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Autolytic Defective Mutant of *Streptococcus faecalis*

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Properties of a variant of *Streptococcus faecalis* ATCC 9790 with defective cellular autolysis are described. The mutant strain was selected as a survivor from a mutagenized cell population simultaneously challenged with two antibiotics which inhibit cell wall biosynthesis, penicillin G and cycloserine. Compared to the parental strain, the mutant strain exhibited: (i) a thermosensitive pattern of cellular autolysis; (ii) an autolytic enzyme activity that had only a slightly increased thermolability when tested in solution in the absence of wall substrate; and (iii) an isolated autolysin that had hydrolytic activity on isolated *S. faecalis* wall substrate indistinguishable from that of the parental strain, but that was inactive when tested on walls of *Micrococcus lysodeikticus* as a substrate. These data indicate an alteration in the substrate specificity of the autolytic enzyme of the mutant which appears to result from the synthesis of an altered form of autolytic enzyme.

Many bacterial species can undergo cellular autolysis when appropriately challenged, apparently due to the action of autolytic peptidoglycan hydrolases (autolysins) on their cell wall peptidoglycans (13, 14). Although potentially dangerous, autolysins are thought to participate in cellular functions related to growth and division (26, 32) such as: (i) turnover of bacterial walls (4, 20, 28, 41); (ii) enlargement of the cell surface (10); (iii) the lethal responses to antibiotics which interfere with wall biosynthesis (2, 25, 27, 39; but see reference 4); (iv) development of a genetically competent state (1, 24, 29, 42); and (v) physical separation of daughter cells (4, 7, 11, 34, 38). Autolytically defective mutants of several gram-positive species such as *Bacillus subtilis, Bacillus licheniformis* (2, 3, 8-12), *Staphylococcus aureus* (4, 15), and streptococci (23, 34, 39) have been described. All of the autolytically defective mutants described thus far seem to possess reduced, but easily detectable, levels of autolytic activity. In only one case (9), the amidase of *B. subtilis* βA0, has evidence been presented suggesting a mutational alteration in the autolytic enzyme itself. Activity of a partially purified preparation of amidase from the mutant was more rapidly inactivated by heat than was a similar preparation from the parental strain.

The autolytic enzyme system of *Streptococcus faecalis* ATCC 9790 (*S. faecium*; SF) appears to be simpler than those found in other bacteria, since a substantial body of evidence indicates the presence of only a single enzyme specificity, an endo-*N*-acetylmuramoylhydrolase (muramidase) activity (33). Muramidase activity can be quantitatively recovered in the cell wall fraction after cell disruption (17), from which it can be obtained in a soluble form by autolysis of wall-enzyme complexes (33) or by extraction with very high salt concentrations (21). This autolysin appears to be synthesized in a latent zymogenic form, which is then transported to appropriate sites on the wall, where it is activated (22). Any of a variety of proteinases can activate latent autolysin in vitro (31, 33).

We report here some properties of an autolytic defective mutant (LYT-14). Compared with the wild type, cells of this strain have a greatly decreased ability to autolyze, especially at elevated temperatures, contain approximately the same level of total autolytic enzyme activity as the wild type, but contain only a very low level of the active form of the enzyme. In addition, soluble preparations of the muramidase of LYT-14, unlike the wild-type enzyme, failed to dissolve walls of *Micrococcus luteus* (ML).

MATERIALS AND METHODS

Bacterial strains and growth conditions. SF was grown either in a chemically defined medium, FCM (30), or in a complex broth (S broth) consisting of (per liter of double-distilled water): yeast extract (Difco), 10 g; tryptone (Difco), 10 g; glucose, 20 g; 0.3 M sodium phosphate, pH 6.5. For solid medium, agar (Difco) (1.5%) was added. Lyophilized ML cells were

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purchased from Miles Research Laboratories, Inc., Kankakee, Ill.

Mutagenesis. Exponentially growing cultures of SF (FCM, 37°C) were filtered (Millipore Corp., Bedford, Mass.; 0.45-μm pore size), washed, resuspended in 0.05 M tris(hydroxymethyl)aminomethane-maleate, pH 6, containing 0.001 M $MgSO_4$ and 0.5% glucose, and exposed to 100 μg of freshly prepared *N*-methyl-*N'*-nitrosoguanidine (NTG) per ml for 30 to 40 min at 37°C. Mutagenized cells were sedimented, suspended in the same buffer, and exposed to 48°C for 30 min (in an effort to increase the potency of the mutagenic treatment [36]), harvested, resuspended in broth, and incubated at 33°C for three to five generations to allow segregation of mutations. This mutagenic treatment was effective, since greater than 90% of the mutagenized population that grew out as colonies at 33°C failed to produce colonies at 43°C.

Enrichment for autolytic deficient mutants. The procedure used was based on the idea that inhibitors of wall biosynthesis kill via the secondary action of autolytic enzymes (27, 39) and that some of the survivors will possess defects in this system.

