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Morphological and Physiological Study of Autolytic-Defective *Streptococcus faecium* Strains

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Three autolytic-defective mutants of *Streptococcus faecium* (*S. faecalis* ATCC 9790) were isolated. All three autolytic-defective mutants exhibited the following properties relative to the parental strain: (i) slower growth rates, especially in chemically defined medium; (ii) decreased rates of cellular autolysis and increased survival after exposure to antibiotics which block cell wall biosynthesis; (iii) decreased rates of cellular autolysis when treated with detergents, suspended in autolysis buffers, or grown in medium lacking essential cell wall precursors; (iv) a reduction in the total level of cellular autolytic enzyme (active plus latent forms of the enzyme); (v) an increased ratio of latent to active forms of autolysin; and (vi) increased levels of both cellular lipoteichoic acid and lipids.

A variety of bacterial species are known to contain enzymes, termed autolysins, which are capable of hydrolyzing their own cell wall peptidoglycan (17, 18). Possible roles for these potentially lethal enzymes in the physiology of bacteria have been the subject of numerous reports (9, 40). Although the autolytic systems characterized so far have not all yielded the same conclusions concerning their functional roles, among the processes implicated have been the following: (i) cellular division (33, 40); (ii) physical separation of daughter cells (2, 10, 30, 43, 48); (iii) cell wall biosynthesis and "remodeling" of the cell wall (9, 12, 41); (iv) peptidoglycan turnover (2, 25, 35); (v) killing by antibiotics that inhibit cell wall biosynthesis (32, 34, 49, 50); and (vi) genetic transformation (31, 48, 55).

Autolytic-defective mutants of various bacterial species have been useful in obtaining experimental evidence in favor of some of the above-mentioned roles of autolysins. The ability of bacteria to autolyze has thus been shown to be affected by the following: (i) changes in the wall substrate (1, 48, 51); (ii) levels of autolytic enzyme activities present (16, 30, 49); (iii) possible alterations in the autolytic enzyme itself (7, 11); and (iv) interactions with lipoteichoic acids, other amphophiles, and lipids (3-5, 20).

Several years ago, two lytic-defective mutants of *Streptococcus faecium* (*S. faecalis* ATCC 9790) were isolated in this laboratory (30). These mutants autolyzed at much slower rates than the wild-type (WT) strain when cells were suspended in 0.01 M sodium phosphate (pH 6.7), whereas they were less resistant to cellular autolysis in 0.3 M sodium phosphate (pH 6.7), a buffer concentration that usually permits maximum expression of cellular autolytic capacity (39). Although these mutants appeared to contain somewhat reduced levels of the active form of the autolytic activity of this species, total autolysin activity (active plus proteinase-activatable, latent activity) was at least as great as that of the WT. A third, more recently isolated autolytic-defective mutant (7) was also found to contain relatively high levels of latent autolysin activity. It was our intention in the present investigation to search for mutants with more pronounced defects in the autolytic system through the use of new screening procedures. In addition to the morphology and the overall growth and lysis characteristics being examined, these isolates were assayed for the cellular content of known inhibitors of the *S. faecium* autolytic enzyme system (3, 4).

MATERIALS AND METHODS

Bacterial strains and cultural conditions. The WT strain of *S. faecium* and the autolytic-defective strains AUT-1, AUT-2, AUT-3, and LYT-14 were stored in the lyophilized state and, when required, were cloned on solid medium before use. Two types of liquid and solid media were used: a chemically defined medium (37) and a broth containing 1% yeast extract, 1% tryptone, 2% glucose, and 0.3 M sodium phosphate, pH 6.5. Solid media contained 1.5% agar. Except where stated otherwise, 37°C was the incubation temperature used throughout this investigation. Commercially prepared lyophilized cells of *Micrococcus luteus* were obtained from Miles Research Laboratories, Inc., Kankakee, Ill.

Autolytic-defective mutants. Exponential-phase populations of *S. faecium* were treated with 100 μg of

N-methyl-*N'*-nitro-*N*-nitrosoguanidine per ml in buffer as described previously (7).

AUT-1 was selected from the survivors of repeated challenge with penicillin G (10 μg/ml) and D-cycloserine (360 μg/ml) (7), after growth on an indicator plate containing an overlay of ethylene-oxide-killed *M. luteus* cells. During growth on such indicator plates, the release of autolysin into the medium resulted in dissolution of the micrococcal cells and a clear halo around the autolysin-producing clones (7, 30). AUT-1 was picked for its failure to produce a halo on the indicator plate.

Enrichment for AUT-2 and AUT-3 was carried out as previously described for AUT-1, except that instead of being challenged with cell wall antibiotics, an exponentially growing culture of the mutagenized cell population was first metabolically poisoned with a reversible concentration of iodoacetate (5×10^{-5} M) (15) and then challenged with Triton X-100 (0.6 mg/mg [dry weight] of cells), a nonionic detergent which induces cellular lysis via the action of the autolytic enzyme system (8). The survivors of this enrichment technique were then plated on solid medium. AUT-2 was subsequently isolated after the transfer of individual clones onto agar plates overlaid with ethylene-oxide-killed *S. faecium* cells and selection for colonies which failed to form a halo on these indicator plates. AUT-3 was selected on the basis of leakage of the intracellular enzyme lactate dehydrogenase (54) from autolytically damaged cells as described below.

Detection of lytic-deficient mutants by the lactate dehydrogenase assay. The previously enriched cell population was diluted to a very low cell density (approximately 5 colony-forming units per ml), and portions of this cell suspension were filtered through sterile membrane filters (48-mm diameter, 0.45-μm pore size). The filters, with their contents, were aseptically transferred onto plates of solid growth medium and incubated overnight (18 to 24 h). Each filter containing approximately 20 colonies was transferred to indicator plates of the following composition: 0.1 M sodium lactate, 0.005% tetranitro blue, 0.0025% phenazine methosulfate, 0.3 M sodium phosphate (pH 7), and 1% agar. Neither lactate dehydrogenase nor NAD was added to the mixture, the rationale being that these two important components of the reaction leading to oxidation of lactate and the reduction of the tetrazolium salt to formazan (53) will be provided from those cellular clones undergoing autolysis. After incubation, those clones exhibiting little or no color reaction were presumed to be autolytic defective (e.g., AUT-3).

Cellular autolysis assays. Exponential-phase cultures at an adjusted optical density at 675 nm (47) between 400 and 800 were harvested by filtration, washed free of the medium with ice-cold distilled water, suspended in 0.3 M sodium phosphate (pH 7.0), and incubated at 37°C. Changes in turbidity of the cell suspensions were monitored with a Bausch & Lomb Spectronic 20 spectrophotometer at 675 nm, and rates of cellular autolysis were determined as described previously (29). The results of such assays for cellular autolysis were recorded as reciprocals of the half-life (in hours^{-1}) of the reaction at the steepest portion of the lysis curve.

