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Characterization of *Bacillus licheniformis* 6346 Mutants Which Have Altered Lytic Enzyme Activities

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Two groups of mutants altered in lytic enzyme activities have been isolated from *Bacillus licheniformis* 6346 MH-1 by screening clones for halo production in agar plates containing cell wall conjugated with Procion brilliant red. In the first group which produced halos during colony formation, two were shown to contain three- and eightfold more muramyl-L-alanine amidase than the parent. These strains liberated amidase and intracellular α-glucosidase into the culture medium during exponential growth in liquid medium. Isolated walls had a normal qualitative composition and in autolysing liberated N-terminal amino acids and reducing groups. Wall preparations from the second group of mutants which did not produce halos lysed very poorly at pH 9.5, the optimal pH for amidase activity, and poorly at pH 5.5 even though they had similar endo-*N*-acetylglucosaminidase activities to the parent. Two of these strains that were also deficient in phosphoglucomutase had only 3 to 5% of the membrane-bound amidase activity compared with that in the parent. Cell walls of the phosphoglucomutase-deficient mutants treated with sodium dodecyl sulfate to inactivate endogenous lytic enzymes were dissolved at 10% of the rate of those from the parent by added amidase, but their sensitivities to lysozyme were similar. Those from one mutant had 10 to 20% of the amidase-binding capacity of parent walls, whereas its isolated mucopeptide was essentially inactive in this respect. The failure of these phosphoglucomutase-deficient mutants to autolyse is likely to be due to the combined effects of both low amidase activity and resistant walls. As a result, daughter cells are unable to separate and long chains are formed during exponential growth.

Bacillus licheniformis and *B. subtilis* are known to have two lytic enzymes, an *N*-acetylmuramyl-L-alanine amidase (11, 14, 33) and a protease-sensitive endo-β-*N*-acetylglucosaminidase (3, 31; Forsberg, unpublished data). The function of these enzymes is still unclear (13, 23). In *B. subtilis*, the amidase is known to participate in cell wall turnover during growth (17), and observations have supported the suggestions that this same enzyme may be involved in the separation of daughter cells (8, 10, 23) and in transformation (34).

A more direct approach to the study of the roles of these enzymes would be to isolate mutants not forming the enzymes; if essential for growth, such mutants would have to be conditional. Preliminary work (9, 10, 12, 20) has been done with non-conditional mutants that autolyse slowly and are therefore deficient in autolytic activity. Two such mutants of *B. licheniformis* 6346 (10, 12) were found to have alterations in wall chemistry attributable to a loss of phosphoglucomutase. These walls were resistant to the action of the principle autolytic enzyme, the amidase. This paper is concerned with the properties and amounts of amidase present in a variety of mutants that we have isolated, including these two.

MATERIALS AND METHODS

Cultures and media. *B. licheniformis* NCTC 6346 His (24) and the lytic enzyme-defective mutants isolated from it (10) are listed in Table 1. The nomenclature of the histidine-requiring parent strain and the mutants has been changed to conform with the recommendations of Demerec et al. (4). Other bacteria included *B. subtilis* 168 *trp* C2 and *Micrococcus lysodeikticus* NCTC 2665.

All cultures were stored in the dried state (28). Strains MH-1 and MH-3 were also kept as spore suspensions in water at 4 C. Cultures grown from these stocks were streaked onto Hedley Wright agar,

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TABLE 1. *Description of Bacillus licheniformis 6346 strains*[a]

Designation	Phenotype	Previous designation
6346	Wild-type prototroph	
MH-1	His^-	His^-
MH-2	His^-, superlytic	$His^- Lyt^+$
MH-3	His^-, poorly lytic	$His^- Lyt^-$-3
MH-4	His^-, poorly lytic	$His^- Lyt^-$-4
MH-5	His^-, poorly lytic	$His^- Lyt^-$-5
MH-6	His^-, poorly lytic	$His^- Lyt^-$-6
MH-7	His^-, superlytic	$His^- Lyt^+$
MH-8	His^-, superlytic	$His^- Lyt^+$

[a] The following abbreviations are used: His^-, histidine requirement; lyt^-, no clear halos produced by colonies on agar media containing cell walls conjugated with Procion brilliant red, or lyt^+ for colonies producing halos during colony formation.

and an isolated colony was used as inoculum. For the experiments, five different media have been used: medium A, a minimal medium without added citrate (21) and containing 0.01% added casein hydrolysate; medium B, Spizizen minimal medium (27) supplemented with 0.3% L-glutamate; medium C, penicillin assay broth; medium D, casein hydrolysate-salts medium (21); and medium E, casein hydrolysate-yeast extract-glucose medium (14).

Unless otherwise indicated, sterile solutions of glucose and L-histidine hydrochloride were added to the sterilized, cooled basal media to give final concentrations of 0.5 and 0.01%, respectively. Maltose (final concentration of 0.5%) was added in place of glucose when required. The media were solidified when required with agar at a final concentration of 1.5%.

All cultures were grown in flasks containing no more than 0.2 of their volume as culture fluid with agitation at 35 C. To monitor the growth rates of cultures, they were usually grown in flasks fitted with side arms. The extinction of a culture at 675 nm was measured at intervals by tipping part of the culture into the side arm and using a Unicam SP-600 spectrophotometer; the readings were corrected for deviations from Beer's law (30). The extinction values for suspensions of both strain MH-1 and of the mutants were shown to be proportional to dry weight. For strain MH-1, an extinction value of 1.0 corresponded to 500 μg/ml and for MH-3 it corresponded to 520 μg/ml, this small difference presumably being due to chain formation by the latter.