Mutagenized populations (50 ml) were incubated at 33°C in FCM to a turbidity (exponential phase) equivalent to 2×10^8 cells/ml, rapidly shifted to 43°C, and challenged with both penicillin G (10 μg/ml) and D-cycloserine (360 μg/ml). Deoxyribonuclease (5 μg/ml) and $MgSO_4$ (0.003 M) were added to reduce the viscosity produced by lysed cells. Surviving cells were sedimented, washed with prewarmed medium, returned to fresh growth medium without antibiotics, and grown at 33°C. This procedure was repeated for seven cycles.

Detection of autolytic defective clones. Surviving cells were plated on solid medium from which individual clones were transferred to duplicate plates that had been previously overlaid with agar growth medium containing ethylene oxide-killed ML or SF cells (2 mg/ml). Zones of dissolution (clear halos) were seen on ML or SF plates around colonies of the parental strain or after spotting crude soluble SF autolysin or another endo-*N*-acetylmuramoylhydrolase, hen egg white lysozyme. Clear zones were not produced in response to spotting proteinases (Pronase and trypsin) at concentrations less than 100 μg/ml. Lack of a halo surrounding a clone after growth at 43°C and a halo around the corresponding clone on the 33°C replica plate was taken as an indication of a possible thermosensitive autolytic defective strain.

Measurement of cellular autolysis. Overnight cultures, inoculated from a single appropriate clone, were diluted 200-fold into fresh medium and incubated at the desired growth temperature. When exponentially growing cultures reached a density of 2×10^8 to 5×10^8 cells/ml, the cells were harvested by filtration, washed three times with ice-cold double-distilled water, resuspended in 0.3 M sodium phosphate, pH 7.0, and incubated at the appropriate temperature. Rates of cellular autolysis were calculated from the linear portion of the curve describing the exponential decrease in turbidity (450 nm; Bausch & Lomb Spectronic 20) with time (22). Rates of cellular autolysis are expressed in units of reciprocal hours describing the time in which the culture decreased in optical density by one-half. Thus, a cell population which loses half of its optical density in 30 min yields a rate of 2.0 h^{-1}.

Preparation of walls. Walls were prepared from exponential-phase cultures of SF and from ML after cell disruption in a Ribi cell fractionator (Sorvall, Norwalk, Conn.) or with plastic beads in a Braun MSK homogenizer as previously described (5).

Preparation of soluble muramidase. Soluble SF autolysin was prepared from autolysates of wall, in the presence or absence of a proteinase activator as described previously (33). Either trypsin (0.1 μg/ml) or the undefined proteinase (40) present in commercial bovine plasma albumin (BPA; 100 μg/ml) was used to activate latent autolysin. These wall autolysates contain soluble wall fragments in addition to the muramidase.

Soluble muramidase was also extracted from walls by the additon of NaOH (0.01 N) to aqueous wall suspension (20 mg/ml) containing bovine serum albumin (100 μg/ml) at 0°C, immediately followed by centrifugation (130,000 × *g*, 10 min), removal of the supernatant, and immediate neutralization (with HCl and 0.01 M sodium phosphate, pH 7) as described by Coyette and Shockman (6). In such preparations, 95% or more of the muramidase activity present was in the latent form and required proteinase treatment for expression.

Preparation of SDS-inactivated wall substrate (SDS-walls). Walls of SF or ML (or alkali-extracted SF walls) were treated with 2% sodium dodecyl sulfate (SDS) and extensively washed as described previously (33).

Assay for muramidase activity. Activity was measured by the rate of dissolution of SDS-walls in 0.01 M sodium phosphate, pH 7.0, at 37°C. One unit of activity was defined as that causing a loss of 0.001 optical density unit per h at 450 nm (Gilford-300 spectrophotometer). Latent autolysin was activated by either trypsin (0.5 to 1.0 μg/ml) or BPA (50 to 100 μg/ml). Activation with BPA did not result in loss of enzyme activity, which sometimes occurred with trypsin during assays using micrococcal wall substrate.

Binding of autolysin to SDS-walls. Alkali-extracted autolysin (50 to 100 μl) in 0.01 M sodium phosphate, pH 7.0, was added to SDS-walls (0.3 mg/ml) in the same buffer in small prewarmed (37°C) tubes (1.0 ml, final volume). In all cases, wall substrate was in excess (17). Conditions were designed so that after various periods of incubation, all tubes were simultaneously rapidly chilled to 0°C and immediately centrifuged in the cold (12,000 × *g*, 10 min). Both activity bound to the SDS-walls (after resuspending the sedimented SDS-wall–enzyme complexes in 1.5 ml of buffer) and unbound activity remaining in the supernatants (by addition of 0.5 ml of the SDS-wall suspensions) were measured as described above. Recovery of activity relative to controls (enzyme plus walls, not centrifuged) varied from 70 to 120%.

Chemical analyses of SDS-walls. Chemical analyses were carried out on SDS-walls that were dried to constant weight over P_2O_5 in vacuo. Hexosamines and free amino groups were measured by the procedures of Ghuysen et al. (14), with glucosamine and aspartic acid as standards, respectively; rhamnose by

the method of Dische and Shettles (35), with rhamnose as the standard; phosphorus by the procedure of Lowry et al. (19), with KH_2PO_4 as the standard; and reducing groups by the procedure of Thompson and Shockman (37), with glucose as the standard. Amino acid analyses were performed with a Beckman single-column automatic amino acid analyzer model 119.