Effects of detergents and wall antibiotics on growth. Cultures of the WT and the mutant strains in midexponential phase of growth in broth were treated with lysis-inducing detergents (Triton X-100, 0.6-mg/mg [cellular dry weight]; and sodium deoxycholate, 1.2-mg/mg [cellular dry weight]) or with specified levels of cell wall antibiotics (penicillin G, bacitracin, vancomycin, and cycloserine), and turbidimetric changes were followed for several hours. In a separate experiment, cultures treated with 5 μg of penicillin G per ml as described above were plated for viable cell counts.

Effects of deprivation of essential nutrients on growth. Cells of exponential-phase cultures (300 adjusted optical density units) in the chemically defined medium were harvested by filtration (0.45-μm-pore size membrane filters) and washed three times with ice-cold, chemically defined medium without lysine (39, 41) or without glucose (39). The cells were then suspended in medium lacking lysine or glucose, respectively, and incubated at 37°C, and changes in turbidity were measured at intervals.

Preparation of cell walls. Cultures in exponential growth were harvested and washed with ice-cold water in a refrigerated centrifuge. Aqueous suspensions of cells were broken by shaking with an equal volume of styrene divinylbenzene beads in a Braun MSK cell homogenizer, and the cell wall fraction was recovered by centrifugation (5). The sediment, consisting of cell wall-autolytic enzyme complexes, was washed twice in ice-cold double-distilled water, once in 0.15 M sodium phosphate (pH 7.5), and four times in ice-cold water before being lyophilized and stored at −70°C. Portions of the aqueous wall suspensions were treated overnight at room temperature with 2% sodium dodecyl sulfate (SDS) to inactivate endogenous autolytic activity and washed thoroughly with distilled water as described previously (42).

Preparation and assay of autolysin. Soluble autolysin was prepared as autolysates of cell walls (6) either in the presence or the absence of trypsin or the proteinase present in bovine plasma albumin (1 and 100 μg/ml, respectively). The lytic activities of the autolysates thus obtained were determined by their ability to dissolve the SDS-inactivated wall substrate in 0.01 M sodium phosphate, pH 7.0 (42). Turbidity changes were measured at 450 nm with a Bausch & Lomb Spectronic 20 equipped with an Arthur H. Thomas absorbance digital readout. One unit of enzyme activity was defined as that amount which reduces the turbidity of the SDS-inactivated wall suspension by 0.001 optical density unit per h at 37°C.

Analytical methods. For chemical analysis, lyophilized SDS-walls were dried to constant weight in vacuo over phosphorus pentoxide. The presence of free N-terminal groups and hexosamine were assayed by the method of Ghuysen et al. (18). Amino acids were determined with a Beckman single-column automatic amino acid analyzer (model 119), after hydrolyzing samples in 6 N HCl at 120°C for 22 h. Phosphorus was measured by the procedure of Lowry et al. (24); rhamnose was measured by the procedure of Dische and Shettles (44), and reducing groups were measured by the procedure of Thompson and Shockman (45).

Chemicals. Triton X-100 and sodium deoxycholate were obtained from Rohm & Haas, Philadelphia, Pa., and Mann Research Laboratories, New York, N.Y., respectively. D-Cycloserine and vancomycin were from Eli Lilly & Co., Indianapolis, Ind.; bacitracin was from Pfizer Inc., New York; and penicillin G was from Wyeth Laboratories Philadelphia, Pa. Phenazine methosulfate and tetrazolium salts were obtained from Sigma Chemical Co., St. Louis, Mo.

Determinations of the amounts of lipoteichoic acid and lipids present in cultures. Cultures were grown for at least six generations in chemically defined medium containing [2-^{14}C]glycerol (0.5 μCi/ml). Total trichloroacetic acid precipitable material was determined by mixing 0.5-ml samples with 4.5 ml of ice-cold 10% trichloroacetic acid. The mixtures were kept in an ice bath for 60 min before the precipitate was collected on glass fiber disks (Whatman GF/C, Whatman, Inc., Clifton Heights, N.J.) and washed twice with 3 ml of ice-cold 10% trichloroacetic acid and twice with 2 ml of ice-cold absolute ethanol. The trichloroacetic acid precipitates on the filters were dissolved in 0.5 ml of 90% NCS solubilizer (Amersham Corp., Arlington Heights, Ill.) for 2 h at 55°C in glass scintillation vials. The samples were first chilled (4°C) and then counted in 5 ml of cold toluene-based scintillation fluid in a Mark I (Nuclear-Chicago Corp., Des Plaines, Ill.) scintillation counter at an efficiency of about 60%. [^{14}C]glycerol incorporated into lipoteichoic acid (LTA), both in the intracellular and extracellular fractions, was determined by the procedures described previously (22, 23, 52). Aqueous samples were counted in Formula 947 (New England Nuclear Corp., Boston, Mass.).

The lipid content of glycerol-labeled cells was estimated by a modification of a method described earlier (46). Samples (7.5 ml) of [^{14}C]glycerol-labeled cells were placed in screw-cap tubes (18 by 150 mm) containing 0.1 ml of 1 M iodoacetate at 4°C. After mixing, cells were harvested by centrifugation (2,000 × g, 30 min, 4°C), and the pellets were lyophilized until used. Lipid extraction was carried out by suspending the pellets in 0.1 ml of water, followed by the addition of 1.0 ml of methanol, and subsequent heating (95°C, 60 min) in tubes tightly closed with Teflon-lined caps. After the tubes returned to room temperature, 2.0 ml of chloroform was added and the samples were stirred overnight. Each sample was then mixed thoroughly with 7 ml of 2 M KCl and 4 ml of neohexane (2,2-dimethylbutane; Phillips Petroleum Co., Bartlesville, Okla.), and the phases were separated by centrifugation (2,000 × g, 20°C, 15 min). Samples (1 ml) were removed from the top organic layer, dried in scintillation vials, dissolved in 0.5 ml of 90% NCS solubilizer, and counted in 5 ml of toluene-based scintillator as described above.

Electron microscopy. Cultures of *S. faecium* in exponential or stationary phase were harvested by filtration and washed free of the medium with ice-cold water. The cells were then suspended in Millonig buffer (26), double fixed with 2% glutaraldehyde, followed by 1% osmium tetroxide in s-collidine, and finally dehydrated through graded levels of acetone. Cells in 100% acetone were transferred onto aluminum foil-covered stumps, dried in a Sorvall critical point drying apparatus, and coated by sputtering with gold (SPI Sputter, West Chester, Pa.). The observation of the prepared specimens was performed with an Etec Autoscan scanning electron microscope (Hayward, Calif.).

RESULTS

Growth properties of the AUT-1, AUT-2, and AUT-3 strains. When grown on solid medium, colonies of the three strains differed from those of the WT only in their smaller size and whitish appearance. When the strains were grown in liquid media, the following differences from the WT were noted. (i) Two of the three mutant cultures grew more slowly than the WT in both growth media used, with the largest difference noted for AUT-3 grown in the chemically defined medium (Table 1). (ii) Although turbidity reached at 24 h by broth cultures of all three mutants was about equal to or slightly higher than that of the WT, all three mutants failed to reach the turbidity attained by the WT strain at 24 h in the chemically defined medium. The decrease in growth rates and turbidities at 24 h for cultures of the three mutants suggest that the growth of each of the strains is stimulated by a nutrient that is either not provided or not present in an adequate amount in the chemically defined medium.