Isolation of mutants. Cultures (20 ml) of *B. licheniformis* MH-1 were grown in medium C (penicillin assay broth) to a density of 0.2 to 0.3 mg (dry weight)/ml. The bacteria were harvested by centrifugation at 20 C and suspended in 5 ml of 0.067 M potassium phosphate buffer (pH 6.5). 1-Methyl-3-nitro-1-nitrosoguanidine (Aldrich Chemical Co., Milwaukee, Wis.) dissolved in water (0.5 mg/ml) was added to give a final concentration of 20 μg/ml. The cell suspension was incubated with shaking for 1 h at 35 C, and all subsequent incubations were at the same temperature. After this treatment, which killed 99.9% of the cells, they were washed once with 5 ml of medium C, diluted in the same medium, and incubated for 3 h. They were then diluted in medium A, spread onto medium A agar plates, and incubated for 30 to 40 h. The colonies were replicated onto agar containing cell walls conjugated with Procion brilliant red (medium A), as previously described (10), and incubated aerobically for 36 h. The parent MH-1 produced gelatinous colonies without zones. From the plates of mutagen-treated cultures, 0.05% of the colonies produced clear halos formed by solution of cell walls by the autolytic enzymes. These were picked and purified by restreaking on cell wall agar plates. The isolates were labeled MH-2, MH-7, and MH-8. The original cell wall agar plates were then incubated under anaerobic conditions in a hydrogen-atmosphere for 36 h. Strain MH-1 produces halos under these conditions. Colonies not doing so were selected and the cultures were purified as described above. These constituted poorly lytic mutants and were found to occur at a frequency similar to that of the superlytic ones. Of these, strains MH-3, MH-4, and MH-5 were studied in detail. The mutants along with their phenotypes are listed in Table 1.

Preparation of cell walls containing autolysin. Autolysin-containing walls were prepared from cultures in the exponential phase of growth (0.25 to 0.3 mg/ml) in medium A. Cultures were harvested rapidly by centrifugation at room temperature and washed once in 0.1 volume of cold water (4 C). All subsequent operations were performed at 4 C. The cells in water (10 to 20 mg/ml in 25 ml of water) were disrupted in a Braun tissue homogenizer by shaking with 20 ml of no. 12 Ballotini beads for 5 min with cooling by vaporization of CO_2. The disrupted suspension was filtered through a no. 2 sintered-glass filter to remove the glass beads; the walls were then sedimented by centrifugation at 36,000 $\times$ g for 20 min and washed five or six times in water. The preparations were then usually lyophilized and stored at -5 C.

Preparation of inactivated walls. Walls were prepared from exponential-phase cultures (0.25 to 0.40 mg/ml) by suspending the harvested bacteria in 4% sodium dodecyl sulfate (SDS) for 2 h or longer, and disrupting them in the presence or absence of SDS depending on which was more convenient. The walls were deposited by centrifugation after the beads were removed, as described for the preparation of autolysin-containing walls. They were then washed once in 4% SDS, three times with 1 M NaCl, and four times with water, and then lyophilized. They will be referred to as SDS-walls.

To remove ester-linked alanine from wall preparations, they were incubated (5 to 10 mg/ml) in 0.1 M NH_4OH at 35 C for 2 h, washed three times, and lyophilized (26).

Preparation of mucopeptide. Mucopeptide was prepared by extracting walls (5 mg/ml) with 5% trichloroacetic acid at 35 C for 24 h, followed by a second 24-h treatment in fresh trichloroacetic acid. The mucopeptide fraction was recovered by centrifu-

gation, washed three times in water, and lyophilized.

Measurement of lytic enzymes in walls containing autolysin, in cells, and in culture supernatant fluids. Lysis of autolysin-containing walls was measured by suspending walls (2 to 3 mg) in 4 ml of either 0.05 M sodium carbonate buffer (pH 9.5) or 0.05 M potassium phthalate buffer (pH 5.5) in a matched tube kept in an ice bath. After mixing, the tube was placed in a water bath at 35 C. At intervals the tube was removed and dried, and the optical density was read at 450 nm in a Unicam SP-600 spectrophotometer. A unit of lytic activity was defined as a linear decrease of 0.001 optical density units per minute.

To measure total cellular lytic enzyme, we harvested 5 or 10 ml of culture by centrifugation at 39,100 $\times$ *g* for 10 min at 15 C. The bacteria were then suspended in 2.5 ml of 0.05 M sodium carbonate buffer (pH 9.5) and incubated at 35 C for 5 h. The reaction mixture was clarified by centrifugation at 12,000 $\times$ *g* for 10 min, and 2 ml of extract was incubated with SDS-treated cell walls. Wall hydrolysis at pH 9.5 was then measured as described above.

The activities of lytic enzymes in the culture supernatant fluids were measured after removal of the bacteria from cultures either by filtration (0.45 μm Millipore membrane filters) or by centrifugation at 39,100 $\times$ *g* for 10 min at 15 C. The supernatant fluids were adjusted to pH 9.5 by the addition of 1.0 M Na_2CO_3 and centrifuged to remove any precipitate, and the enzyme present was measured at pH 9.5 as described above.

Wall and membrane amidase extracts. Cell wall amidase from walls containing autolysins and membrane amidase were prepared as previously described (11). The disaggregated membrane suspensions were centrifuged at 44,000 $\times$ *g* for 30 min, and the supernatants were saved and stored in a frozen state. The units of enzyme are as previously described.

