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Some Properties of Two Autolytic-Defective Mutants of *Streptococcus faecalis* ATCC 9790

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The isolation and some properties of two mutants of *Streptococcus faecalis* ATCC 9790 (*S. faecium*) which autolyze at a much slower rate than the wild type are described. Compared with the wild type, mutant E71 autolyzed more slowly, contained less active but more latent autolysin in the isolated wall fraction, and possessed a wall of very similar chemical composition and degree of cross-bridging. Ultrastructural studies of exponential phase cells showed that cells of E71 were on the average slightly longer and had slightly thickened walls compared to the wild type. Mutant E81 autolyzed much more slowly, grew exponentially in long chains (8 to 40 cells compared with mainly diplococci), contained much less active and latent autolysin in the wall, and possessed a wall of very similar chemical composition but with about twice the content of *N*-terminal groups. Mutant E81 walls were more susceptible to isolated autolysin but possessed an autolysin of the same specificity as the wild type. Ultrastructurally E81 cells were, on the average, significantly longer and had thicker walls than the wild type. Mutant E71 may be partially blocked at either transport of autolysin to the wall or in conversion of latent to active autolysin. The pleitropic effects noted in mutant E81 have been taken to suggest a possible membrane defect and to support the role of the autolysin in cell separation.

A number of biological roles for peptidoglycan hydrolases have been proposed. These include participation in (i) the process of cell wall growth (13, 17, 24); (ii) cell separation (10, 23); (iii) peptidoglycan turnover (1, 12); and (iv) providing gaps or holes in the cell surface large enough to admit large macromolecules which cannot pass through the spaces in the cell wall (25).

The cleavage of the polysaccharide backbone of the rigid peptidoglycan polymer of the cell wall by *N*-acetylmuramidases could allow for the insertion of new disaccharide units resulting in cell wall growth. The autolysin of *Streptococcus faecalis* could perform such a role, although it is difficult to imagine how this role could be achieved by autolytic activities cleaving certain other bonds in the cell wall (20; M. L. Higgins and G. D. Shockman, CRC Crit. Rev. Microbiol, *in press*). Support for the involvement of the *S. faecalis* autolysin in the cell wall growth comes from the association of the active form of this enzyme with the highly localized region engaged in enlargement of the wall surface (7, 18).

A role for an autolysin in cell separation in streptococci has long been suggested (10). Recently, support for such a role in *Bacillus subtilis* comes from the finding that the addition of an autolytic amidase or hen egg-white lysozyme to cultures of *B. subtilis* growing in long chains leads to the unlinking of such chains and the release of separated cells (4).

The replacement of choline by ethanolamine in the cell wall teichoic acid of pneumococcus resulted in several striking changes (23). The cells became simultaneously unable to be transformed and unable to undergo deoxycholate-induced lysis, and grew as long chains of unseparated cells, while the cell walls became insensitive to the action of the pneumococcal autolysin. One possible explanation for these results is that all the changes in properties are consequences of the insensitivity of the ethanolamine-containing walls of the autolysin.

Lytic-deficient mutants of *B. licheniformis* have been reported with only 3 to 5% of the

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wild-type level of lytic *N*-acetylmuramyl-L-alanine amidase activity. In addition, the cell walls had changes in composition which resulted in their being less sensitive to this activity. Such cells grow as long chains of unseparated bacilli (15).

A mutant of *Staphylococcus aureus* has been described (2) which shows a number of pleitropic effects including phage resistance, a reduced content of teichoic acid in the cell wall, changes in the distribution of the autolytic activity, and a greater tendency than the parent organism to grow in large clusters. These findings were used to support a role for autolysin in cell separation.

We have isolated and studied autolytic-defective mutants of *S. faecalis* in the belief that they may help to provide insight into the role(s) of the autolysin in this organism.

MATERIALS AND METHODS

Growth. The organism used in the present study was *Streptococcus faecalis* ATCC 9790 and two mutants designated E71 and E81. Cells were grown on a chemically defined liquid medium (17, 22) at 37 C. Cultures were harvested in the mid or late exponential phase (LOG); growth was arrested by pouring the cultures onto ice.