Cultures of both AUT-2 and AUT-3 showed a marked tendency to grow in long chains of 10 to 25 cells per chain (AUT-2) and 10 to 50 cells per chain (AUT-3) (Fig. 1A). In undisturbed cultures, substantially longer chains were seen by light microscopy, and the cells had a tendency to settle to the bottom of the tube, leaving the upper portion of the medium clear (Fig. 2). Upon reaching the stationary phase, extensive clumps of cells of AUT-3 (Fig. 1B) that were not seen in cultures of the WT or AUT-1 could be observed with scanning electron microscopy. In contrast,

TABLE 1. *Growth of S. faecium strains in two different media*

Strain	Broth		Chemically defined	
	T_D[a] (min)	Culture turbidity[b] (AOD)	T_D (min)	Culture turbidity (AOD)
WT	33 ± 1	2,410	34 ± 1	2,074
AUT-1	35 ± 1	2,992	46 ± 1	1,666
AUT-2	50 ± 2	2,930	65 ± 3	950
AUT-3	65 ± 3	2,834	71 ± 3	1,138

[a] These values on doubling times (T_D) represent the average of five independent determinations.

[b] Measurements of culture turbidity at 675 nm after 24 h were taken with a Coleman model 14 spectrophotometer. The absorbance was corrected for deviations from Beer's Law (47), yielding adjusted optical density units (AOD).

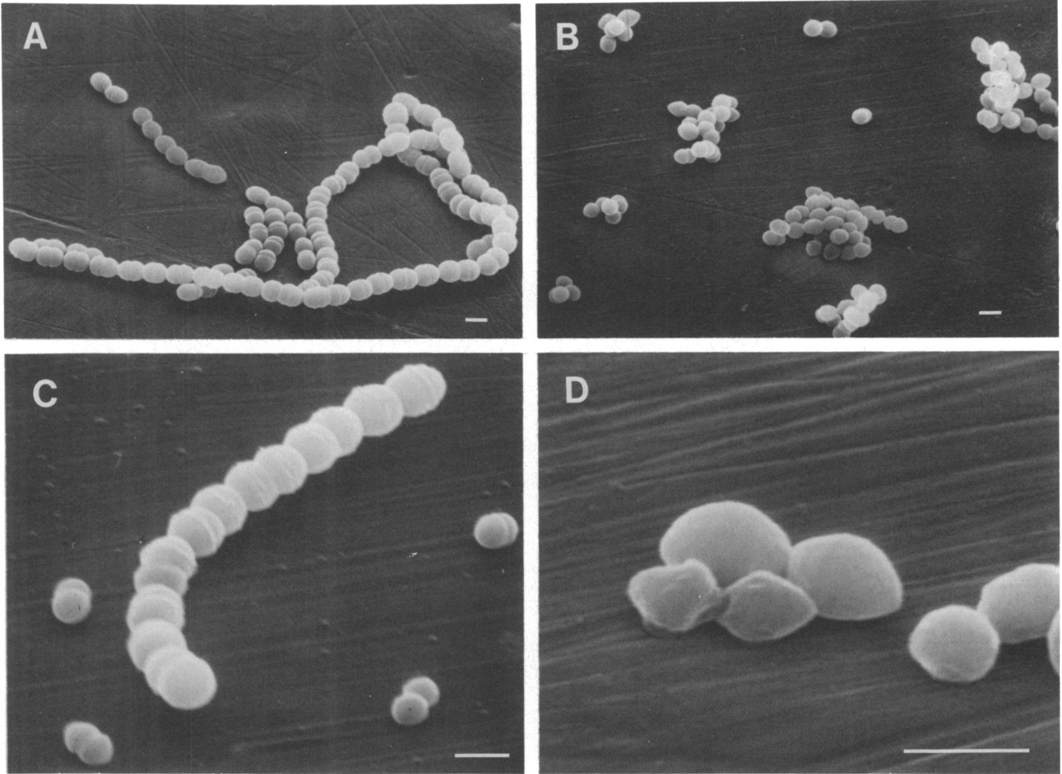

FIG. 1. *Scanning electron micrographs of WT and mutant strains of S. faecium showing: (A) a long chain of cells from an exponential-phase culture of AUT-3; (B) clumping of AUT-3 cells in the stationary phase; (C) the relative sizes of the parental strain (in pairs) and AUT-3 (chain); (D) the relative sizes of the WT cells (larger) and AUT-1 cells (smaller). Each bar represents 1 μm.*

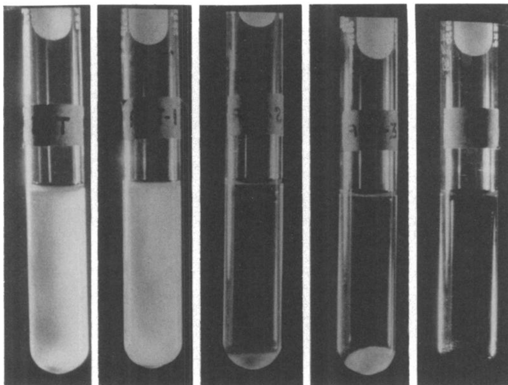

FIG. 2. *Growth of the four strains of S. faecium plus the uninoculated control (C) in broth showing the clumping and sedimentation phenomenon in AUT-2 and AUT-3. The cultures were left undisturbed during incubation (18 h). From left to right: WT, AUT-1, AUT-2, AUT-3, uninoculated control.*

cultures of AUT-1 grew mainly as individual cells and pairs of cells; only rarely were chains of six or more cells of AUT-1 observed. In addition to their growth in chains, individual cells of AUT-3 had about twice the diameter of WT cells (Fig. 1C). In contrast, cells of AUT-1 had about one-half the diameter of WT cells (Fig. 1D).

Capacity of cells to autolyze. All three mutant strains were isolated on the basis of their inability to autolyze when grown on solid media. The mutant strains also showed a decreased capacity to autolyze when cells from exponentially growing liquid cultures were washed and suspended in 0.3 M sodium phosphate, pH 7.0, in either the presence or the absence of trypsin (Table 2). Expression of the active form of the autolysin (in the absence of trypsin) in cellular autolysis assays ranged between 2.4 and 10% of that of the WT, whereas expression of total cellular autolytic activity ranged from 1.3 to 53% of that of the WT. The AUT-3 strain failed to show a significant increase in cellular autolytic capacity in the presence of trypsin.