Extraction of cell walls with LiCl. Autolysin-containing cell walls were extracted with LiCl by the method of Fan (7), diluted to less than 0.1 M in LiCl, and assayed at pH 9.5 for the presence of amidase.

Binding of amidase to walls. The mixture contained the following components in a total volume of 2 ml: 10 to 15 units of amidase-containing extract that had been centrifuged (18,000 rpm for 30 min), 3 mg of SDS-cell walls or mucopeptide, and 0.2 mmol of sodium-carbonate buffer (pH 9.5). Prior to addition, the cell walls (5 mg/ml in water) were subjected to sonic oscillation to dissociate clumps. The components were mixed briefly at 0 C, incubated for 5 min, and then centrifuged at 45,000 $\times$ *g* for 5 min. The supernatant was poured off and the amidase remaining in solution was measured.

Preparation of membranes and mesosomes. Membrane and mesosomes were prepared as described by Forsberg and Ward (11).

Enzyme assays. *N*-acetylmuramyl-L-alanine amidase, lysozyme (EC 3.2.1.17), and succinate dehydrogenase (EC 1.3.99.1) in membranous preparations were assayed as described previously (11), with the exception that the final concentration of pH 9.5 sodium carbonate buffer was 0.05 M in the amidase assay. α-Glucosidase (EC 3.2.1.20) was measured by the hydrolysis of *p*-nitrophenol-α-D-glucoside (18). The specific activities of succinate dehydrogenase and α-glucosidase were expressed as either micromoles of 2,6-dichlorophenolindophenol reduced or *p*-nitrophenol produced per minute per milligram of protein. For the assay of phosphoglucomutase (EC 2.7.5.1), the bacteria were grown and the extracts were prepared and assayed under conditions outlined previously (12). One unit of enzyme activity was defined as the amount of enzyme utilizing 1 μmol of substrate per minute. Specific activity is expressed as units per milligram of protein.

Analytical methods. Amino acids and amino sugars were determined by S. Lathwell of this Institute with a Beckman-Spinco automatic amino acid analyzer after hydrolysis of the samples in 4 N HCl for 16 h at 100 C unless stated otherwise. Protein was determined by the method of Lowry et al. (16) with bovine plasma albumin as a standard; reducing groups produced during autolysis of walls were determined by the method of Thompson and Shockman (29) after centrifugation (34,000 $\times$ *g* for 15 min) of the digest to remove residual walls. Glucose was used as the standard. The release of free amino groups after lysis of purified cell walls was assayed as before (11). Hexuronic acid was determined on unhydrolyzed walls by the Dische method (5) with D-glucuronic acid as the standard. Phosphorus was determined by the method of Ames and Dubin (1). Glucose in cell walls was determined with glucose oxidase (EC 1.1.3.4) (2) after acid hydrolysis in 2 N HCl for 2 h at 100 C. Carbohydrate was estimated on unhydrolyzed walls either by the phenol-sulfuric acid method (6) or by modification of the anthrone method (22) with glucose as the standard.

RESULTS

Phenotypes of mutants. Two groups of mutants modified in lytic enzyme production were found. One group was tentatively identified as liberating lytic enzyme (superlytic), because on cell wall agar they produced clear halos (1 to 3 mm in diameter) encircling each colony after 36 to 45 h at 35 C. These mutants (MH-2, MH-7, MH-8) are listed in Table 1. Strain MH-1 did not form halos during colony formation, but treatment of the plates by spraying either with D-cycloserine (100 μg/ml), or treatment with chloroform vapor (10 min) and subsequent incubation, or by incubation under anaerobic conditions (48 h) caused halos to form gradually around the colonies. The other group was poorly lytic, and mutants were detected by the absence of clearing around the colonies after anaerobic treatment.

Content and release of lytic enzyme from superlytic mutants. The amounts of lytic enzymic activity at pH 9.5 present in cells of strains MH-1, MH-2, and MH-8 were determined on cell autolyzates prepared from cultures of different ages. Figure 1 shows that

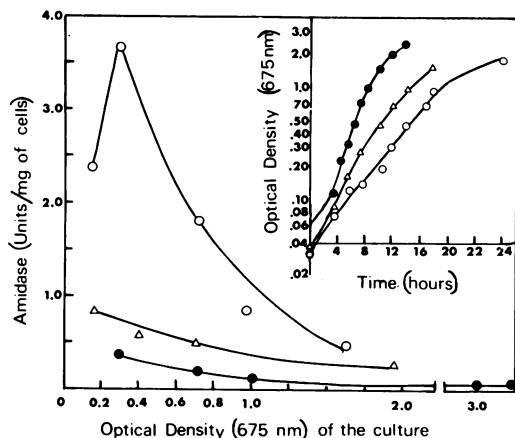

FIG. 1. *Amidase content of MH-1, MH-2, and MH-8 at various times during growth. Cultures were grown in medium A with maltose as the carbon source. The insert illustrates the growth curves of the cultures used in this experiment. Symbols: ●, MH-1; △, MH-2; ○, MH-8.*

strains MH-2 and MH-8 contained approximately three and eight times the amount present in strain MH-1. The times during the growth of the cultures at which the samples were taken are shown in the inset (Fig. 1), and their doubling times during exponential growth are shown in Table 2. The lytic activity was always greatest during the exponential phase of growth. Strains MH-2 and MH-8 released lytic enzyme and α-glucosidase into the culture medium during the exponential phase, and the amounts found in the medium from strain MH-8 were similar whether the bacteria were removed by centrifugation (39,000 $\times$ g for 10 min) or filtration with a 0.45-μm membrane. The release of amidase activity was directly proportional to that of α-glucosidase (Fig. 2). In some experiments, as much as 69% of the total α-glucosidase in the culture was found in the cell-free culture medium during the early exponential phase of growth.