Chemicals and reagents. Triton X-100 was purchased from Rohm & Haas, Philadelphia, Pa.; penicillin G and cycloserine were gifts from Wyeth Laboratories and Lilly Laboratories, respectively; trypsin (2× crystallized) was purchased from Grand Island Biological Co., Grand Island, N.Y.; bovine albumin fraction V powder was purchased from Pentex, Inc., Kankakee, Ill.; and BPA was purchased from Armour Pharmaceutical Co., Kankakee, Ill. (lot B70411).

RESULTS

Isolation of LYT-14. A population of *S. faecalis* ATCC 9790 was treated with NTG and enriched for autolytic defective mutants as described in Materials and Methods. During the enrichment procedure, the cell population became progressively more resistant to cellular autolysis at 43°C. After seven consecutive cycles of enrichment, the rate of autolysis at 43°C had decreased from a normal rate of 6.0 h^{-1} to 0.21 h^{-1}. This population was plated for single colonies, which were screened for autolysin production on indicator plates as described in Materials and Methods.

Of the various autolytic defective clones detected in this population by the indicator plate assay, one variant (LYT-14) which failed to form a halo on the indicator plates at 43°C was selected for further study.

Growth characteristics of LYT-14. When cultured in S broth at 43°C, LYT-14 was not thermosensitive for growth. In fact, the mutant strain grew faster at 43°C than the parental strain. In representative measurements, the doubling times (T_D) for the mutant strain in S broth at 30, 37, and 43°C were 52, 34, and 36 min, respectively, compared with T_D values of 46, 33, and 43 min at the corresponding temperatures for the parental strain, respectively.

Cellular autolysis of LYT-14. In comparison to the parental strain, exponential-phase cells (37°C) of LYT-14 showed a markedly decreased capacity to autolyze in 0.3 M sodium phosphate, pH 7, at 33°C (Fig. 1B). In addition, rather than the comparatively higher rate of autolysis at 43°C observed with the parental type, LYT-14 showed substantial resistance to autolysis at 43°C (Fig. 1B). This pattern of cellular autolysis was exhibited by LYT-14 when tested under other lysis conditions. For example, exponential-phase cells of LYT-14 autolyzed more slowly than the parental strain in 0.04 M ammonium acetate, pH 7.0 (not shown), or upon the addition of 40 μg of penicillin G per ml to growing cultures. Regardless of whether cellular autolysis was induced in lysis buffers or with penicillin G (Fig. 2), increases in the rate of lysis with increasing incubation temperatures were not observed, indicating a thermosensitive phenotype.

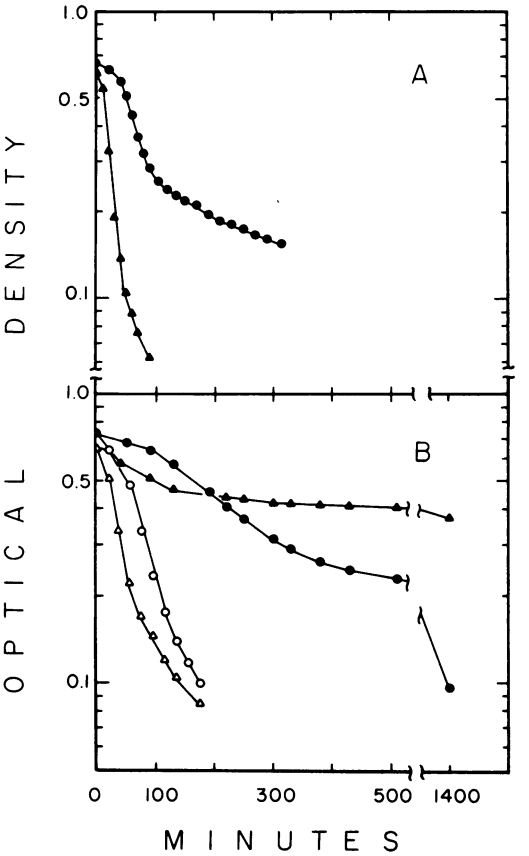


FIG. 1. *Cellular autolysis of SF LYT-14. Exponentially growing cultures (S broth, 37°C) were harvested by filtration, washed, and resuspended in 0.3 M sodium phosphate, pH 7, as described in Materials and Methods. (A) Autolysis of LYT-14 in the presence of trypsin (0.5 μg/ml, added at time zero) at 33 (●) or 43°C (▲). (B) Autolysis of LYT-14 (solid symbols) and the parental strain (open symbols) in the absence of trypsin at 33 (circles) and 43°C (triangles).*

In contrast to the results described above, the addition of trypsin (an activator of latent autolytic enzyme) consistently resulted in rates of cellular autolysis of LYT-14 similar to those observed with the parental strain and a temperature response not consistent with thermosensitivity. For example, in the presence of trypsin, the capacity of exponential-phase cells of LYT-