Mutagenesis. Fifteen milliliters of liquid medium was inoculated and grown overnight into stationary phase. A 0.3 ml-amount of ethylmethane sulfonate was added, and, after mixing, 10 samples (1 ml) were immediately withdrawn. All samples were incubated for 45 min at 37 C and then for 15 min at 48 C. Each tube was diluted 1 to 10 with fresh medium and incubated at 37 C for 5 hr. Dilutions of each tube were made and plated on Trypticase soy agar (TSA) and incubated overnight at 37 C. Plates containing from 100 to 300 colonies were chosen for selection of possible autolytic mutants.

Selection procedure. A suspension of heat-killed *Micrococcus lysodeikticus* cells [approximately 2 mg (dry weight) per ml] in melted TSA, called hereafter ML agar, was prepared by autoclaving at 110 C for 15 min. An 8-ml amount of this suspension was poured onto a solidified basal layer of 15 ml of TSA in a petri dish. In some cases, trypsin (100 μg/ml) was incorporated into the melted ML agar. To screen for possible autolytic mutants, a replica of a TSA plate containing colonies to be tested was made onto an ML agar plate, which was incubated for 24 hr at 30 C and then placed in a cold room. The plates were inspected daily. A zone of clearing of the slightly turbid upper layer (ML agar) appeared around the edges of the colonies of the wild type after 24 to 48 hr in the cold. This clearing indicated disolution of the *M. lysodeikticus* cells presumably by hydrolytic enzyme action. Colonies isolated from the mutagenized culture which showed a very small or no "halo" of clearing were selected and recloned for further testing for autolytic activity when grown in liquid medium.

Cell wall isolation and autolysis. Exponential phase cells were broken and the wall fraction (LOG walls) was isolated as previously described (18). Cell walls were assayed for active and total autolytic activities in 0.01 M buffer, *p*H 6.7, as previously described (18).

Substrate properties of the cell wall. Cell walls isolated from mutant or wild-type cells were inactivated by treatment with 2% sodium dodecyl sulfate (SDS) as previously described (19). LOG walls were allowed to autolyze by the action of the active autolysin. The autolysate, containing approximately 600 units of total autolysin activity, was added to the SDS walls on ice for 60 min when almost all of the autolysin became bound to the walls (16). The rate of dissolution of the SDS walls attributable to the action of the active and total autolysin activities was determined turbidimetrically (16) or by the release of radioactivity to the soluble fraction from SDS walls labeled with ^{14}C-lysine, as previously described (19). One unit of autolysin activity equals a decrease of 0.001 OD per hr (18).

Analytical procedures. The isolated cell wall fractions were lyophilized and dried in vacuo over phosphorus pentoxide. Measurement of *N*-terminal groups, hexosamine and *N*-acetylhexosamine, was performed by the method of Ghuysen et al. (6). Amino acid analysis was carried out after hydrolysis in 6 N HCl at 120 C for 22 hr in a sealed tube. Phosphorus was measured by the method of Lowry et al. (11). Rhamnose was estimated by the method of Dische and Shettles (3).

Electron microscopy. Cells were fixed with glutaraldehyde-osmium, embedded in Epon 812, sectioned, and poststained with uranylacetate-lead citrate as previously described (7, 9).

Glutaraldehyde-fixed cells were freeze-fractured and etched for 3 min with a Balzers BA360M freeze-fracture microtome. The basic techniques outlined in the Balzers instruction manual (no. All-3992e) were used.

RESULTS

Isolation and cell autolysis of autolytic defective mutants. After 3 days of incubation of the ML agar plates in the cold, four or five colonies which gave no or very little clearing of the turbid ML agar were selected out of a total of about 10^3 colonies. These colonies were recloned, transferred to liquid medium, and assayed for cellular autolysis, as previously described (14). Since trypsin alone (approximately 100 μg/ml) caused noticeable clearing of the heat-killed cells in the ML agar, it was necessary to confirm that failure to clear the ML agar was a result of some autolytic deficiency. Two cultures (originating from separate mutagenic events), designated E71 and E81, were found to autolyze only very slowly. When tested in 0.01 M phosphate (Fig. 1A), mutant E81 lost only about 25% of its initial turbidity after 20 hr. In the case of mutant E71, the turbidity dropped by over 80% in this period but only about 10% in 2 hr. Since the

wild type had autolyzed completely in 2 hr, E71 was still relatively resistant to autolysis. Both mutants were less resistant to autolysis when tested in 0.3 M phosphate (the concentration of phosphate in the growth medium) than they were in 0.01 M buffer (Fig. 1B), but both still lysed significantly more slowly than the wild type.

