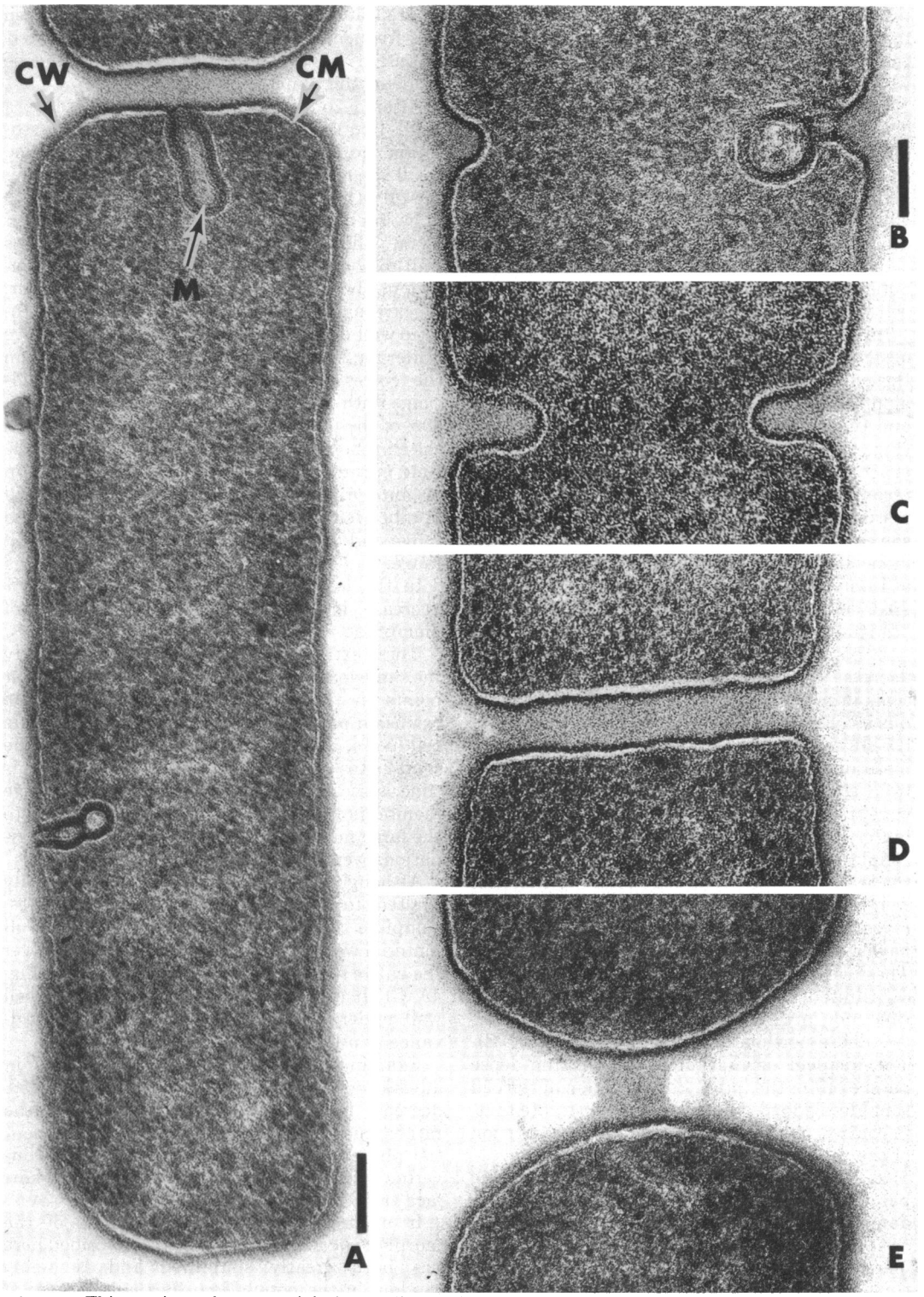

FIG. 2. *Thin sections of exponential-phase cells showing some of the observed mesosome attachment points (A and B) and the proposed cross wall development cycle (B–E). Relevant structures are indicated as follows: CW, cell wall; CM, cell membrane; M, mesosome. Bars equal 100 nm. The bar in B also applies to C–E.*

mid-laminar sector of the cross wall beginning from the outside (Fig. 2A, D, E) and proceeding centripetally towards the center of the cross wall.