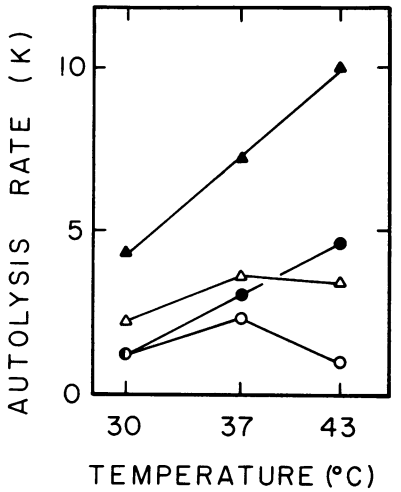


FIG. 2. *Effect of increased temperature of incubation on the rates of cellular autolysis of exponential-phase cells of LYT-14 in lysis buffer and induced in exponentially growing cultures by penicillin G. Exponentially growing cultures of LYT-14 (S broth, 37°C) received penicillin G (40 μg/ml) (triangles) or were transferred to 0.3 M sodium phosphate, pH 7.0, lysis buffer (circles), and the rates (K, h^{-1}) of cellular autolysis were determined as described in Materials and Methods. Assays were performed both in the absence (open symbols) and presence (closed symbols) of trypsin (0.4 μg/ml).*

14 to autolyze in 0.3 M sodium phosphate buffer, pH 7.0 (Fig. 1A and 2), or upon the addition of penicillin G directly to the exponentially growing cultures (Fig. 2) failed to show the thermosensitive phenotype. In fact, in the presence of trypsin, lysis patterns of LYT-14 were very similar to those of the parental strain (not shown) when tested at different incubation temperatures.

The phenomena observed were related to the temperature at which cellular autolytic capacity was measured rather than temperature of growth. Comparisons of LYT-14 and the parental strain for cellular autolysis at 33 and 43°C after growth at 33, 37, or 43°C consistently showed a decreased autolytic capacity and a thermosensitive pattern of lysis for LYT-14 in the absence of a proteinase activator (not shown). These results were taken to suggest that LYT-14 possessed a decreased level of the active form of the autolysin and that the naturally active (in contrast to trypsin-activated) form of the autolytic enzyme in the cells was thermolabile.

Determination of autolysin content in isolated cell wall preparations. The unusually strong affinity of the autolysin for binding to walls (17, 21) was used to compare the autolytic enzyme content of LYT-14 with that of the parent. Wall-autolytic enzyme complexes were isolated from both strains, and the rates of autolysis in the presence and absence of trypsin were compared (Fig. 3). In the presence of trypsin, very similar rates of wall hydrolysis were observed, indicating equivalent amounts of total autolytic activity in both the mutant and parental wall-enzyme complexes. However, assays for the active form in the absence of trypsin showed the presence of very low levels of the active form of the enzyme in LYT-14 and indicated that only about 2% of the total autolytic activity of LYT-14 walls was in the active form, compared with 20 to 25% in parental walls. Since LYT-14 enzyme also has a high affinity for binding to walls (see below), these results can be interpreted as indicating that LYT-14 contained at least as much total autolysin as the parent, but that much less of this autolysin was in the active form.

Nature and composition of LYT-14 walls. Both LYT-14 and parental autolysin hydrolyzed SDS-walls from either strain with equal efficiency (not shown). In fact, with either substrate, increasing amounts of LYT-14 autolysin yielded proportionally increasing rates of wall hydrolysis up to at least 400 U of enzyme activity (not

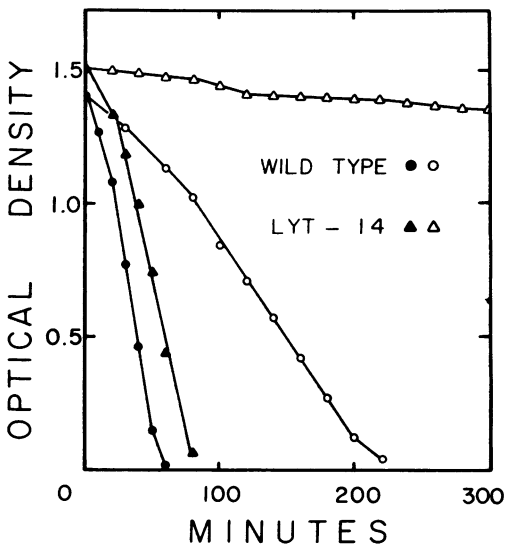


FIG. 3. *Autolysis of SF wild-type and LYT-14 cell wall-enzyme complexes. Cell wall-enzyme complexes were incubated at 37°C in 0.01 M sodium phosphate, pH 7, containing 100 μg of bovine serum albumin per ml in the presence (closed symbols) or absence (open symbols) of trypsin (0.1 μg/ml) added at zero min. Soybean trypsin inhibitor (Worthington) was added to 0.5 μg/ml 40 min after the addition of trypsin. Optical density was monitored at 675 nm with a Bausch & Lomb Spectronic 20.*

shown). Thus, by this biological criterion both types of walls were equivalent. Also, there were no significant differences in the chemical composition of SDS-walls of LYT-14 and parental strain (Table 1). Furthermore, when SDS-walls of the parental and LYT-14 strains were hydrolyzed by soluble autolysin extracted from either strain, the products released to the supernatant after hydrolysis were quantitatively indistinguishable (Table 2).