Wall autolysis. The overall yield of cell wall, isolated from exponential phase cultures, was about 50% higher (dry weight) per unit mass of culture in the case of E81 than in E71 or the wild type.

In the standard 0.01 M phosphate buffer, LOG walls isolated from E81 and E71 autolyzed at about 0.3 to 0.4 of the wild-type rate when assayed for the active form of the autolysin (Table 1). The absolute loss of turbidity leveled off after about 400 min and was less extensive (22 and 42%, respectively) than the wild-type walls (53%). Thus, in the absence of significant substrate differences, both mutants appeared to contain less active enzyme. When assayed for latent autolysin, walls of E81 again autolyzed more slowly than the wild type (0.6 of the rate), whereas walls of E71 dissolved more rapidly (1.6 times the wild-type rate). For both mutants, the ratio of latent to active form of the autolysin was higher (three times higher in the case of E71) than that in the wild type. This suggests disturbances in the mechanism of activation of latent autolysin.

Substrate properties of the mutant walls. The apparent decrease content of the active form of the autolysins shown in Table 1 assumes the absence of a significant change in susceptibility of the wall substrate of the mutants. Accordingly, the walls of the wild type and both mutants were inactivated with SDS, and each inactivated wall preparation was then tested for its ability to serve as substrate for autolysin isolated from wild-type or mutant walls (Table 2). The same preparation of autolysin dissolved SDS-inactivated walls of either the wild type or E71 at virtually the same rate (Table 1, experiment 1). In contrast, autolysin prepared from any of the three sources (wild type, E71, or E81) dissolved E81 SDS walls more rapidly than either wild-type or E71 walls by a factor of 1.6 to 2.1 (Table 1, experiments 1 and 2). In view of the greater sensitivity of E81 walls to autolysin from all three strains, the levels of active and latent autolysin in the cell walls of E81 are actually about 50% lower than those indicated by the results in Table 1.

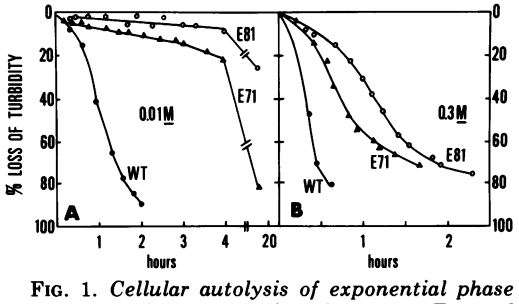


FIG. 1. *Cellular autolysis of exponential phase cells of the wild type (WT) and mutants E81 and E71 in 0.01 and 0.3 M sodium phosphate buffer, pH 6.7.*

Growth properties. Both mutants grew well in the chemically defined liquid medium, with mass doubling times of 32 to 34 min (E71) and 41 to 43 min (E81), compared with 31 to 32 min for the wild type. When grown in liquid medium, E71 had a more yellowish color than either the wild type or E81; this might be related to an increased amount of cell membrane, since centrifugation of broken cells of E71 resulted in larger, yellow membrane layer. This suggests that the lesion in E71 could involve some aspect of membrane synthesis or composition. Both mutants tended to revert readily to the wild-type phenotype. This necessitated monitoring for reversion and recloning when indicated.

Cells of exponential phase cultures of both mutants appeared slightly larger than the wild type under the phase microscope. Cells of E71,

TABLE 1. *Active and latent autolysin in the cell wall fractions of wild type and mutants*

Walls	Autolysin assay[a]	Units per mg	Active autolysin relative to wild type	Ratio of latent to active autolysin[b]
Wild type	Active	283[c]	(1)	4
	Total	1,350		
	Latent	1,067		
E81	Active	77	0.3	9
	Total	752		
	Latent	675		
E71	Active	115	0.4	15
	Total	1,835		
	Latent	1,720		

[a] Activity of the active form was based on the rate of wall autolysis in the absence of a proteinase; total activity in the presence of trypsin (1 μg/ml); latent activity values were obtained by difference.