In thin section, the cell membranes of *L. acidophilus* had the usual asymmetric structure observed in most bacterial membranes. That is, the outer electron-dense layer of the membrane stained much more strongly than the inner electron-dense layer (Fig. 2A-E). Since the protoplast membrane is closely opposed to the cell wall, it is difficult to determine where the outer layer of the membrane ends and where the cell wall begins.

In exponential-phase cells, mesosomes were observed at three locations. These were (i) at the cell poles (Fig. 2A), (ii) at subpolar positions along the cylindrical axis of the cell (Fig. 2A), and (iii) at the point of cross wall invagination (Fig. 2B). It is impossible to comment on the exact morphology of these mesosomes without reconstruction of serial sections. However, from a study of random sections, most of the mesosomes of *L. acidophilus* appear to be either (i) large single-layered tubules, frequently pinched at intervals (Fig. 2A), (ii) single- or double-layered vesicles (Fig. 2B), or (iii) double-layered "tongues" of invaginated membrane.

In populations of cells examined after an 8% decrease in initial turbidity, two early alterations in morphology were observed. First, complexes of multitubular mesosomes were seen in the submural zone (Fig. 3A). This alteration in mesosome structure and location could result from either a change in osmolarity when cells were transferred from the growth medium to the buffer, or from an early manifestation of lytic cellular damage. Second, along the periphery of the wall, patches of densely staining material were seen (Fig. 3A-E). These patches probably reveal zones of wall which have been altered to have a high affinity for the electron-dense stain. These electron-dense patches were randomly distributed over the wall surface and usually (but not always) observed along the outer, less-dense portions of the cell wall (Fig. 3B-E). The frequent separation of the electron-dense patches from the inner, dense portion of the cell wall gives added credence to the postulate that, in control, nonautolyzing cells, the outer portion of the wall can be seen only with difficulty.

It seems likely that these densely staining patches of wall are associated with autolytic damage. They were not seen on walls of growing or stationary-phase cells (1) or on walls of cells merely exposed to the autolysis buffer (zero time controls in 0.05 M citrate buffer, pH 5.0). At very late stages of autolysis, the densely stained sites on the outer wall surface were seen less frequently (Fig. 4A-B). A definitive interpretation of these stain-binding sites cannot be made at this time.

After a 20% decrease in initial turbidity, a third, frequently found alteration was the dissolution, from the outside, of nascent cross walls, in all stages of development (Fig. 3A-E). Under the growth conditions used, the cells tended to grow in chains of three and more cell units per chain. The ultrastructural observations of cross wall dissolution were correlated with cell separation. By using the Coulter particle counter, the average cell size of cells undergoing autolytic wall digestion in an osmotically protective buffer for 200 min was compared with cells from the corresponding exponential-phase population. With the exponential-phase population, 60% of the total number of cell units fell within the threshold range of 5 to 50 units, whereas 40% were in the 50- to 105-unit range. After 200 min of autolytic damage, 90% of the total cell number fell within the 5- to 50-unit threshold range and only 10% were in the 50- to 105-unit range.

In the latter phases of autolysis (after 40% decrease in initial turbidity), extrusions of membrane were frequently noted (Fig. 4).

These extrusion points were at approximately the same locations as the mesosome attachment points in the exponential-phase cells. These extrusion points were observed primarily at the cell poles, at subpolar positions, and at the septal sites (Fig. 4A, B, C, respectively). At this time, a section usually contained a single large membranous extrusion. However, in addition to the large membranous extrusion, smaller evaginations were frequently observed (Fig. 4B).

Although membrane extrusion was usually limited to one of these three general sites, complete removal of short lengths of cell wall material was observed at irregular intervals over the entire length of cylindrical cell surface (Fig. 4A-C). It appears that some points of the cell wall undergo complete dissolution well in advance of others.

Examination of freeze fractures of cells in advanced stages of autolysis (after about a 35% decrease in initial turbidity) showed that the rather smooth cell wall surface of the exponential-phase cell (Fig. 1A) was frequently converted into a rough "pebble-like" textured surface (Fig. 1B).