Activity of LYT-14 autolysin on walls of ML. The autolysin of the parental strain hydrolyzes ML walls at about one-half the rate of SF walls, in a nonlinear manner (33). The inability of clones of LYT-14 to produce halos on ML indicator plates suggested that this strain had lost autolytic activity directed against ML. Comparisons of the activity of soluble enzyme (Fig. 4) on SF and ML SDS-walls showed that, indeed, the LYT-14 autolysin had lost nearly all of its ability to hydrolyze ML walls. In most preparations of LYT-14 autolysin, activity against ML walls (ML activity) was 1 to 3% of the activity against SF walls (SF activity). To test this point further, the activities of alkali-extracted (and wall lysate) LYT-14 and wild-type autolysins were titrated on ML SDS-walls (Fig. 5). With parental autolysin, as expected, the rate of hydrolysis of ML walls increased in proportion to the amount of enzyme added. In contrast, increased amounts of LYT-14 autolysin failed to increase the rate of hydrolysis on ML walls. Thus, in these assays employing high levels of autolysin, the ML activity of the LYT-14 enzyme was actually less than 0.5% of the SF activity. This very low but constant level of ML

TABLE 1. *Composition of SF wild type and LYT-14 SDS-walls*

Component[a]	Wild type		LYT-14	
	μmol/mg	Molar ratio	μmol/mg	Molar ratio
I. Rhamnose	1.09		1.02	
Phosphorus	0.40		0.46	
Hexosamines	0.92		0.89	
II. Glutamic acid	0.39	(1.0)	0.37	(1.0)
Aspartic acid	0.38	0.99	0.32	0.87
Alanine	0.64	1.7	0.62	1.7
Lysine	0.36	0.92	0.34	0.91
III. Reducing groups	0.027		0.021	

[a] Hydrolysis conditions: (I) 3.0 N HCl, 95°C, 4 h; (II) 6.0 N HCl, 120°C, 22 h; (III) no hydrolysis. Determinations were carried out by the procedures described in Materials and Methods.

TABLE 2. *Products of SDS-treated wild type and SDS-treated LYT-14 walls after digestion by autolysin*[a]

Product	Amt of product (μmol solubilized/mg of starting material)			
	Wild type[b]		LYT-14	
	Wild type[c]	LYT-14	Wild type	LYT-14
Reducing groups	0.51	0.55	0.46	0.50
Free amino groups (FDNB)	0.18	0.18	0.15	0.16
Hexosamines	0.20	0.21	0.18	0.20
Rhamnose	1.36	1.31	1.33	1.31

[a] SDS-treated walls were incubated with the indicated alkali-extracted (latent) autolytic enzyme in 0.01 M sodium phosphate, pH 7, to yield the following final concentrations: wild-type and LYT-14 SDS-walls, 1.64 mg/ml; wild-type autolysin, 120 U; LYT-14 autolysin, 200 U; trypsin, 20 μg/ml. Autolysin-wall mixtures were incubated at 37°C for 16 h. The undigested material was sedimented at 12,000 × *g* for 20 min, and the supernatants were analyzed by the procedures listed in Materials and Methods.

[b] Wall type.

[c] Source of autolysin.

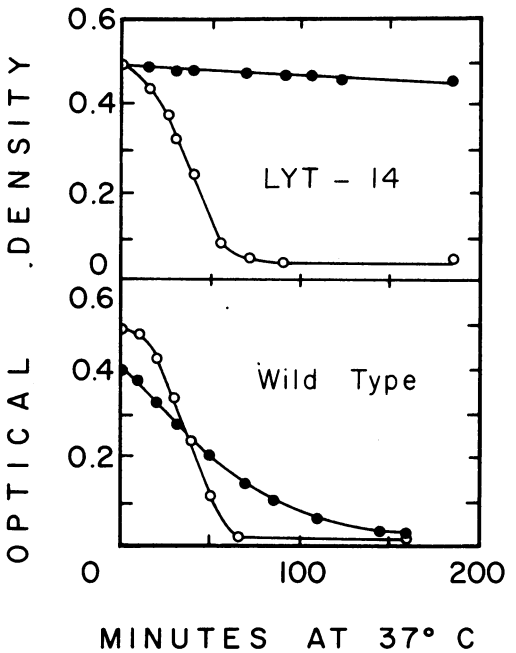


FIG. 4. *Dissolution of SF and ML wall substrates by SF wild-type and LYT-14 autolysin. Equal volumes of soluble (alkali-extracted) autolysin of LYT-14 (upper panel, 570 U) or the wild-type (lower panel, 720 U) strain were added to suspensions of SDS-treated ML (●) or SF (○) walls in 0.01 M sodium phosphate, pH 7, at 37°C containing 100 μg of BPA per ml.*