[b] Ratio was determined for autolysin present in cell walls of each strain.

[c] The figures for active enzyme were an average based on the initial 90 min of autolysis, since assay for active autolysin is not usually linear (14).

like the wild type, consisted mostly of diplococci, although there appeared to be a greater number of chains of four or six cells. Cells of E81 grew in relatively long chains. The majority of the population was found in chains of 8 to 40 cells with very few diplococci present.

Chemical analyses of cell walls. No difference in content of peptidoglycan amino acids (Table 3) or of rhamnose, phosphorus, or total hexosamines (Table 4) was detected. No difference in peptidoglycan amino acid content of untreated and SDS-treated walls was noted. As shown in Table 5, autolysis of walls of all three strains resulted in the release of about the same quantity of Morgan-Elson positive groups (*N*-acetylamino sugars). However, autolysis of E81 walls resulted in the release of about twice the quantity of *N*-terminal groups as did autolysis of the walls of the other two strains. This could be attributable to the presence of an additional autolytic enzyme with a different specificity, such as a peptidase or amidase, or to the presence of additional *N*-terminal groups in the walls. Therefore SDS-inactivated walls of E81 and the wild type were exposed to autolysin obtained from wall autolysates of E81 and wild-type walls prepared in the presence of trypsin (19). Wall lysis was allowed to come to completion, and the release of *N*-acetylamino sugars and *N*-terminal groups to the supernatant fluid (after centrifugation) was determined. A significant difference in the release of *N*-acetylamino sugars was not observed with either wall substrate when walls of either strain were used as a source of autolysin (Table 6). The action of E81 autolysin on SDS-inactivated wild-type walls resulted in the release of the same quantity of *N*-terminal groups (0.17 μmole/mg), as did the action of the wild-type enzyme on the same substrate (0.16 μmole/mg), or as released by autolysis of wild-type walls (0.19 μmole/mg; Table 5). This quantity of released *N*-terminal groups from wild-type walls (0.16 to 0.19 μmole/mg) closely approximated that previously found in intact walls of the wild type (0.19 to 0.22 μmole/mg; references 5 and 19). In contrast, when SDS walls of E81 were used as a substrate for either wild-type or E81 autolysin, a higher level of *N*-terminal group release was observed (0.4 and 0.36 μmole/mg, respectively). This was approximately the same as the *N*-terminal release observed after autolysis of E81 walls (0.39 μmole/mg; Table 5). These results suggest that the autolysin of E81 does not contain an amidase or peptidase and that

TABLE 2. *Susceptibility of SDS-inactivated walls of mutants and wild type to autolysin from mutants and wild type*

Source of SDS walls	Expt 1		Expt 2		
	Autolysin[a] from wild type (units)		Autolysin[a] wild type (units)	Autolysin[a] from E71 (units)	Autolysin[a] from E81 (units)
		(0.3 M)			
Wild type	220 (1)[b]	168 (1)	7.7 (1.0)	3.7 (1.0)	10.9 (1.0)
E71	219 (1)	248 (1.5)			
E81	415 (1.9)	346 (2.1)	12.3 (1.6)	6.2 (1.7)	20.4 (1.8)

[a] Autolysins were obtained from autolysates (-trypsin) of the respective wall preparations. Autolysates were assayed (16) on sodium dodecyl sulfate (SDS)-inactivated walls at 37 C in the presence of trypsin (1.0 μg/ml) in 0.01 M sodium phosphate buffer (pH 6.7), except where 0.3 M phosphate is indicated. One unit of activity equals a decrease of 0.001 optical density per hr (18).

[b] Numbers in parentheses refer to activity relative to activity on wild-type walls.

TABLE 3. *Amino acid content of cell walls of wild type and mutants E81 and E71*

Amino acid	Wild type[a]		E71[a]		E81[b]	
	Amount (μmole/mg)	Ratio[c]	Amount (μmole/mg)	Ratio[c]	Amount (μmole/mg)	Ratio[c]
Aspartic acid	0.36	0.7	0.39	0.8	0.29	0.7
Glutamic acid	0.52	(1)	0.52	(1)	0.47	(1)
Alanine	0.76	1.5	0.79	1.5	0.70	1.5
Lysine	0.52	1	0.47	0.9	0.44	0.9

[a] Average of duplicate determinations.