It must be noted that the timing of the morphological stages of autolysis described here has been greatly simplified and is highly random in the population. Particular stages of autolysis were observed in some cells well in

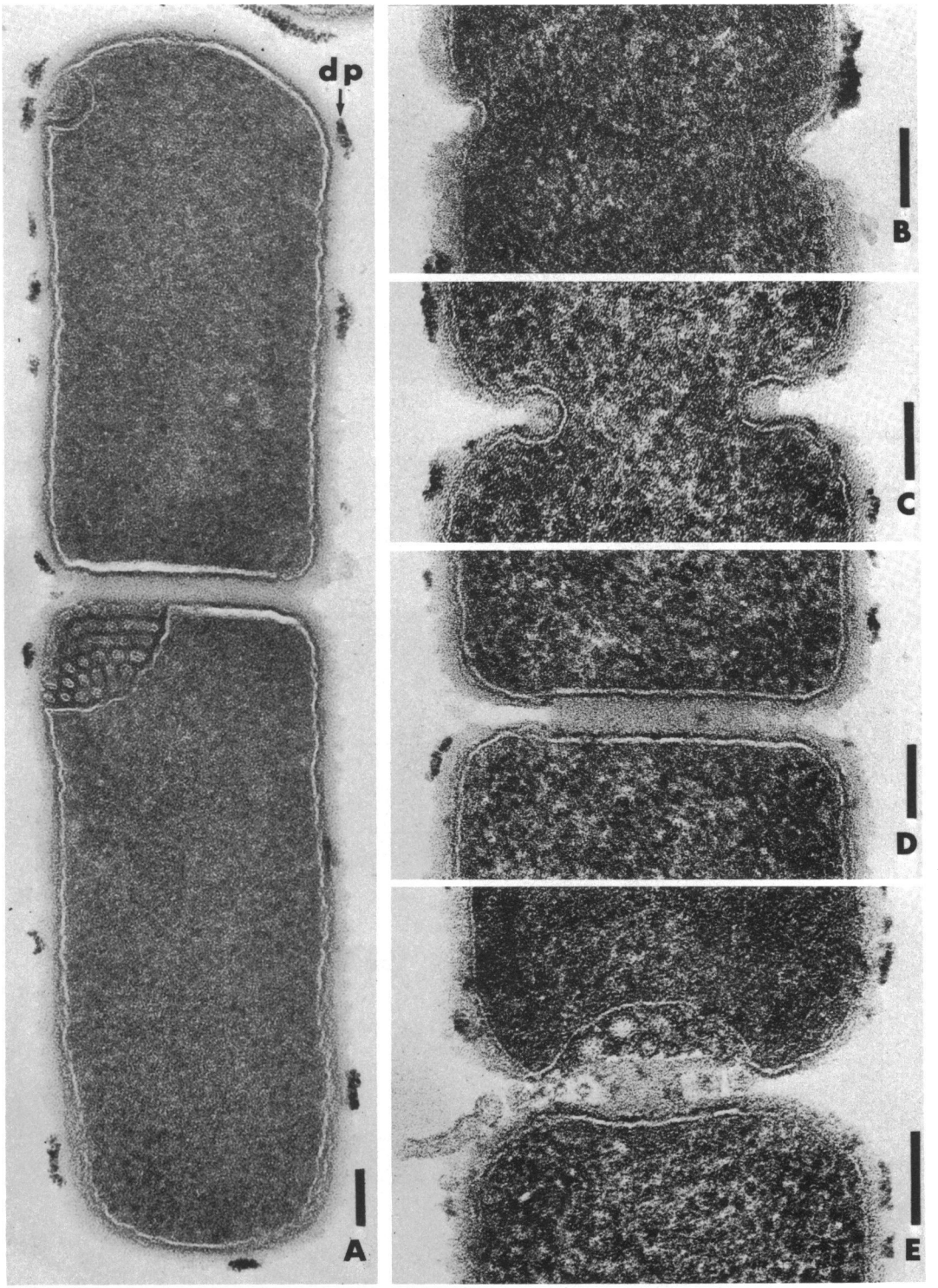

FIG. 3. *Examples of early points of autolytic attack. Micrographs were selected to allow direct comparison with Fig. 2. A longitudinal cell section taken from a culture that had lost 8% of its initial turbidity is shown in A. The intense areas of staining (dp) along the cell wall and the presence of the multitubular, submural, but extracytoplasmic, mesosomes represent the earliest observed departures from the normal cell morphology. In cultures that had lost 20% of their initial turbidity, dissolution of the mid-laminar section of all stages of cross wall development was observed (B–E). In all cases the bars equal 100 nm.*