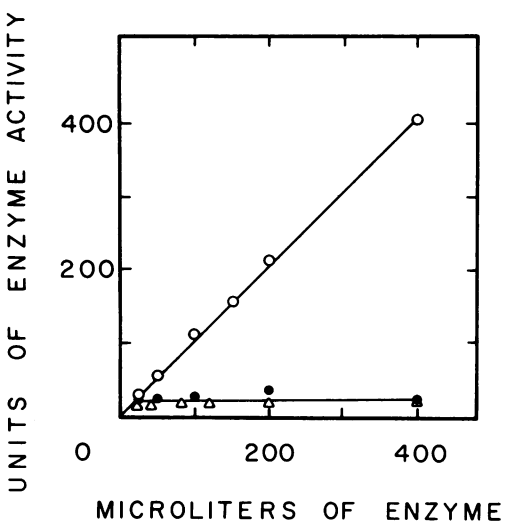


FIG. 5. *Hydrolytic activity with increasing amounts of wild-type and LYT-14 autolysin with ML wall substrate. Increasing volumes of alkali-extracted wild-type (○, 5,000 U/ml) or LYT-14 (△, 12,200 U/ml; or ●, 20,000 U/ml) autolysin were added to ML walls in a final volume of 1.50 ml. All assays were carried out at 37° C in 0.01 M sodium phosphate, pH 7, containing 100 μg of BPA per ml. The units of autolysin activity were determined as described in Materials and Methods, using SF wall substrate.*

activity was significantly above the essentially zero rate of dissolution of ML walls in the absence of added enzyme. The nature of this slow rate of dissolution by ML walls by both alkali-extracted and wall lysate LYT-14 autolysin, which failed to increase with enzyme concentration, is not known.

Binding of autolysin to SDS-walls. Both wild-type and LYT-14 autolysins bound rapidly and efficiently to SF SDS-walls (Fig. 6). The rate and extent of binding of LYT-14 autolysin to SF walls were indistinguishable from those of the parental autolysin. Neither LYT-14 nor wild-type autolysin bound very efficiently to the ML wall substrate. In fact, a smaller fraction of wild-type autolysin was bound to ML walls than of the LYT-14 autolysin. Since binding of two different levels of mutant autolysin activity (420 and 840 U) resulted in the same fractions bound in each case (Fig. 7A), binding of only about 50% of LYT-14 autolysin to ML walls did not seem to be due to substrate limitation.

Thermosensitivity of LYT-14 autolysin. Both the cellular autolysis phenotype of LYT-14 and the difference in substrate specificity of LYT-14 autolysin suggested that the mutant enzyme might be less thermostable than the parental enzyme. However, wall-hydrolytic activity of LYT-14 autolysin bound to SF SDS-walls had the same temperature profile as parental autolysin bound to the same walls (Fig. 7A). In addition, exposure of soluble enzyme preparations to a series of elevated temperatures for 5 min, followed by assays at 37°C (Fig. 7B), showed that soluble LYT-14 autolysin was only slightly less stable to brief exposure to elevated temperatures. Similarly, exposure of soluble enzyme preparations for increasing time intervals at 50°C (Fig. 7C) also showed a somewhat greater thermolability of LYT-14 autolysin activity relative to the parental-type autolysin. The slight thermolability observed with the LYT-14 enzyme under these conditions was not altered by substituting LYT-14 SDS-walls for parental walls as the substrate for measuring the autolytic activity (Fig. 7C). The same degree of thermosensitivity seen for the alkali-extracted

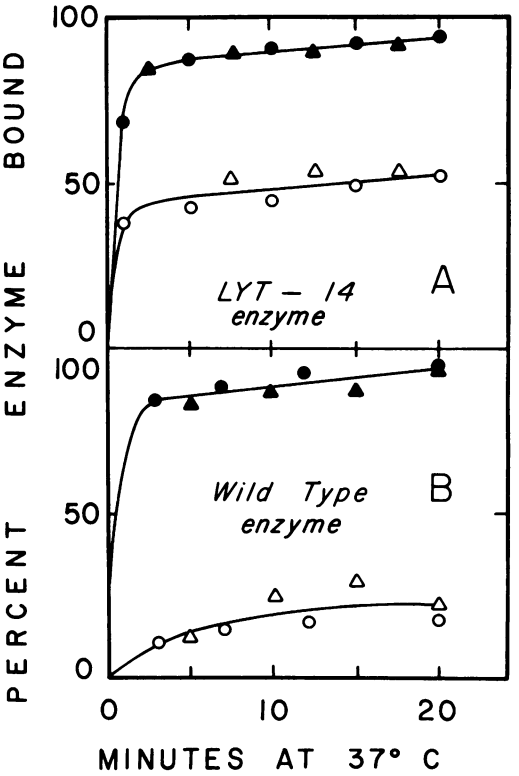
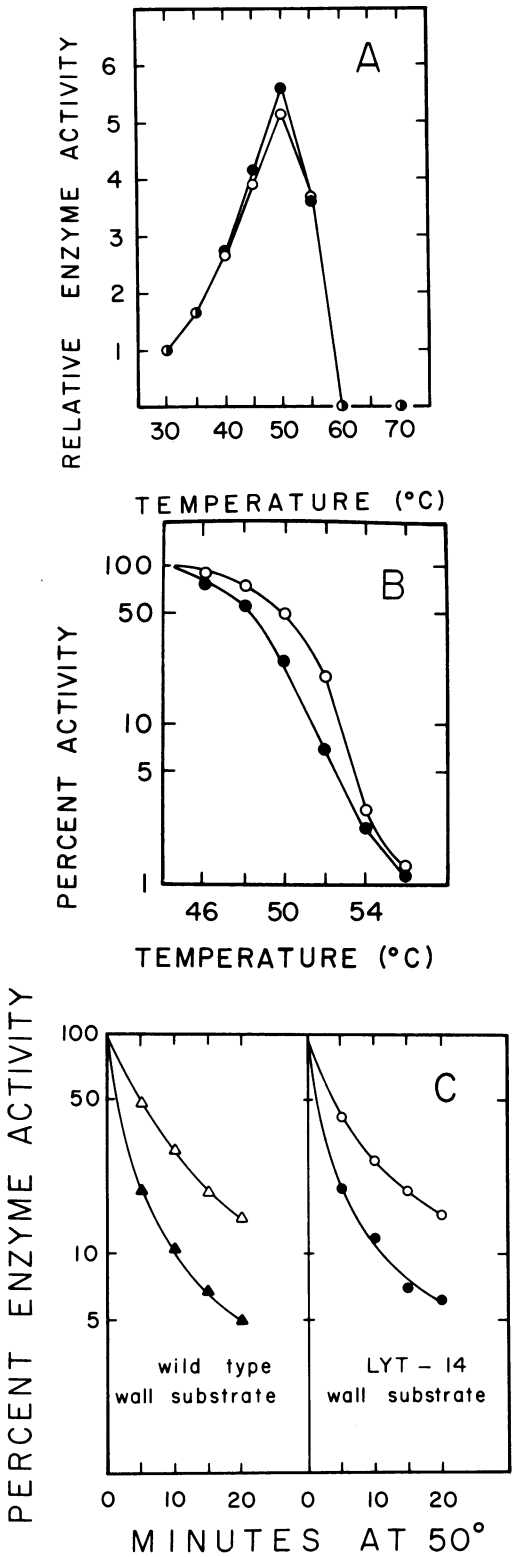