[b] Average of four determinations.

[c] To glutamic acid.

TABLE 4. *Phosphorus, rhamnose, and hexosamine content of walls*

Walls	Phosphorus (μmole/mg)	Rhamnose (μmole/mg)	Hexosamine[a] (μmole/mg)
Wild type	0.32	0.93	0.81
E71	0.32	0.83	0.82
E81	0.31	0.86	0.81

[a] As glucosamine equivalents. Each value represents the average of duplicate determinations.

TABLE 5. *Release of N-acetylamino sugars and N-terminal groups after wall autolysis*

Walls[a]	*N*-acetylamino sugars[b] (μmole/mg)	*N*-terminal groups[c] (μmole/mg)
Wild type	0.27 ± 0.01	0.19 ± 0.01
E81	0.27 ± 0.01	0.39 ± 0.03
E71	0.26[d]	0.16 ± 0.01

[a] Walls, 0.08 mg/ml; trypsin, 1.0 μg/ml.
[b] As cross-bridged, disaccharide-peptide units. Corrected for the reduced color yield of peptide-substituted, cross-bridged units (5). Average of at least two determinations.
[c] As aspartic acid equivalents.
[d] Single determination.

E81 walls are less cross-bridged than are wild-type or E71 walls. In fact, based on the glutamic acid content of 0.47 μmole/mg (Table 3), the presence of 0.36 to 0.4 μmole/mg of *N*-terminal groups (Tables 5 and 6) indicates that 75 to 85% of the disaccharide peptide units in the wall of E81 are not cross-bridged. It remains possible that, in the growing cells, an amidase or, more likely, peptidase action that is not wall-bound and isolated with the wall fraction could be responsible for the *N*-terminal groups found in the walls of all three strains.

Ultrastructure of mutants E71 and E81. Electron micrographs of thin sections of rapidly growing exponential phase cells of the two mutants are shown in Fig. 2. The mutants were found to differ from the wild type in that: (i) they have slightly thicker walls. Average wall thickness of E71 and E81 was found to be 29.9 nm (±9% average deviation; 36 cells measured) and 31.0 nm (±6% average deviation; 49 cells measured), respectively, compared to 27.2 nm (±8% average deviation; reference 9) for wild-type walls. (ii) Mutant cells have a greater tendency to stick together and form chains. This was much more pronounced for E81 and is illustrated by the micrograph of a freeze-fractured specimen of E81 shown in Fig. 2C. Except for the increased chaining of E81,

TABLE 6. *Release of N-acetylamino sugars and N-terminal groups from SDS-inactivated walls resulting from the action of autolysin from either the wild type or mutant E81*[a]