Release of lytic enzymes and α-glucosidase also occurred in strain MH-2, but the amounts were less and were decreased by using rapid filtration rather than centrifugation to harvest the cells. Under identical conditions, neither strain MH-1 nor strain MH-7 released lytic enzymes, and only traces of soluble α-glucosidase were found in the cultures.

Distribution of lytic enzymes in the superlytic mutants. Wall preparations from the superlytic mutants MH-2 and MH-8 without SDS treatment did not appear to undergo enzymatic attack as rapidly as the parent walls at either pH 9.5 (Fig. 3) or pH 5.5. In the case of strain MH-8, the failure of its wall suspensions to decrease in extinction value was an artifact due to contamination of the preparations with protein, but if the walls were washed a sufficient number of times to remove protein they were lost owing to autolysis during the extended process. During the autolysis of preparations from both strains at pH values of 9.5 and 5.5, there were increases in N-terminal amino acids. Increases in reducing power during autolysis of all strains were barely detectable at pH 9.5. At pH 5.5 the glycosidase activity of MH-2 and MH-8 walls was greater than that of MH-1 walls (Table 3), but the significance for MH-8 was difficult to evaluate owing to the presence of protein in this preparation. The enhanced glycosidase activity of strain MH-2 might be related to its lower proteolytic activity (see later), but strain MH4, which was poorly autolytic at pH 9.5, also had an increased glycosidase activity. When cells were disrupted and the walls were removed, the extracts from strains MH-2 and MH-8 had 2.4 and 32 times the amount of amidase, respectively, compared with the activity in extracts of MH-1, using SDS MH-1 walls in the assay. The activities of amidase in the cytoplasmic and mesosomal

TABLE 2. *Growth rates of MH-1 and the superlytic mutants in minimal and complex media*

Strains	Doubling time (min)		
	Medium A plus glucose	Medium A plus maltose	Medium C plus glucose
MH-1	80–96	111	32
MH-2	138	207	89
MH-7	147	111	35
MH-8	288	210–240	79

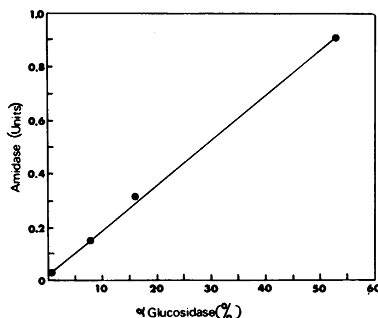

FIG. 2. *Relationship between the release of amidase and α-glucosidase from MH-8 during growth. Growth conditions were identical to those of Fig. 1. Samples were removed from the cultures after 1, 6, 12 and 24 h of growth; the ratio of amidase/α-glucosidase increase linearly with time.*

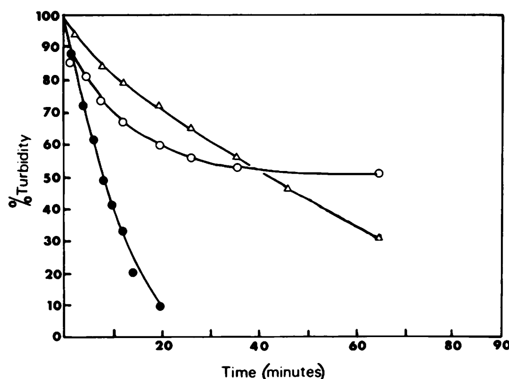

FIG. 3. *Lysis at pH 9.5 of autolysin-containing walls prepared from MH-1, MH-2, and MH-8 cells grown in medium A. The activity contained in MH-1 walls was 30 U. Symbols: ●, MH-1; △, MH-2; ○, MH-8.*

TABLE 3. *Rates of increase in reducing power during the lysis of autolytic cell walls at pH 5.5*[a]

Expt	Walls	Increase in reducing power (nmol/h/mg of walls
1	MH-1	0.8
	MH-2	3.9
	MH-8	2.0
	MH-4	2.0
2	MH-1	1.1
	MH-3	0.68
	MH-5	1.13

[a] Freshly prepared or freeze-dried walls at a concentration of 2 to 3 mg/ml were incubated in 0.05 M phthalate buffer (pH 5.5) at 35 C. Samples were removed at intervals for optical density readings and reducing power measurements.

membranes from strain MH-8 were similar to those in the same fractions from strain MH-1 (Table 4).

Wall chemistry of the mutants. Brief accounts of the wall chemistry of the poorly lytic mutants grown in batch culture have already appeared (10). The analysis of walls prepared from exponentially growing batch cultures of all the present mutants (Table 5) shows that the walls of the two poorly lytic strains (MH-3 and MH-5), now known to be phosphoglucomutase negative, are clearly distinguished, as would be expected, from the rest by their very small content of glucose, glucuronic acid, and galactosamine, and by their larger amount of phosphorus. This is consistent with the absence of teichuronic acid and of glycosylated teichoic acid. Strain MH-4, that has phosphoglucomutase despite its poorly lytic properties, has normal amounts of teichuronic acid components but a relatively small amount of glucose and a large amount of phosphorus, suggesting that it too may share with strains MH-3 and MH-5 the presence of poorly glycosylated teichoic acids in its wall. The walls of the three superlytic mutants show differences in their content of non-mucopeptide polymers, but each is quantitatively different. All would appear, however, to be qualitatively similar to the parent MH-1 walls, having both teichuronic acid and glycosylated teichoic acid polymers but in different proportions. The walls of strain MH-2, like those of strains MH-3 and MH-5, have a very high mucopeptide content, but instead of a high content of phosphate polymers have only about half that present in the walls of strain MH-1.