FIG. 6. *Binding of wild-type and LYT-14 autolysin to SF and ML SDS-treated walls. Autolysin was bound to ML (open symbols) or SF (closed symbols) walls as described in Materials and Methods; different symbols correspond to separate experiments. Percentage of enzyme bound was calculated from the amount of SF activity remaining in the supernatant fractions after sedimenting the wall-enzyme complexes. (A) LYT-14 autolysin; (○, ●) 840 U of enzyme; (△, ▲) 420 U of enzyme. (B) Wild-type autolysin: 300 to 350 U of enzyme.*



(latent form) autolysin of LYT-14 was also observed with activated LYT-14 autolysin prepared from trypsin or BPA-speeded wall lysates (results not shown).

DISCUSSION

The mutant selection procedure was designed to obtain thermosensitive mutants in a function(s) of the autolytic system. True to the selective conditions employed, the ability of strain LYT-14 to autolyze under a variety of conditions indicated a lytic-defective phenotype that was also thermosensitive.

Clearly, when tested in the absence of proteinase activation, cellular autolysis of LYT-14 failed to show the normally expected increase in rate characteristic of biological reactions (Fig. 2). However, several types of tests failed to show pronounced decrease in thermostability for the mutant autolysin. Although when tested in solution LYT-14 autolysin was somewhat more heat labile than the parental autolysin (Fig. 7B and C), when either LYT-14 or parental-type autolysin was first bound to wall substrate (which most closely approximates the in vivo

FIG. 7. *Thermolability of SF wild-type and LYT-14 autolysin activity. (A) Thermolability of wall-bound autolysin. Constant volumes of alkali-extracted wild-type and LYT-14 autolysin were bound to SF walls at 0°C in 0.01 M sodium phosphate, pH 7, prior to shifting the cell wall enzyme complexes to the indicated temperatures for assay of hydrolytic activity. Units of enzyme activity are described in Materials and Methods, which for the relative value of 1.0 equals 112 U for wild-type autolysin* (○) *and 142 U for LYT-14 autolysin* (●). *All assays contained 100 μg of BPA per ml and therefore determine total autolysin activity. (B) Thermolability of soluble autolysin with increasing temperature. Preparations of alkali-extracted wild-type and LYT-14 autolysin containing 100 μg of bovine serum albumin per ml were incubated in 0.01 M sodium phosphate, pH 7, for 5 min at the indicated temperatures prior to removing increasingly larger volumes of autolysin to ice-cold tubes containing SF wall substrate plus 100 μg of BPA per ml. Hydrolytic activity was measured at 37°C, which for 100% activity is 314 U for wild-type autolysin* (○) *and 366 U for LYT-14 autolysin* (●). *(C) Thermolability of soluble autolysin with increasing time of heating. Preparations of alkali-extracted wild-type and LYT-14 autolysin containing 100 μg of bovine serum albumin per ml were incubated in 0.01 M sodium phosphate, pH 7, at 50°C for the times indicated prior to removing samples to ice-cold tubes containing wild-type (triangles) or LYT-14 (circles) SDS walls plus 100 μg of BPA per ml. Hydrolytic activity of the heat-treated autolysin was subsequently measured at 37°C, which for 100% is 133 U for wild-type autolysin* (○, △) *and 163 U for LYT-14 autolysin* (●, ▲).

condition of cellular autolysis), the heat stabilities of the enzymes from the two sources were indistinguishable (Fig. 7A). Additionally, autolysin extracted from LYT-14 did not differ from the parent autolysin in: (i) ability to bind to cell wall substrate (Fig. 6); (ii) ability to dissolve LYT-14 or parental walls; or (iii) the products of wall hydrolysis (Table 2). Moreover, the chemical composition of the mutant or wild-type walls was the same (Table 1) as was the total amount of autolytic enzyme activity present in either strain (Fig. 3).