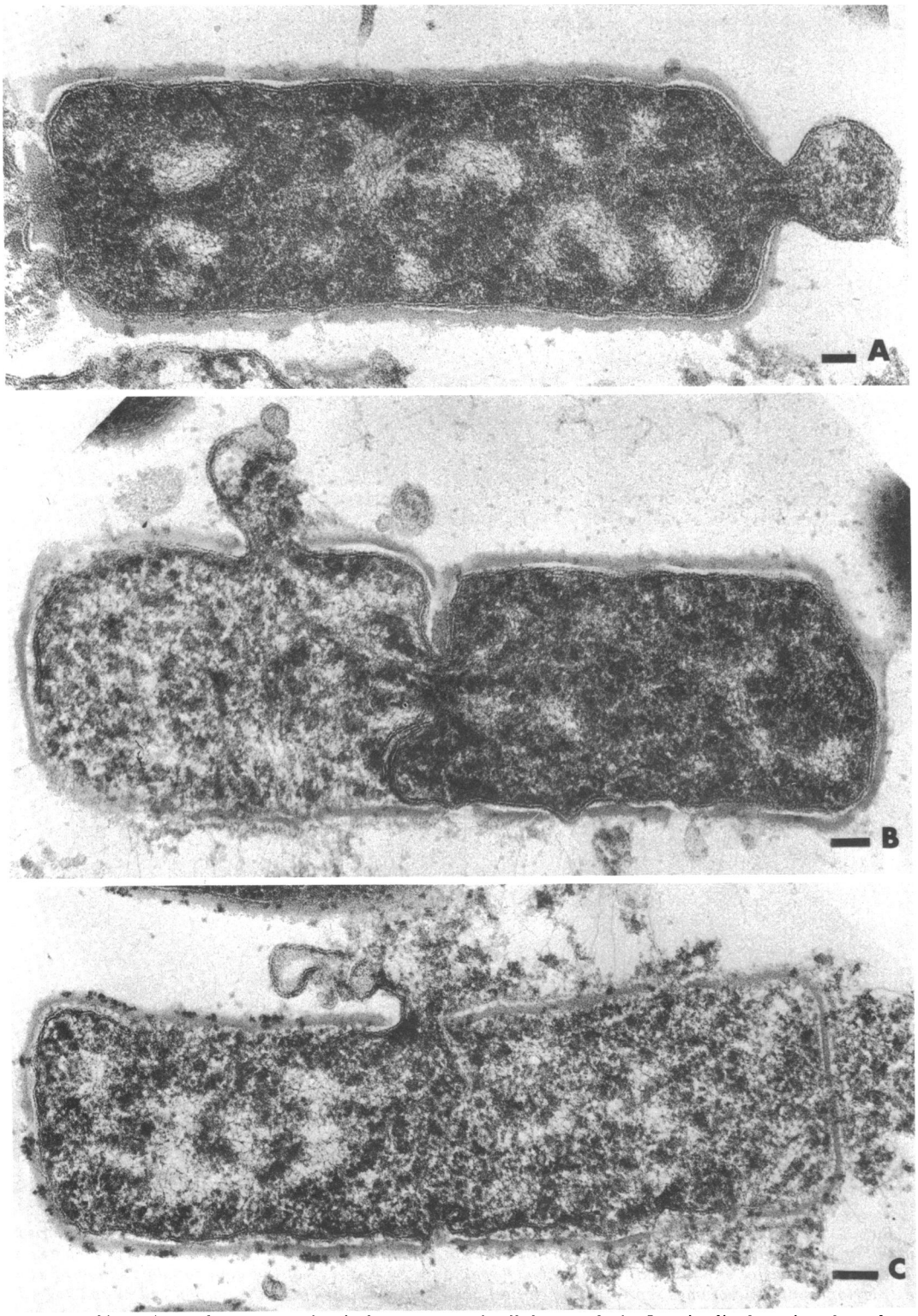

FIG. 4. *Sites of membrane extrusion in later stages of cellular autolysis. Longitudinal sections have been selected to show polar (A), subpolar (B), and septal eversion of the cytoplasm (C). Bars equal 100 nm.*

advance of other cells. In fact, a significant number of cells appeared to be resistant to the autolytic process. The sensitivity and site of autolytic attack in a given cell could easily be a function of individual cell age. Such a variation in individual cell sensitivity to autolysis has also been observed previously in both *S. faecalis* (9) and a group H streptococcus (14).