Lytic enzymes on walls and in cytoplasm of poorly lytic mutants. Cell walls containing autolysin that were prepared from the poorly lytic mutants MH-3, MH-4, and MH-5 lysed more slowly at pH 9.5 than did preparations from the parent bacterium (Fig. 4). With MH-3 and MH-5, lysis was not complete even after 72 to 84 h. There were always at least minor increases in N-terminal amino groups indicative of limited amidase activity. At pH 5.5 there were small increases in reducing sugar in both the mutant and parent walls (Table 3). No increases in amino groups were observed in MH-3 or MH-5 walls at this lower pH.

The cytoplasmic contents of strains MH-4 and MH-5 contained no lytic enzyme activity. The addition of 1 ml of MH-5 extract (5 mg of protein/ml) to an equal volume of either a similar extract of strain MH-1 or of cell wall amidase (11 U, 0.2 ml), followed by incubation for 60 min at 35 C, did not inhibit or stimulate

TABLE 4. *Specific activities of enzymes in cytoplasmic and mesosomal membrane of the parent strain and various mutants*

Strain	Sp act (U/mg of protein)			
	Cytoplasmic Membranes		Mesosomal Membrane	
	Amidase	Succinic dehydrogenase	Amidase	Succinic dehydrogenase
MH-1	26	0.66	8.6	0.0094
MH-3	0.86	0.91	0.47	0.001
MH-4	3.5	0.61	[a]	[a]
MH-5	1.1	0.66	[a]	[a]
MH-8	19.7	0.54	11.6	0.023

[a] Not examined.

TABLE 5. *Chemical analyses of SDS-treated cell walls prepared from Bacillus licheniformis MH-1 and its lytic enzyme-defective mutants*[a]

Component	Amount (µmol/100 mg of cell wall)						
	MH-1	MH-2	MH-7	MH-8	MH-3	MH-4	MH-5
Glucuronic acid	36.4	36.0	48.4	29.9	2.5	35.0	2.6
Phosphorus	103.0	49.0	91.0	138.0	172.0	145.0	170.0
Glucose	30.6	12.2	26.6	15.5	3.0	9.0	3.1
Carbohydrate[b]	130.0	46.7	105.4	83.3	2.7	38.8	4.7
Muramic acid	10.9	20.2	19.0	11.8	23.5	11.1	22.4
Glucosamine	24.6	32.5	26.3	26.0	35.0	30.4	30.0
Galactosamine	12.3	20.6	26.0	16.1	<0.1	8.9	<0.1
Diaminopimelic acid	34.9	67.0	37.7	32.3	57.0	41.0	64.0
Glutamic acid	28.2	55.8	33.2	25.6	39.5	29.6	43.8
Alanine	71.3	113.0	84.6	96.2	153.0	116.0	125.0

[a] Cell walls were prepared from exponential-phase batch cultures grown in medium A.
[b] Phenol sulfuric acid-positive material.

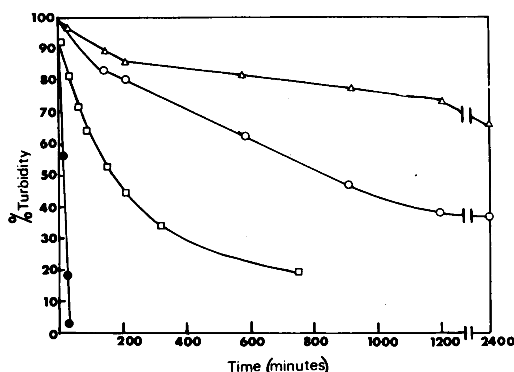

FIG. 4. *Lysis of autolysin containing walls prepared from MH-1, MH-3, MH-4, and MH-5 cells grown in medium A. Symbols:* ●, *MH-1;* ○, *MH-3;* □, *MH-4;* △, *MH-5.*

amidase activity on SDS-walls from strain MH-1.

Separation of amidase from walls of MH-1 by using LiCl. The walls of strains MH-3 and MH-5 were found to be very resistant to amidase action (see below). To obtain unambiguous estimates of the amounts of amidase in autolytic walls of these poorly lytic mutants, compared with those of strain MH-1, we hoped to use LiCl solutions (7) to extract the enzymes from walls and then to assay the extracts on MH-1 walls. Concentrations of LiCl between 4 and 6 M applied to MH-1 walls removed or inactivated the amidase. No activity was demonstrable in the extracts when they were diluted and incubated in the presence of SDS-walls from strain MH-1.

Amidase in membranes and mesosomes of poorly lytic mutants. The amidase activities of cytoplasmic and mesosomal membranes of the parent MH-1 were similar to those recorded previously (11; Table 4). The amidase activity present in the mesosomal fraction, which was distinguished from the cytoplasmic membrane by its lower succinate dehydrogenase activity (21), was considerably lower than that in cytoplasmic membranes. In strains MH-3, MH-4, and MH-5 the activities of the cytoplasmic membrane fraction were 3.3, 13.5, and 4.2%, respectively, of that in strain MH-1. The mesosomal activity of strain MH-3 was even lower than that in the cytoplasmic membrane. To confirm the specificity of the lytic activities in the various samples, we mixed membranes dissociated by Triton X-100 with SDS-walls of strain MH-1 under conditions (see Table 6) where 60% of the amidase was bound to the walls. After these walls had been separated by centrifugation and washed once, they were allowed to autolyze in 0.05 M carbonate buffer (pH 9.5). Lysis of MH-1 walls by an MH-1 enzyme was accompanied by the release of N-terminal L-alanine (Fig. 5). Similarly, there were increases in N-terminal L-alanine with MH-4 and MH-5 membrane extracts, but at much lower rates. There were no detectable increases in other N-terminal amino acids or in reducing power.