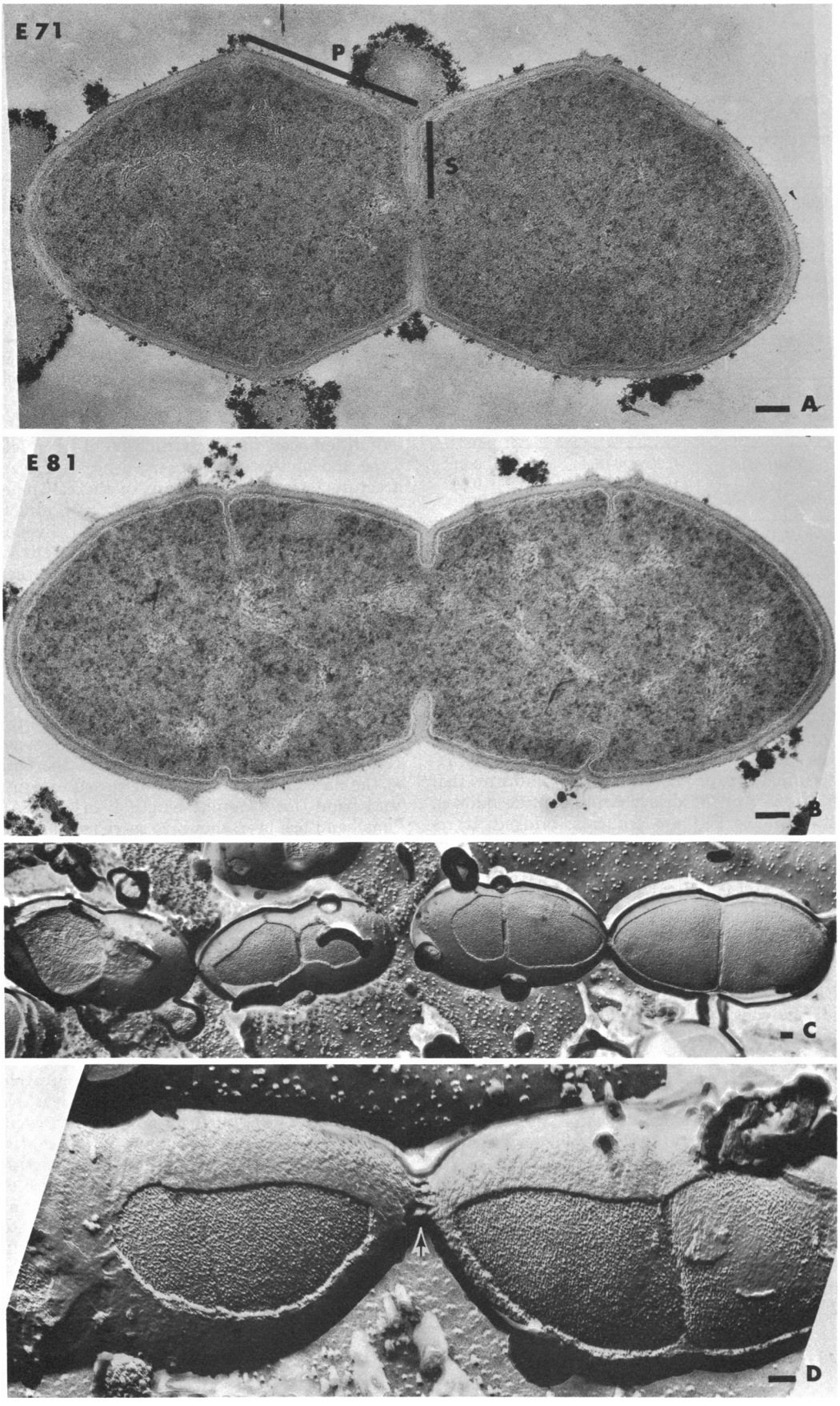
Source of enzyme	Source of SDS-wall substrate	*N*-acetylamino sugars (μmoles/mg)	*N*-terminal groups (μmoles/mg)
Wild type	Wild type	0.32 ± 0.05	0.16 ± 0.02
E81	Wild type	0.33 ± 0.03	0.17 ± 0.01
Wild type	E81	0.26 ± 0.01	0.4 ± 0.03
E81	E81	0.28 ± 0.01	0.36 ± 0.05

[a] Experimental conditions and methods of determination are so described in the footnotes to Table 5. SDS, sodium dodecyl sulfate.

no differences in ultrastructure of the wild type and mutants was observed by the freeze-fracture technique. The caps of the cell poles appear to adhere together by a stringy, almost capsular-like material, well after cell division has been completed (Fig. 2D). (iii) Cells of both mutants were significantly longer than those of the wild type. This again was more pronounced for E81.

These differences in cell dimensions were quantitated by measuring and comparing cross wall length (S) and peripheral wall length (P) on a large number of central, antitangential, longitudinal sections, showing the typical tri-banded wall profile (9). The length of new peripheral wall (P) was measured from the base of the nascent cross wall to the raised external wall band (for example, length P in Fig. 2A). This band has been shown to serve as a marker which separates old polar wall from new equatorial wall made in the most recent generation (8). The length of the nascent cross wall was measured from its tip to its base (for example, length S in Fig. 2A). In the case of a completed cross wall, the entire cross wall length was measured and divided by two. Cell symmetry was assumed, and in this way each pair of measurements represented a stage of wall growth of one half of a cell produced in a single cell cycle.