The decreased capacity of cells from exponential-phase cultures of the three mutants to autolyze was not due to a different optimal ionic

strength for expression of autolytic capacity. All three strains autolyzed much more slowly than did the WT in sodium phosphate concentrations of up to 0.8 M, pH 7.0 (Fig. 3). However, none of the mutants exhibited the dual optimal phosphate buffer concentrations (at 0.05 to 0.01 and 0.3 M) for cellular autolysis characteristic of the WT (39) (Fig. 3). AUT-1 exhibited an optimal rate of cellular autolysis at 0.3 M, whereas the rates of cellular autolysis for the other two mutants continued to increase slightly with increased buffer concentration. The greatest rate of turbidity decrease occurred between pH 6.7 and 7.0 for the WT and for all three mutant strains (data not shown). Furthermore, the rates of autolysis for exponential-phase cells of all three mutants were substantially less than those for the WT when examined in 0.05 M Tris, pH 7.0, and 0.05 M sodium cacodylate, pH 7.0 (Table 3).

TABLE 2. *Cellular autolysis of exponential-phase cells of S. faecium strains in 0.3 M sodium phosphate, pH 7.0.*

Strain	With trypsin (3 μg/ml)		Without trypsin	
	Rate (h^{-1})	% of WT rate	Rate (h^{-1})	% of WT rate
WT	5.5	(100)	3.4	(100)
AUT-1	2.9	53	0.17	5.0
AUT-2	1.3	24	0.33	9.7
AUT-3	0.07	1.3	0.08	2.4

For all three mutants, the capacity of exponentially growing cultures to autolyze upon the addition of detergents or antibiotics that inhibit cell wall peptidoglycan synthesis or upon deprivation of precursors of cell wall peptidoglycan from the growth medium was substantially less than that of the WT. Except in two cases, bacitracin treatment and autolysis in the absence of lysine or glucose, AUT-3 was most resistant to cellular lysis (Tables 2 and 3). AUT-1 was somewhat more resistant than AUT-3 to bacitracin-induced lysis and to lysis induced by lysine or glucose deprivation. Rather than autolysis, a slow increase in culture turbidity was observed

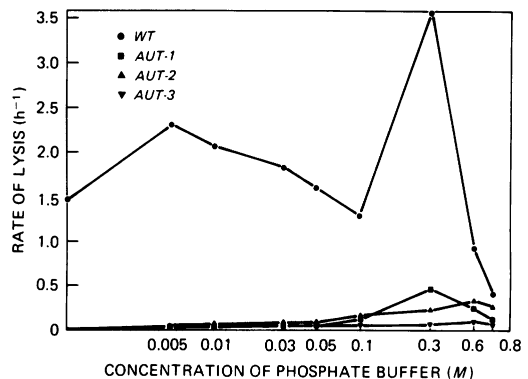

FIG. 3. *Effect of varying the concentrations of the phosphate buffer (pH 7.0) on cellular autolysis of the various S. faecium strains without activation by trypsin.*

TABLE 3. *Cellular lysis profiles of strains of S. faecium under a variety of conditions*

Treatment	WT (rate of lysis [h^{-1}])	AUT-1		AUT-2		AUT-3	
		Rate (h^{-1})	% of WT	Rate (h^{-1})	% of WT	Rate (h^{-1})	% of WT
Lysis buffer (without trypsin)							
Tris (0.05 M, pH 7)	2.4	0.06	2.5	0.05	2.1	0.01	0.4
Cacodylate (0.05 M, pH 7)	2.0	0.09	4.5	0.03	1.5	0.01	0.5
Detergents (added to growth medium)							
Triton X-100 (0.6 mg/mg [dry weight] of cells)	4.3	2.53	58.8	0.93	21.6	—[a]	
Sodium deoxycholate (1.2 mg/mg)	5.7	1.33	23.3	1.29	22.6	0.39	6.8
Antibiotics							
Cycloserine (90 μg/ml)	3.3	1.00	30.3	1.46	44.2	0.73	22.1
Vancomycin (2.5 μg/ml)	2.7	0.16	5.9	0.36	13.3	0.16	5.9
Bacitracin (2.5 μg/ml)	1.5	—[a]		0.08	5.3	0.01	0.7
Penicillin G (20 μg/ml)	4.0	1.46	36.5	1.12	28.0	0.49	12.2
Depletion of essential nutrients							
Lysine	0.13	0.02	15.4	0.04	30.8	0.03	23.1
Glucose	0.62	0.04	6.4	0.06	9.7	0.06	9.7

[a] —, Slow increase in turbidity rather than lysis was observed.

when Triton X-100 was added to exponentially growing cultures of AUT-3.

Effects of penicillin. The minimal inhibitory concentrations and minimal bactericidal concentrations (MICs and MBCs, respectively) of benzylpenicillin for the three strains were not significantly different from those for the WT (Table 4). It should be noted, however, that the MBCs for all three mutants were slightly higher than the corresponding MICs. Examination of the effect of penicillin on the viability of growing cultures (Fig. 4A) of the AUT-1 and AUT-2 strains showed that the number of colony-forming units decreased more slowly than for the WT strain. This effect was particularly pronounced for the AUT-2 strain which, after exposure to 5 μg of penicillin G per ml for 2 h, retained 44% of the initial number of CFU compared with 1.6% for the WT. However, the conclusion that AUT-2 is more resistant to penicillin-induced killing than is the WT should be viewed with some caution since, as discussed above, AUT-2 tends to grow in chains and a single viable cell per chain is capable of producing a colony. The even more extensive chaining of the AUT-3 strain precluded interpretable determinations of killing rates. The slow rate of killing of the mutant strains in the presence of 5 μg of penicillin G per ml was accompanied by a very slow loss of

TABLE 4. *MICs and MBCs of penicillin G on S. faecium strains*[a]

Strain	20-h incubation MIC (μg/ml)	20-h incubation MBC (μg/ml)	44-h incubation MIC (μg/ml)	44-h incubation MBC (μg/ml)
WT	4	5	5	6
AUT-1	8	15	8	15
AUT-2	6	15	8	15
AUT-3	1	5	1	5

[a] Tubes containing serial twofold dilutions (geometric progression) of the freshly prepared antibiotic in broth (5 ml) were inoculated with cells from a midexponential-phase culture (approximately 10^7 colony-forming units per ml) and incubated overnight. After the initial MIC determination (last completely clear tube in the series), the experiment was repeated using a narrower range of concentrations. To determine MBCs, the medium in the clear tubes of the MIC assay was streaked onto solid media. The MBC was the lowest concentration of the antibiotic that killed the organism, as determined by the absence of colony formation.