Susceptibility of MH-3 and MH-5 walls and mucopeptides to amidase and lysozyme. When SDS-walls from strains MH-3 and MH-5 were treated with exogenous amidase, they underwent turbidity decrease at approximately 10% of the rate of those from strain MH-1 (Fig. 6A), but the rates of solution by lysozyme were similar (Fig. 6B).

Isolated mucopeptide from strain MH-1 was dissolved at 22% of the rate of the original walls (Fig. 7A) that, from strain MH-5 (Fig. 7B) and

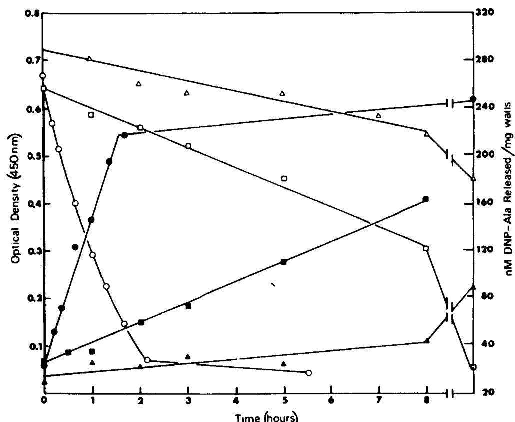

FIG. 5. *Decrease in optical density and increase in N-terminal alanine during the lysis of MH-1 walls by membrane amidases from: MH-1 (○, ●); MH-4 (□, ■), and MH-5 (△, ▲). Open symbols, Decrease in optical density; closed symbols, increase in N-terminal alanine.*

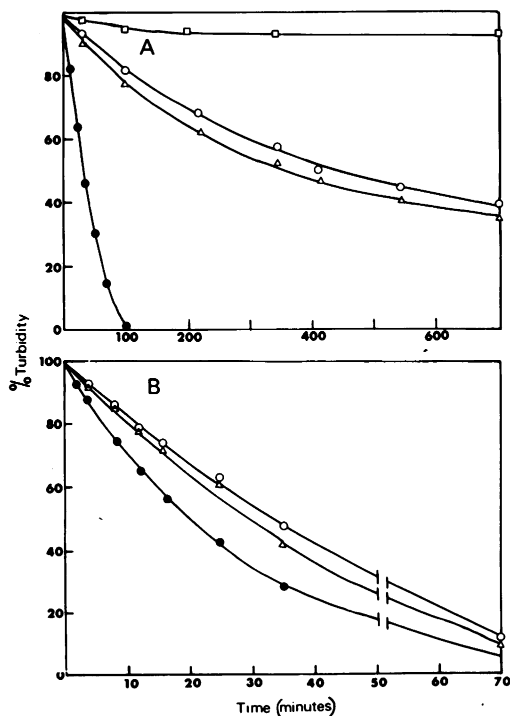

FIG. 6. *Lysis of SDS-walls from MH-1, MH-3, and MH-5 by cell wall amidase (A) and by lysozyme (B). The amounts of cell wall amidase and lysozyme added to each assay gave activities of 5.0 and 7.5 U, respectively, on MH-1 walls. Symbols: ●, MH-1; ○, MH-3; △, MH-5; □, walls without added enzyme.*

strain MH-3 (not shown), were hydrolyzed at slightly faster rates than whole walls. Lysis of neither the mucopeptide nor the walls of the mutants showed zero-order kinetics. Walls from strain MH-4 were more susceptible to amidase action than those from the phosphoglucomutase-deficient mutants.

Binding of amidase to walls. When amidase in a cell wall digest of strain MH-1 was mixed with MH-1 SDS-walls, it bound rapidly at 0 C (Table 6) and was not removed by water washing. Dissociated membrane amidase behaved similarly, but the amounts bound were 30 to

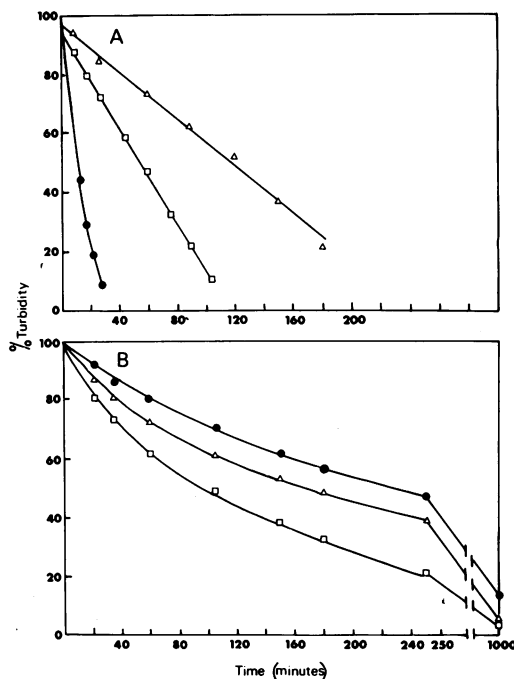

FIG. 7. *Lysis of SDS-treated cell walls and mucopeptides from MH-1 (A) and MH-5 (B) by cell wall amidase: cell walls, 28 U of amidase (●); mucopeptide, 14 U (△) and 28 U (□) of amidase.*

TABLE 6. *Effect of time on the binding of cell wall and membrane amidases to SDS-walls from MH-1*[a]

Binding time (min)	Amidase bound (%)	
	Cell wall amidase	Membrane amidase
0	83.1	43.0
5	88.0	46.3
20	92.2	59.0
60	93.2	56.0

[a] The MH-1 cell walls were from bacteria growing exponentially in medium D.