An approximation of the cell division cycle can be reconstructed from S and P measurements. A cell just starting a new division cycle would have very little or no new peripheral (P) or cross wall (S). A cell just completing its septation would normally have large values of both parameters although the S values of complete cross walls decrease as the cells separate and the cross wall is converted into polar wall. The mean and range of measurements for wild-type cells from a rapidly exponentially growing culture are shown by solid and dashed


E 71
P
S
A
E 81
B
C
D

lines in Fig. 3A and B, respectively. For mutant E71 (Fig. 3A), the S and P values were generally within or near the range of those of the wild type, but many of the S measurements tended to be lower than the mean throughout most of the cell cycle. For mutant E81 (Fig. 3B), many of the S values at corresponding P values were significantly lower than those for the wild type. At larger P values (for example, over 300 nm for P in Fig. 3B), most of the S values were well below the mean for the wild type. In addition, only a very small percentage of the observed cells of either mutant had completed cross walls (open circles in Fig. 3B). With the wild type, most cells with over 550 nm of new peripheral wall had completed septation. These measurements suggest that both mutants, but especially E81, construct more new peripheral wall, relative to cross wall, than does the wild type. Our model for wall growth of *S. faecalis* states that wall elongation results from wall synthesis at the leading edges of the nascent cross wall, where it pushes out new wall which peels apart at the base of the cross wall to form peripheral wall (8; M. L. Higgins and G. D. Shockman, CRC Crit. Rev. Microbiol., *in press*). According to this model, the decrease in S relative to P in both E71 and E81 would be consistent with a more rapid peeling apart of new wall at the base of the cross wall, which in turn would inhibit centripetal cross wall penetration and stimulate peripheral wall elongation. This results in longer cells which require more wall surface to complete a cell division cycle. In both mutants, but more pronouncedly in E81, many more cells than in the wild-type culture were observed to have over 550 nm of new peripheral wall without having completed septation (Fig. 2B and 3).

DISCUSSION

The bulk of the free *N*-terminal groups in the cell walls is found on the D-isoasparagine which forms the cross bridge of the peptidoglycan of *S. faecalis* (5). Approximately 30% of this amino acid is un-cross-bridged in the walls of exponential phase cells of the parent organism (5). The greater content of *N*-terminal groups in the walls of the mutant E81 suggests a less cross-bridged peptidoglycan. This conclusion is supported by the finding that, as a substrate for the autolysin, the cell walls of E81 are approximately twice as susceptible as those of the wild type. In the ab-

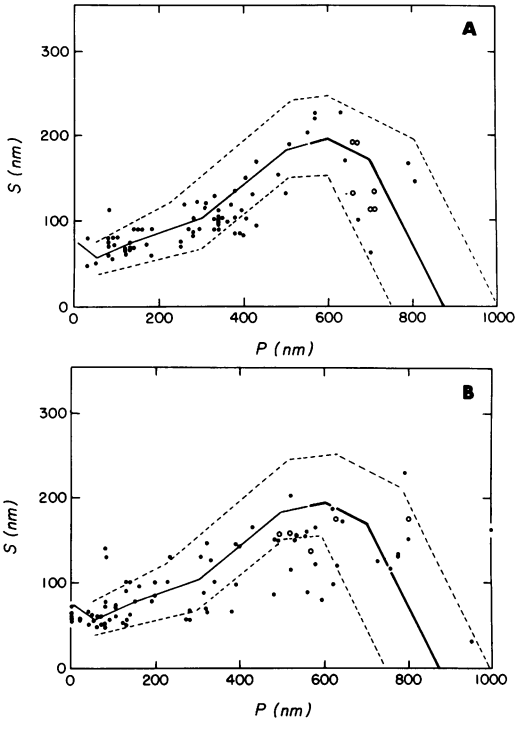


FIG. 3. *S (length of cross wall) to P (length of peripheral wall) measurements made from central longitudinal sections of mutant E71 (A) and mutant E81 (B) taken from exponentially growing cultures. In both parts of the figure, the mean and range of S and P measurements of exponential phase, wild-type cells are shown by the solid and dashed lines, respectively, and represent the analysis of the data from three separate experiments. The mean S and P line for the wild-type cell was established by dividing the P ordinate into classes (every 50 nm through 500 nm and every 100 nm after 500 nm) and calculating an average S value for each class. The break in the solid line followed by the heavier line indicates measurements made from cells that had completed cross walls. The experimental determinations are shown by solid circles and open circles, for cells that have incomplete and completed cross walls, respectively.*