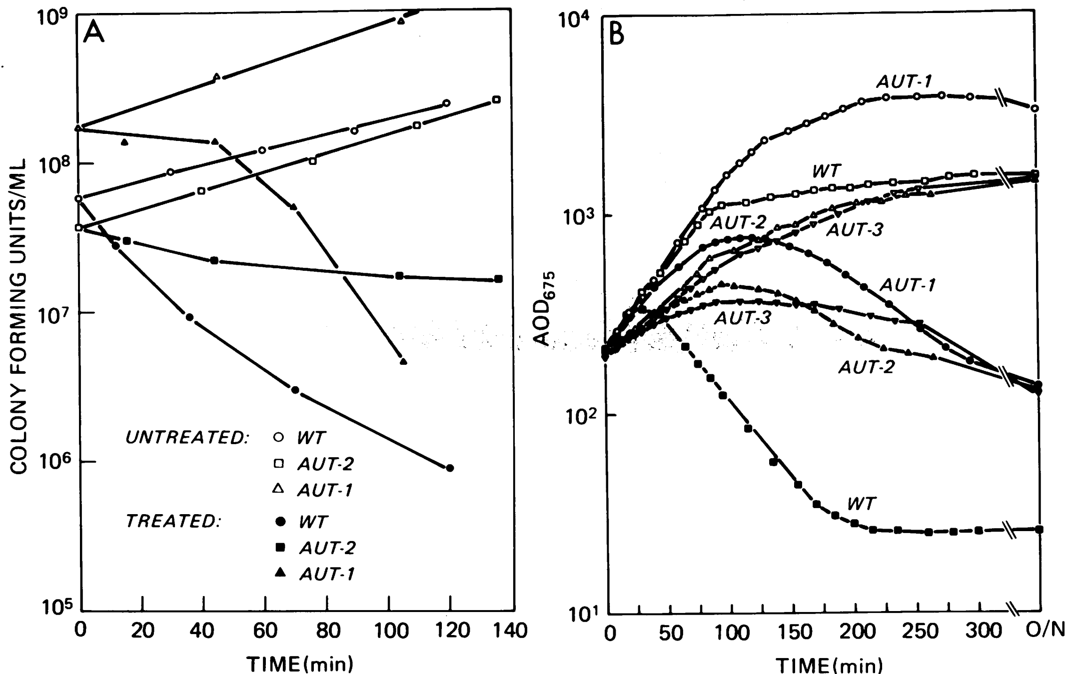

FIG. 4. *(A) Bactericidal effect of penicillin G on S. faecium strains. Cells growing exponentially in broth were treated with penicillin G (5 μg/ml) at time zero. Samples of treated and untreated cultures were withdrawn at given intervals, appropriately diluted in sterile water, and plated for viable cell counts. (B) Effect of penicillin G on the growth of S. faecium. Portions of broth cultures of S. faecium strains in midexponential growth (open symbols) were treated with penicillin G (5 μg/ml, closed symbols), and changes in adjusted optical density at 675 nm (AOD_{675}) over a period of time were monitored.*

culture turbidity for all three mutant strains (Fig. 4B).

Autolysin activity present in isolated cell walls. The autolysin of *S. faecium* has a very high affinity for binding to cell walls (21, 28). Thus, the autolysin activity present in carefully isolated cell wall-enzyme complexes after cell disruption can serve as an index of the effective level of autolysin activity present in intact cells (38). Walls isolated from exponential-phase cultures of all three mutants autolyzed more slowly than did walls from the parent strain when assayed in either the presence or the absence of trypsin (Table 5). Total autolysin activity (measured in the presence of trypsin) in walls from all three strains was 53 to 65% of the WT level, and the active form (measured in the absence of trypsin) was 20 to 37% of the WT value. These differences did not seem to be due to changes in the cell wall substrate. In fact, SDS-inactivated walls from all three mutants were somewhat more susceptible to dissolution by autolysin obtained from the WT and two of the mutants (Table 6). Furthermore, the chemical compositions of SDS-inactivated walls from all three mutant strains were very similar to that of the WT (Table 7). Dissolution of all four SDS-wall preparations via the action of WT autolysin yielded the same number of reducing groups (Table 7). Yields of reducing groups were consistent with the hydrolysis of 75 to 92% of the susceptible glycosidic linkages present in the walls (42, 45).

TABLE 5. *Autolytic activity (total, active, and latent) in isolated cell wall preparations of WT and mutants*[a]

Strain	Activity	U	% of WT	Active (% of total)
WT	Total[b]	432	—	
	Active[c]	95	—	22
	Latent[d]	337	—	
AUT-1	Total[b]	282	65	
	Active[c]	35	37	12
	Latent[d]	247	73	
AUT-2	Total[b]	231	53	
	Active[c]	16	17	7
	Latent[d]	215	64	
AUT-3	Total[b]	228	53	
	Active[c]	30	32	13
	Latent[d]	198	59	

[a] Wall lysis was carried out in 0.01 M sodium phosphate, pH 7.0.

[b] Assayed in the presence of trypsin (1 μg/ml).

[c] Assayed in the absence of trypsin.

[d] Determined as the difference between *b* and *d*.

TABLE 6. *Susceptibility of SDS-walls to autolysins from the WT and mutants*[a]

Autolysin from walls of:	Relative activity on SDS-walls from: WT	AUT-1	AUT-2	AUT-3
WT	(1.0)	1.4	1.3	1.6
AUT-1	(1.0)	1.4	1.3	1.6
AUT-2	(1.0)	0.9	0.7	0.8
AUT-3	(1.0)	1.3	1.4	1.7

[a] Wall hydrolysis was carried out with the respective autolysin preparations in 0.01 M sodium phosphate, pH 7.0, at 37°C in the presence of 0.2 μg of trypsin per ml. For comparison, the amount of autolysin from walls of each strain was adjusted to 112 to 118 U, when tested on SDS-treated, WT walls.

Relative amounts of LTA and lipids in the mutant strains. LTA and certain lipids have been shown to inhibit the activity of the autolytic enzyme system of *S. faecium* (3, 4). Therefore, the amounts of [^{14}C]glycerol incorporated into chloroform-soluble lipids and into intracellular LTA and extracellular LTA were determined (Table 8). Since labeled glycerol is known to be incorporated exclusively into the LTA and lipid fractions of this species (14, 27) and fully equilibrated labeling conditions were used, the data obtained indicate that all three mutants isolated in this study and a previously isolated autolytic-defective mutant (7) contained increased amounts of lipid and intracellular LTA. In addition, except for AUT-2, the mutant cultures contained increased levels of extracellular LTA. In each case, all of the extracellular LTA was in the deacylated (22) form (data not shown).