The LYT-14 strain did differ from the parental strain in that only 2% of the total autolysin activity was in the active form, in contrast to the parental strain, in which this value was 20 to 25% (Fig. 3). It is possible that this low level of the naturally active (in contrast to trypsin- or BPA-activated) form of autolytic enzyme in the mutant strain contributes to the temperature-sensitive cellular autolytic phenotype. For example, the slight thermosensitivity of the mutant enzyme, in conjunction with the low level of naturally active autolysin, may reduce the level of autolytic activity in the cells incubated at 43°C to the point where cellular autolysis eventually stops. This explanation adequately describes the kinetics of LYT-14 cellular autolysis at 43°C in the absence of trypsin (Fig. 1B). Consistent with this view is that the temperature-sensitive phenotype of LYT-14 was not observed when the proportion of active autolysin was experimentally increased by the addition of trypsin to cells during autolysis in buffer (Fig. 1A) or during autolysis following treatment with penicillin G (Fig. 2). Alternate interpretations include the possibilities that: (i) increased temperature of incubation causes increased enzymatic (e.g., proteinase) degradation of the active form of the LYT-14 autolysin; and (ii) the active form of the autolysin actually present in LYT-14 cells differs in thermostability from the enzyme preparations tested. Naturally active LYT-14 autolysin is a product of the conversion of the latent form, presumably via the action of a proteinase present in SF that is likely to differ in enzymatic specificity from trypsin or the proteinase in BPA. Because of the very low level of naturally active autolysin present in wall-enzyme complexes of the mutant strain, we were unable to test the thermolability of naturally activated LYT-14 autolysin.

An additional phenotypic property of LYT-14 was the inability to form halos on ML indicator cells while retaining the halo-forming activity on SF indicator plates. These results suggested that the ML activity exhibited by the wild-type autolytic enzyme preparations was lost or at least enormously decreased in the LYT-14 strain. Although the expected linear relationship between increased amount of enzyme and hydrolytic action on SF walls was observed for the autolysin from LYT-14 (not shown), such was not the case for ML activity (Fig. 5). These data indicated that the ML activity of soluble LYT-14 autolysin is actually 0.5% or less of the SF activity and is therefore reduced by more than 100-fold relative to the ML activity of the parental autolysin. This inability of the mutant autolysin to hydrolyze ML walls could not be attributed to the failure of the enzyme to bind to the ML wall substrate (Fig. 6A). Additionally, the minimal level of ML activity does not appear to result from the unusually low level of naturally active autolysin found in the LYT-14 strain, since preparations of soluble wild-type autolysin in which 95% of the enzyme was in the latent form still showed high levels of ML activity under the same assay conditions (Fig. 5). Moreover, LYT-14 autolysin obtained from proteinase (BPA)-speeded wall lysates, containing only proteolytically activated autolysin, had no greater ML activity than mutant autolysin prepared by alkali extraction (not shown).

The data demonstrating that the LYT-14 mutant produces an autolytic enzyme lacking ML activity while retaining SF activity could be interpreted to suggest that different proteins are responsible for the ML and SF activities. Although a variety of data indicate that SF has only a single specificity of a peptidoglycan hydrolase activity (33), it remains possible that more than one enzyme protein of the same specificity may be present. For example, evidence for the presence of two fractions of amidase activity in *B. subtilis* W23 has been reported (18): a major fraction which adsorbs to an ion-exchange resin and a minor fraction which does not adsorb, both of which had the same molecular weight and pH optimum. An answer to this question awaits further purification and characterization of the enzyme(s).

All of the data obtained so far can be interpreted as being the pleiotropic effects of a single mutation that results in the synthesis of an altered form of the latent (zymogenic) form of the autolysin. This modified zymogen could: (i) lack hydrolytic activity on ML walls and be slightly thermolabile in solution; and (ii) be less effectively activated by the proteinase of SF to produce a lower level of the naturally active form that is more thermolabile than the wild-type enzyme. In contrast to the action of the native proteinases on the modified zymogen, trypsin or the proteinase present in BPA activate efficiently to produce a more thermostable enzyme. However, NTG mutagenesis (16) and the strong selection methods employed in isolat-

ing this mutant strain are likely to result in clones that carry more than a single mutation. Repeated attempts to genetically transform *S. faecalis* ATCC 9790 have failed. Thus, it has not been possible to obtain information on the nature of the genetic alteration(s) that has occurred in the LYT-14 strain. It is certainly not surprising to find pleiotropic effects from a mutation that has altered the cell envelope or a cell wall-hydrolytic enzyme(s). Autolytic defective mutants of *B. subtilis* have been described as carrying a single mutation which results in several phenotypic alterations in addition to the loss of two (11) or more (2) detectable enzyme activities. It does appear that the soluble autolysin extracted from this mutant strain has undergone an alteration which has changed the substrate specificity of the autolytic enzyme. Physical and chemical characterization of the wild-type and mutant autolytic enzymes is currently in progress to define the putative alteration and to extend this conclusion beyond the biological criteria reported here.

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