DISCUSSION

In contrast to the situation in the coccal-shaped *S. faecalis*, wherein a highly localized primary attack at the inner edges of nascent cross walls was observed (9), the initial events which occur during cellular autolysis of the rod-shaped *L. acidophilus* seem to be much more varied and complex. This could be due to one or more of a number of both technical and physiological factors. For example, the "fuzzy" appearance of the outer contour of the wall of exponential-phase cells in antitangential sections might result from a portion of the wall having a low affinity for the electron-dense stain, or to the collapse of the wall during the dehydration steps of the embedding process. The fairly smooth appearance of the wall in freeze-fracture preparations (Fig. 1A) is consistent with one or both of these interpretations. On the other hand, irregular external margins were seen on sections of exponential-phase cells but not on sections of stationary-phase, valine-deprived cells (1). In exponential-phase cells extensive peptidoglycan turnover was found to accompany cell wall enlargement (1). After valine deprival, walls became thickened and smooth, and peptidoglycan turnover was below detectable levels.

The first observable autolytic damage to the wall appears to be (i) the appearance of electron-dense zones along the cell wall, and (ii) loss of wall material from the external and mid-laminar sector of nascent and completed cross walls (Fig. 3). The apparent shortening of average chain length during autolysis and the observations of the separation of completed cross walls in exponential-phase populations (Fig. 2A, D, E) suggest that wall hydrolysis in the cross wall might be a normal part of the cell separation procedure.

This process of forming cross walls in exponential-phase cultures of this species differs greatly from that seen previously in *S. faecalis* (10, 12), in that the cross wall virtually completes the septation of the cytoplasm before the cross wall begins splitting into two cell poles. In this regard it is similar to other rods that have been studied, such as *Bacillus megaterium* (5) and *L. plantarum* (13). Wall thickness appears to decrease as cells round up their poles and separate (Fig. 2D, E). Cell wall thickening upon amino acid starvation in *L. acidophilus* results in unusually thick pads of wall between many cells in chains (1). The loss of wall material from the external and central portions of the cross wall during cellular autolysis (Fig. 3A–D) seems to be an exaggeration of the normal cell separation process with the exception of the absence of the accompanying rounding up of the poles.

Interpretation of these observations of the very early stages of cellular autolysis in terms of enzyme localization is difficult without considerable additional knowledge concerning both the wall substrate and the autolytic enzyme activity. On the one hand, autolysin molecules could be "built into" cross walls and then somehow activated when needed on the outside of the cell. On the other hand, it also seems possible that the external attack on cross walls is due to the action of extracellular, excreted autolysin. The latter would presuppose a differential susceptibility of a portion of the cross wall compared with not only the rest of cross wall but also with peripheral wall. Such an effect could result from either a "built-in" inhibitor of autolysin activity in much of the wall (e.g., an oxidation [reduction] of sulfhydryl groups) or some kind of protection from accessibility to extensive autolysin activity. In support of such an hypothesis, the autolysin of *S. faecalis* fails to bind to the external surface of intact exponential-phase cells of *S. faecalis* in the absence of autolytic wall damage (R. Joseph and G. Shockman, manuscript in preparation). Such a difference in wall substrate susceptibility has been proposed by Mosser and Tomasz after the incorporation of choline in place of ethanolamine in the walls of *Diplococcus pneumoniae* (19). Such cells resist autolysis and grow in long chains, because they either fail to divide or separate, or both. Refractoriness of most of the external wall surface, except for part of the cross wall, to autolysins and other wall hydrolytic enzymes could explain the dechaining activities of hen egg white lysozyme or the autolysin of *B. subtilis*. Growth of *S. faecalis* is unaffected by the presence of a high concentration (1 mg/ml) of lysozyme (18), whereas both cells (16) and isolated walls (17) are dissolved by low concentrations (1 to 4 μg/mg, dry weight) of the same enzyme.

The later stages of cellular autolysis (Fig. 4) suggest that membrane extrusion occurs primarily at one point per cell. This can be either a polar (Fig. 4A), subpolar, (Fig. 4B) or central (Fig. 4C) location along the cylindrical portion of the wall. Such sites could be associated with points of recent cross wall completion (Fig. 4A)

or new cross wall initiation (Fig. 4C), or could be due to geometrically "weak" zones along the cylindrical cell (12). By using light or phase-contrast microscopy, sites at which a protoplast or spheroplast begins to emerge from rod-shaped cells have been used as an index of primary wall damage or of sites of nascent cross wall formation (4). The danger of such an interpretation is evident in the electron micrographs shown in Fig. 4. For example, in Fig. 4A wall loss can be observed at many sites along the cylindrical portion of the cell while the protoplast is emerging from a pole. Also, the section shown in Fig. 4B shows wall loss at the central nascent cross wall as well as along the cylindrical portion of the wall, but the protoplasts appear to be bursting out at only two areas along the periphery of the cell.

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