DISCUSSION

Newly devised enrichment and selection procedures permitted the successful isolation of three additional (independent) mutants of *S. faecium* containing defects in their autolytic enzyme system. Although AUT-1 was isolated on the basis of its resistance to cellular autolysis induced by the combined action of penicillin and cycloserine, AUT-2 was isolated on the basis of its resistance to Triton X-100-induced cellular autolysis, and AUT-3 was isolated on the basis of both its resistance to Triton X-100 and its lack of leakage of lactate dehydrogenase from colonies, cells of all three mutants had several phenotypic properties in common. Cells from exponential-phase cultures of all three mutants autolyzed more slowly than did the parental

TABLE 7. *Chemical composition of SDS-walls from the WT and mutants*

Component[a]	WT (μmol/mg)	AUT-1 (μmol/mg)	AUT-2 (μmol/mg)	AUT-3 (μmol/mg)
I. Total hexosamines	0.80	0.91	0.78	0.81
Phosphorus	0.40	0.37	0.33	0.36
Rhamnose	0.91	0.88	0.73	0.85
II. Glutamate	0.42 (0.88)[b]	0.47 (0.84)	0.43 (0.86)	0.46 (0.87)
Aspartate	0.46 (0.96)	0.53 (0.95)	0.57 (1.14)	0.50 (0.94)
Alanine	0.87 (1.8)	1.04 (1.8)	0.89 (1.8)	0.91 (1.7)
Lysine	0.48 (1.0)	0.56 (1.0)	0.50 (1.0)	0.53 (1.0)
III. Free N-terminal groups	0.18	0.14	0.18	0.16
Reducing groups after autolytic hydrolysis	0.44	0.44	0.37	0.45

[a] Hydrolysis conditions were as follows: (I) 3.0 N HCl, 95°C, 4 h; (II) 6.0 N HCl, 120°C, 22 h; (III) SDS-treated walls were hydrolyzed overnight at 37°C with the WT autolysin in 0.01 M sodium phosphate (pH 7.0), with 0.2 μg of trypsin added per ml. The autolysates were then centrifuged at 10,000 × *g* (20 min), and the supernatants were used for analyses.

[b] Numbers within parentheses refer to molar ratios relative to lysine.

TABLE 8. *Levels of [^{14}C]glycerol-labeled compounds in the WT and mutant strains*[a]

Strain	Lipids (dpm/μg [cellular dry weight])	Cellular LTA (dpm/μg [cellular dry weight])	Extracellular LTA (dpm/μg [cellular dry weight])
WT	162 (1)[b]	145 (1)	11 (1)
AUT-1	307 (1.9)	254 (1.8)	40 (3.6)
AUT-2	239 (1.5)	195 (1.3)	11 (1)
AUT-3	275 (1.7)	214 (1.5)	23 (2.1)
LYT-14	317 (2.0)	452 (3.1)	59 (5.4)

[a] Cultures were grown in medium containing 0.5 μCi of [2-^{14}C]glycerol per ml for six generations to late exponential phase.

[b] Numbers within parentheses are relative to the WT value.

strain: (i) in each of a series of different lysis buffers (Tables 2 and 3), (ii) over a wide range of sodium phosphate concentrations (Fig. 3), (iii) after the addition of Triton X-100 or sodium deoxycholate, (iv) after the addition of each of four antibiotics known to inhibit cell wall peptidoglycan synthesis, and (v) upon lysine or glucose deprivation.

However, the three mutant strains showed qualitative and quantitative differences. Although all three grew somewhat more slowly than the parental strain (Table 1), in broth AUT-1 grew at nearly the same rate as the parent. Cells of AUT-2 and AUT-3, but not of AUT-1, showed distinct tendencies to settle rapidly in liquid cultures (Fig. 2), to grow in long chains and clumps (Fig. 1A and B), and to form individual cells that were about twice the diameter of the WT cells (Fig. 1C). In contrast, AUT-1 cells were much smaller than the WT (Fig. 1D). The three strains also differed in the ability of trypsin to increase the rate of cellular autolysis in 0.3 M sodium phosphate, pH 7.0 (Table 2). For example, upon trypsin addition the low rate of cellular autolysis of AUT-1 in phosphate buffer was substantially increased, whereas the rate of autolysis of AUT-3 remained at about the same slow rate. Although these data suggest that AUT-3 had very low levels of both the active and the latent forms of the autolysin, examination of the enzyme activity present in isolated walls showed this not to be the case (Table 5). Walls of AUT-3 contained significant levels of latent activity that could be activated by trypsin or the proteinase present in commercial preparations of bovine plasma albumin but that was not expressed upon autolysis of whole cells in buffers containing proteinase (Table 2). Thus, AUT-3 differs from the parental strain and the other mutants described here and elsewhere (7) in the inability of externally added proteinase to activate latent autolysin activity of AUT-3.

Consistent with the hypothesis of Rogers (32), autolysin activity appears to be closely related to the killing action of penicillin on *S. faecium*. Both AUT-1 and AUT-2 are more resistant than the WT to cell death (Fig. 4A and Table 4) and cellular lysis (Fig. 4B) in the presence of 5 μg of penicillin G per ml. Growth of AUT-3 in long chains and clumps precluded similar experiments with this strain. Qualitatively similar resistance to lysis induced by three other antibiotic inhibitors of cell wall assembly (Table 3) is consistent with a role for autolysins in the killing action of such antibiotics and with the induction of "tolerance to killing" (49) by defects in bacterial autolytic systems. It should also be noted that MIC levels of the mutants were not significantly higher than that of the WT (Table 4 and data not shown) and that tolerance (measured

as resistance to antibiotic-induced lysis) was relative and not absolute (Table 3), varied with the antibiotic treatment used (Table 3), and was dependent on antibiotic concentration (data not shown). Although other mechanisms of tolerance could exist, variations in rates of antibiotic-induced cellular lysis with drug and drug concentration could explain the lack of cross-tolerance between vancomycin, nafcillin, and cephalothin observed in some strains of *Staphylococcus aureus* (36).

For the WT strain, Shockman et al. (39) observed that when lysine, a major component of the cell wall peptidoglycan, or glucose, a presumptive precursor of cell wall polysaccharides and an energy source, was depleted, wall synthesis stopped and the prevailing degradative action of the autolysin resulted in cell lysis. All three autolytic-defective mutants isolated in the course of this investigation were markedly more resistant than the WT to lysis induced by the deprivation of either of these two cell wall precursors (Table 3). This series of observations lends strong support to the role of autolysins in the lethal action of antibiotics which prevent cell wall synthesis and to the view that during bacterial growth degradative processes are normally counterbalanced by synthetic processes.

The mutation(s) involved does not greatly affect the chemical composition (Table 7) or the substrate properties of the walls (Table 6). Overall, the wall composition of the four strains was very similar, and hydrolysis of the walls via the WT autolysin yielded very similar values for released free N-terminal and reducing groups. The released reducing group values were consistent with the hydrolysis of essentially all the susceptible bonds in the wall (42, 45), and the number of free N-terminal groups was consistent, with the same extent of peptide cross-linking in the walls of all four strains. SDS-inactivated walls of all three mutants were more rapidly hydrolyzed than walls of the WT, suggesting that the mutant walls were in some way better substrates for the enzymes from all the strains tested. However, the relative activities of all three autolytic enzymes tested were the same on each substrate (Table 6), suggesting that the mutations involved were not in the structural gene(s) for the enzyme.