40% lower and were less reproducible. Although not shown, the binding in sodium carbonate buffer at pH 9.5 was higher than in phosphate or tris(hydroxymethyl)aminomethane buffers, of lower pH.

The binding abilities of walls and mucopeptide preparations are shown in Table 7; several of the experiments were repeated a number of times, and good agreement of results was obtained. Considerable specificity is apparent. For example, the walls of *B. subtilis* 168 *trp* C2 bound only 0 to 22% as much and those of *Micrococcus lysodeikticus* bound only 0 to 5% of the activity bound by preparations from strain MH-1 itself. Mucopeptide preparations seem to bind less than the corresponding walls. The walls of the mutant MH-5 bind only 20% as much activity as those of the parent strain MH-1. Amidase from a crude autolyzate is bound better by walls of strain MH-1 than when the enzyme is present in disaggregated membrane, but this is not so for the walls of mutant MH-5. The very poor binding capacity of the mucopeptide from the mutant can not be explained in terms of chemical composition which is identical to that of strain MH-1 within analytical error (Table 8).

Some morphological and physiological properties of the mutants. Strain MH-2 grew as short chains of unseparated cells, and there were mini-cell-like structures interspersed in

TABLE 7. *Binding of cell wall and membrane amidases to cell walls and mucopeptide from various sources*

Treatment	Medium cells grown in	Amidase bound (%)	
		Expt 1: cell wall amidase	Expt 2: membrane amidase
MH-1 walls	D	[a]	53
MH-1 walls	B	89.5	61
MH-1 mucopeptide	B	61.0	39
MH-5 walls	B	9.0	10
MH-5 mucopeptide	B	0	5
M. lysodeikticus walls	E	0	3.3
B. subtilis walls	B	0	12.0
MH-1 walls	Phosphate limitation[b]	[a]	79.0

[a] Not examined.

[b] These cell walls contained 1.6 μmol of glucuronic acid per mg (dry weight) and no detectable teichoic acids. The conditions of growth were as described by Forsberg et al. (12).

TABLE 8. *Analyses of mucopeptides prepared from MH-1 and MH-5 walls*[a]

Component	Amount (μmol/100 mg of mucopeptide)	
	MH-1	MH-5
Glucuronic acid	0	0
Phosphorus	16	15
Muramic acid	41	43
Glucosamine	49	49
Galactosamine	<2	<2
Diaminopimelic acid	80	78
Glutamic acid	61	61
Alanine	96	99

[a] The analyses are on the samples recorded in Table 7. The amino sugars and amino acids were analyzed after hydrolyses in 4 N HCl for 4 h at 100 C.

the chains. The other superlytic strains had normal morphologies, but strains MH-2 and MH-8 had slower growth rates than the parent (Table 2). Infrequently, revertants appeared in MH-8 cultures and rapidly outgrew the original mutant, presumably owing to a more rapid growth rate. Strains MH-3 and MH-5 grew as long chains of unseparated daughter cells during exponential growth at rates similar to strain MH-1 (10, 12). Strain MH-4 grew as single cells associated in large clumps at a rate about half that of the parent. When this strain, but not strains MH-3 and MH-5, was streaked onto the low-salt agar of Rogers et al. (25) the cells grew as spheres. All the mutants except strain MH-5 were able to form spores. When grown on skim milk agar, all the mutants were found to be proteolytic, but strain MH-2 was less so than the others.

Since *B. licheniformis* 6346 is notoriously susceptible to anaerobic shock (18), the poorly lytic strains MH-3 and MH-5 were compared with strain MH-1 to see whether the susceptibility was a consequence of rapid amidase action. Exponentially growing cultures of strain MH-3 (Fig. 8) were placed in sealed tubes for various intervals of time and then allowed to grow aerobically again. They grew exponentially at the same rate immediately after removal from anaerobic conditions. Similar results were obtained with strain MH-5. This treatment had a considerable effect on strain MH-1; there was a lag in growth and some bacterial lysis before the resumption of exponential growth. Such a resistance to anaerobic shock which is inherent in many manipulative procedures makes this type of mutant extremely useful in a variety of physiological experiments.