FIG. 2. *Electron micrographs of sections of typical cells of mutants E71 and E81 taken from exponentially growing cultures. (A) A thin section of mutant E71. The bars along the wall illustrate the method used to measure the length of peripheral wall (P) and length of cross wall (S). (B) A thin section of mutant E81. (C) A freeze-fracture preparation of mutant E81, showing the typical chaining of the cells of this mutant. (D) Another freeze-fracture preparation of E81, illustrating the "stringy" material (shown by an arrow) that prevents cells from separating long after cell division has been completed. The bar in the lower right corner of each figure indicates 100 nm. The particulate material seen to be clinging to the external surface of the cell sections is a precipitate of tryptone which is added to cells during osmium tetroxide fixation.*

sence of any other differences in cell wall composition, it would be expected that a wall with a lower degree of cross-bridging should be more readily dissolved by enzymes attacking the glycan backbone. This view is supported by the finding that the peptidoglycan extracted from penicillin-treated cells of *S. aureus* are much more readily hydrolyzed than normal cell walls by the autolytic amidase of this organism (21). Such walls are presumably less extensively cross-bridged. The greater substrate susceptibility of the E81 walls to both the wild-type and E71 autolysins indicates that the level of autolysin in E81 is even lower than it appears to be from the rate of dissolution of E81 walls. The levels of active and latent autolysin in E81 walls appear to be lower than those of the wild type by a factor of approximately 6 and 3, respectively. This assumes that there is no difference in the properties of the autolysin of E81 from that of the wild type. Support for this view comes from the finding that enzyme from both wild type and E81 shows the same relative activity towards SDS E81 walls and SDS wild-type walls.

The mutant E71 appears to be partially affected at the activation step (converting latent to active autolysin) or in the transport of the autolysin to the cell wall, since E71 contains a considerably higher level of latent autolysin but less active autolysin than the wild type (the ratio of the two forms was almost four times higher in the mutant). If the activation occurs outside the permeability barrier as has been proposed (14), both of these possibilities are consistent with the suggestion that there exists a lesion affecting protein transport across the membrane. The apparently normal degree of cross-bridging reaction may, at least relatively, be independent of the level of active autolysin bound to the walls. The growth properties of the mutant E81, which contain the lowest levels of autolytic activity, are significantly altered. The growth rate of E81 is somewhat slower than the wild type; but a more notable change, perhaps, is the tendency of the organism to grow in long chains. This result supports the involvement of the autolysin in cell separation following cell division in agreement with previous suggestions (4, 15, 23; M. L. Higgins and G. D. Shockman, CRC Crit. Rev. Microbiol., *in press*). Mutant E81 also appears to possess a thicker cell wall, and it is possible that this may also reflect the reduced autolysin activity. If the autolysin plays a role in cell wall biosynthesis by cleaving the existing glycan backbone, a reduced rate of such cleavage may result in a greater proportion of the synthesis going into wall thickening at the expense of expansion of cell wall area.

Although it is possible that the phenotype of either or both mutants resulted from more than one mutational event, the simultaneous finding of an apparently less cross-bridged wall, together with a low level of active autolysin in E81, is of interest and has been taken to suggest that the cross-bridging reaction and the mechanism controlling the level of active autolysin may interact. It is one way in which the cell could integrate the biosynthetic and autolytic activities in the processes of cell wall synthesis and cell division. An alternative explanation comes from the suggestion that the cross-bridging reaction takes place outside the cell's permeability barrier (4). If the enzyme activities involved both in peptidoglycan cross-bridging and autolysis have their sites of action outside this barrier (since these two activities are both diminished in E81), a partial transport block provides a possible explanation for the properties of this organism. Thus a lesion which affects protein transport across the membrane in E81 could explain the finding that both activities appear to be diminished, although it would still not explain why there was a diminution in the level of the latent autolysin activity within the cell.

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