Evidence that the mutation(s) did not seem to be in the enzyme protein itself and the observation of substantial, although reduced, levels of autolysin activity in isolated walls led to a series of experiments concerning factors known to regulate autolysin activity. LTAs and certain lipids are known to inhibit autolysin activity (3–5, 20). Therefore, levels of LTA and lipids were measured in these three mutants and in a previously isolated (7) lytic-defective mutant, LYT-14. All four mutant strains were found to incorporate larger amounts of [^{14}C]glycerol into their cellular lipids and LTA fractions (Table 8). In addition, three of the isolates (AUT-1, AUT-3, and LYT-14) excreted substantially larger amounts of [^{14}C]glycerol-labeled deacylated LTA into the culture medium than did the parental strain (Table 8). We have interpreted these findings as suggesting that the increased synthesis of lipid and/or LTA is, at least in part, responsible for the observed lytic-defective phenotypes of AUT-1 and LYT-14. This interpretation is consistent with the very low rates of cellular autolysis of the three mutants in comparison with the WT (Tables 2 and 3) and the presence of 20 to 37% of the WT level of the active form of the autolysin in isolated and well-washed walls of the three mutants (Table 5).

Unfortunately, the dearth of genetic techniques for this bacterium prevents a closer definition of the defect(s) present in these mutants. It is not possible to distinguish between pleiotropic effects resulting from a single mutation and multiple genetic lesions. Also, indirect effects could easily result in the observed phenotypes. For example, growth rate is known to affect the cellular autolytic capacity of this species (19), and two of the mutants, AUT-2 and AUT-3, grow at significantly slower rates than the WT. Also, slower growth rates and lower turbidity values after overnight incubation in the chemically defined medium suggest additional growth requirements which may or may not be related to the mutation(s) responsible for the autolytic defect. However, these mutants will be useful for studies of the action and regulation of autolysin activity. The nature and the significance of the observed increased levels of LTA and lipids in these autolytic-defective mutants of *S. faecium* are currently being investigated more closely.

It is of some interest to note that in this and several other attempts (7, 30), we have not been successful in isolating a truly lytic-negative mutant, even using techniques that should have yielded conditional (temperature-sensitive) mutations (Shungu and Cornett, unpublished data). Thus, we prefer to call these "AUT" mutants to avoid possible mis- or overinterpretations of the "LYT" or "LYT^{-}" designations. A similar lack of success in isolating lytic-negative (as contrasted to lytic-defective) mutants of bacilli (1, 13, 16) and *S. aureus* (2) is consistent with, but certainly does not prove, the idea that such an activity is essential to bacterial cell growth and division.

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LITERATURE CITED

1. **Brown, W. C., C. R. Wilson, S. Lukehart, F. E. Young, and M. A. Shiflett.** 1976. Analysis of autolysins in temperature-sensitive morphological mutants of *Bacillus subtilis*. J. Bacteriol. **125**:166–173.
2. **Chatterjee, A. N., W. Wong, F. E. Young, and R. W. Gilpin.** 1976. Isolation and characterization of a mutant of *Staphylococcus aureus* deficient in autolytic activity. J. Bacteriol. **125**:961–967.
3. **Cleveland, R. F., L. Daneo-Moore, A. J. Wicken, and G. D. Shockman.** 1976. Effect of lipoteichoic acid and lipids on lysis of intact cells of *Streptococcus faecalis*. J. Bacteriol. **127**:1582–1584.
4. **Cleveland, R. F., J.-V. Holtje, A. J. Wicken, A. Tomasz, L. Daneo-Moore, and G. D. Shockman.** 1975. Inhibition of bacterial wall lysins by lipoteichoic acids and related compounds. Biochem. Biophys. Res. Commun. **67**:1128–1135.
5. **Cleveland, R. F., A. J. Wicken, L. Daneo-Moore, and G. D. Shockman.** 1976. Inhibition of wall autolysis in *Streptococcus faecalis* by lipoteichoic acid and lipids. J. Bacteriol. **126**:192–197.
6. **Conover, M. J., J. S. Thompson, and G. D. Shockman.** 1966. Autolytic enzyme of *Streptococcus faecalis*: release of soluble enzyme from cell walls. Biochem. Biophys. Res. Commun. **23**:713–719.
7. **Cornett, J. B., B. E. Redman, and G. D. Shockman.** 1978. Autolytic defective mutant of *Streptococcus faecalis*. J. Bacteriol. **133**:631–640.
8. **Cornett, J. B., and G. D. Shockman.** 1978. Cellular lysis of *Streptococcus faecalis* induced with Triton X-100. J. Bacteriol. **135**:153–160.
9. **Daneo-Moore, L., and G. D. Shockman.** 1977. The bacterial cell surface in growth and division, p. 597–715. *In* G. Poste and G. L. Nicholson (ed.), Cell surface reviews, vol. 4. Elsevier/North Holland Publishing Co., Amsterdam.
10. **Fan, D. P.** 1970. Autolysin(s) of *Bacillus subtilis* as dechaining enzyme. J. Bacteriol. **103**:494–499.
11. **Fan, D. P., and M. M. Beckman.** 1973. Mutant of *Bacillus subtilis* with a temperature-sensitive autolytic amidase. J. Bacteriol. **114**:798–803.
12. **Fan, D. P., M. M. Beckman, and W. P. Cunningham.** 1972. Ultrastructural studies on a mutant of *Bacillus subtilis* whose growth is inhibited due to insufficient autolysin production. J. Bacteriol. **109**:1247–1257.
13. **Fein, J. E., and H. J. Rogers.** 1976. Autolytic enzyme-deficient mutants of *Bacillus subtilis* 168. J. Bacteriol. **127**:1427–1442.
14. **Fine, D. H., R. E. Kessler, L. A. Tabak, and G. D. Shockman.** 1978. Limulus lysate activity of lipoteichoic acids. J. Dent. Res. **56**:1500.
15. **Fitzgerald, R. J., and H. U. Jordan.** 1953. The *in vitro* effects of antibiotics and other inhibitory agents on representative oral *Lactobacilli*. Antibiot. Chemother. (Washington, D.C.) **3**:231–240.
16. **Forsberg, C. W., and H. J. Rogers.** 1974. Characterization of *Bacillus licheniformis* 6346 mutants which have altered lytic enzyme activities. J. Bacteriol. **118**: 358–368.
17. **Ghuysen, J.-M.** 1968. Use of bacteriolytic enzymes in determination of wall structures and their role in cell metabolism. Bacteriol. Rev. **32**:425–464.
18. **Ghuysen, J.-M., J. Tipper, and J. L. Strominger.** 1966. Enzymes that degrade bacterial cell walls. Methods Enzymol. **8**:685–699.
19. **Hinks, R. P., L. Daneo-Moore, and G. D. Shockman.** 1978. Cellular autolytic activity in synchronized populations of *Streptococcus faecium*. J. Bacteriol. **133**:822–829.
20. **Holtje, J.-V., and A. Tomasz.** 1975. Lipoteichoic acid: a specific inhibitor of autolysin activity in pneumococcus. Proc. Natl. Acad. Sci. U.S.A. **72**:1690–1694.
21. **Joseph, R., and G. D. Shockman.** 1976. Autolytic formation of protoplasts (autoplasts) of *Streptococcus faecalis*: location of active and latent autolysin. J. Bacteriol. **127**:1482–1493.
22. **Kessler, R. E., and G. D. Shockman.** 1979. Precursor-product relationship of intracellular and extracellular lipoteichoic acids of *Streptococcus faecium*. J. Bacteriol. **137**:869–877.
23. **Knox, K. W., and A. J. Wicken.** 1973. Immunological properties of teichoic acids. Bacteriol. Rev. **37**:215–257.
24. **Lowry, O. H., N. R. Roberts, K. Y. Leiner, M. L. Wu, and A. L. Farr.** 1954. The quantitative histochemistry of brain. I. Chemical methods. J. Biol. Chem. **207**:1–17.
25. **Mauck, J., L. Chan, and L. Glaser.** 1971. Turnover of the cell wall of Gram-positive bacteria. J. Biol. Chem. **246**:1820–1827.
26. **Millonig, G.** 1961. Advantages of phosphate buffer for OsO_4 solutions in fixation. J. Appl. Phys. **32**:1637.
27. **Pieringer, R. A., and R. T. Ambron.** 1973. A method for the specific labeling of the glycerol in glyceride containing lipids of *Streptococcus faecalis* ATCC 9790. J. Lipid Res. **14**:370–372.
28. **Pooley, H. M., J. Porres-Juan, and G. D. Shockman.** 1970. Dissolution of an autolytic enzyme-cell wall complex by treatment with unusually high concentrations of salt. Biochem. Biophys. Res. Commun. **38**:1134–1140.
29. **Pooley, H. M., and G. D. Shockman.** 1969. Relationship between the latent form and the active form of the autolytic enzyme of *Streptococcus faecalis*. J. Bacteriol. **100**:617–624.
30. **Pooley, H. M., G. D. Shockman, M. L. Higgins, and J. Porres-Juan.** 1972. Some properties of two autolytic-defective mutants of *Streptococcus faecalis* ATCC 9790. J. Bacteriol. **109**:423–431.
31. **Ranhand, J. M., C. G. Leonard, and R. M. Cole.** 1971. Autolytic activity associated with competent group H streptococci. J. Bacteriol. **106**:257–268.
32. **Rogers, H. J.** 1967. Killing of staphylococci by penicillins. Nature (London) **213**:31–33.
33. **Rogers, H. J.** 1970. Bacterial growth and the cell envelope. Bacteriol. Rev. **34**:194–214.
34. **Rogers, H. J., and C. W. Forsberg.** 1971. Role of autolysins in the killing of bacteria by some bactericidal antibiotics. J. Bacteriol. **108**:1235–1243.
35. **Rogers, H. J., H. M. Pooley, P. F. Thurman, and C. Taylor.** 1974. Wall and membrane growth in bacilli and their mutants. Ann. Microbiol. (Paris) **125B**:135–147.
36. **Sabath, L. D., N. Wheeler, M. Laverdiere, D. Blazevic, and B. J. Wilkinson.** 1977. A new type of penicillin resistance of *Staphylococcus aureus*. Lancet **i**:443–447.
37. **Shockman, G. D.** 1963. Amino acids, p. 567–673. *In* F. Kavanagh (ed.), Analytical microbiology. Academic Press Inc., New York.
38. **Shockman, G. D., and M. C. Cheney.** 1969. Autolytic enzyme system of *Streptococcus faecalis*. V. Nature of the autolysin-cell wall complex and its relationship to properties of the autolytic enzyme of *Streptococcus faecalis*. J. Bacteriol. **98**:1199–1207.
39. **Shockman, G. D., M. J. Conover, J. J. Kolb, P. M. Phillips, L. S. Riley, and G. Toennies.** 1961. Lysis of *Streptococcus faecalis*. J. Bacteriol. **81**:36–43.
40. **Shockman, G. D., L. Daneo-Moore, and M. L. Higgins.** 1974. Problems of cell wall and membrane growth, enlargement and division. Ann. N.Y. Acad. Sci. **235**:

161-197.

41. **Shockman, G. D., J. J. Kolb, and G. Toennies.** 1958. Relations between cell wall synthesis, growth phase and autolysis. J. Biol. Chem. **230**:961-977.
42. **Shockman, G. D., J. S. Thompson, and M. J. Conover.** 1967. The autolytic enzyme of *Streptococcus faecalis*. II. Partial characterization of the autolysin and its substrate. Biochemistry **6**:1054-1065.
43. **Soper, J. W., and C. G. Winter.** 1973. Role of cell wall autolysin in chain formation by a mutant strain of *Streptococcus faecalis*. Biochim. Biophys. Acta **297**: 333-342.
44. **Spiro, R. G.** 1966. Analysis of sugars found in glycoprotein. Methods Enzymol. **8**:3-26.
45. **Thompson, J. S., and G. D. Shockman.** 1968. A modification of the Park and Johnson reducing sugar determination suitable for the assay of insoluble materials: its application to bacterial cell walls. Anal. Biochem. **22**:260-268.
46. **Toennies, G., D. N. Das, and F. Feng.** 1968. New observations on the determination of bacterial lipid phosphorus. Can. J. Microbiol. **14**:484-485.
47. **Toennies, G., and D. L. Gallant.** 1949. The relation between photometric turbidity and bacterial concentration. Growth **13**:7-20.
48. **Tomasz, A.** 1968. Biological consequences of the replacement of choline by ethanolamine in the cell wall of pneumococcus: chain formation, loss of transformability and loss of autolysin. Proc. Natl. Acad. Sci. U.S.A. **59**: 86-93.
49. **Tomasz, A., A. Albino, and E. Zanati.** 1970. Multiple antibiotic resistance in a bacterium with suppressed autolytic system. Nature (London) **227**:138-140.
50. **Tomasz, A., and S. Waks.** 1975. Mechanism of action of penicillin: triggering of the pneumococcal autolytic enzyme by inhibitors of cell wall synthesis. Proc. Natl. Acad. Sci. U.S.A. **72**:4162-4166.
51. **Tomasz, A., and M. Westphal.** 1971. Abnormal autolytic enzyme in a pneumococcus with altered teichoic acid composition. Proc. Natl. Acad. Sci. U.S.A. **68**:2627-2630.
52. **Wicken, A. J., and K. W. Knox.** 1970. Studies on the group F antigen of lactobacilli: isolation of a teichoic acid-lipid complex from *Lactobacillus fermenti*. J. Gen. Microbiol. **60**:293-301.
53. **Wilkinson, J. H.** 1970. Isoenzymes, p. 40-57. J. B. Lippincott Co., Philadelphia.
54. **Wittenberger, C. L., and N. Angelo.** 1970. Purification and properties of a fructose-1,6-diphosphate-activated lactate dehydrogenase from *Streptococcus faecalis*. J. Bacteriol. **101**:717-724.
55. **Young, F. E., and D. L. Strominger.** 1964. Autolysis of cell wall of *Bacillus subtilis*: mechanism and possible relationship to competence. J. Biol. Chem. **239**:3600-3602.