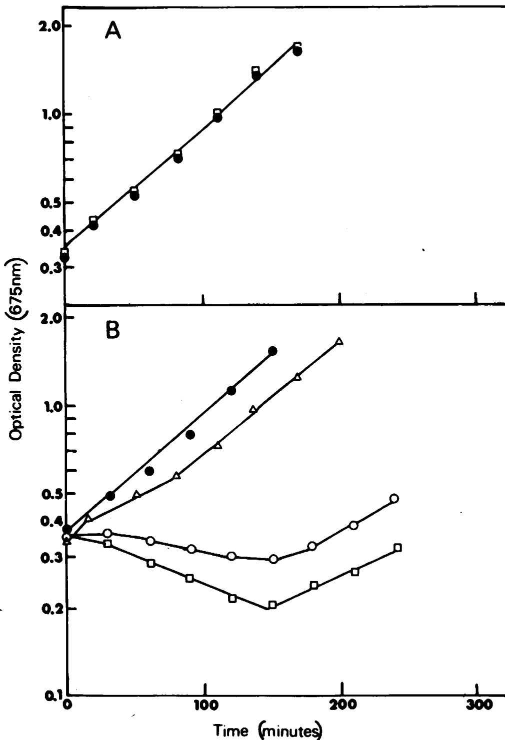

FIG. 8. *Effect of various intervals of anaerobiosis on exponentially growing cultures of MH-3 (A) and MH-1 (B). Volumes (10 ml) of exponential-phase cultures (0.13 mg/ml) were added aseptically to tubes until full. They were stoppered with rubber bungs and incubated stationary at 35 C. At each indicated time, the contents of a tube were poured into a side-arm flask (100 ml) and the culture was incubated aerobically with shaking. The optical density (675 nm) was measured at intervals. Symbols: ●, untreated; △, 15 min; ○, 30 min; □, 60 min.*

Examination of revertants isolated from the phosphoglucomutase-deficient mutants. Revertants of strains MH-3 and MH-5 were isolated by plating washed cells onto medium A agar plates by using galactose as the sole carbon source. The mutants are unable to use this carbohydrate and revertants able to grow were isolated. The reversion rate was found to be about 10^{-7} for strain MH-3. Nine of the revertants examined by streaking on Procion brilliant red cell wall agar plates produced halos like those produced by strain MH-1 after anaerobic treatment.

Two such revertants (R1 and R2) from each of strains MH-3 and MH-5 were examined for phosphoglucomutase, wall autolysis at pH 9.5, and cell wall composition. The revertants were corrected either partially or completely in these characteristics (Tables 9 and 10).

DISCUSSION

The method used for selection of the mutants is unambiguous in as much as only mutants disturbed in their autolytic ability would be selected. Agar containing whole, killed cells rather than cell walls of the parent organism would not necessarily exclude mutants forming altered amounts of other hydrolytic enzymes such as exo-cellular protease (10). The use of cell walls from even closely related but not identical species might cause mutants disturbed in autolysin formation to be overlooked because of the considerable specificity of the autolysins. The method does not distinguish, however, organisms disturbed only in autolytic enzyme formation from those showing the same phenotypic response resulting from some other lesion, for example, a disturbed wall composition, structure, or biosynthesis.

Two types of poorly lytic mutants can be recognized among those studied in detail in the present paper: those having a disturbed wall composition due to a phosphoglucomutase deficiency with walls that are resistant to the main autolytic enzyme, the muramyl-L-alanine amidase, but not to lysozyme; and the other with

TABLE 9. *Phosphoglucomutase and cell wall autolysis activities of the parent Bacillus licheniformis MH-1, the phosphoglucomutase-deficient mutants, and revertants derived from them*

Strain	Phosphoglucomutase sp act[a]	Wall autolysis units
MH-1	172	40
MH-3	<1	<0.5
MH-3R1	123	14
MH-3R2	54	10
MH-5	<1	<0.5
MH-5R1	119	31
MH-5R2	101	45

[a] Expressed as units × 10^3 mg of protein in the extracts.

TABLE 10. *Chemical analysis of autolysin-containing cell walls prepared from Bacillus licheniformis MH-1 and revertants isolated from MH-3 and MH-5*

Component	Amount (μmol/100 mg of cell wall)				
	MH-1	MH-3R1	MH-3R2	MH-5R1	MH-5R2
Glucuronic acid	43	39	40	29	29
Phosphorus	105	104	120	85	72
Carbohydrate[a]	104	111	102	90	79

[a] Determined by the anthrone procedure.

walls containing normal components, except for the usual amount of glucose, and which are sensitive to externally applied amidase. It appears, however, that the resistance to amidase of the walls in the first group is not the whole explanation of their phenotype, since they also have a grossly reduced amount of autolytic enzyme associated with their membranes. This is due neither to the greater avidity of the walls for the enzyme nor to its accumulation as soluble enzyme inside or outside the bacteria. It would seem that they are truly deficient in active amidase function. They have the same low activity of the autolysin with a different specificity (i.e., the endo-β-*N*-acetylglucosaminidase) as the parent microorganism. Genetic evidence, although incomplete (C. W. Forsberg and H. J. Rogers, unpublished data), suggests that only one genetic lesion causes both the deficiency in phosphoglucomutase and that in amidase. Moreover, both activities are restored or partially restored by growth in media containing galactose (12) or by reversion. The interrelationship is difficult to appreciate, especially since L-forms of strain MH-3 (32), which have no wall, also have little or no phosphoglucomutase and amidase compared with L-forms derived from the parent (C. W. Forsberg, unpublished data). Amidase production is not, therefore, a consequence of the possession of a wall of abnormal composition. A further complication is the observation that mucopeptide preparations from walls of these mutants will not bind amidase as do those from the parent. This suggests possible differences in the mucopeptide itself, yet the composition, the glycan chain length (31), and the degree of cross-linking of the peptides (J. B. Ward, unpublished observation) is the same as in preparations from the parent.

Another type of autolytic-deficient mutant is represented by strain MH-4. This organism has a deficiency in glucose in the wall but has teichuronic acid and phosphoglucomutase (12), which suggests the possibility that it is partly deficient in glucosyl transferase, especially since such a mutant of *B. subtilis* is also deficient in amidase activity (C. W. Forsberg and H. J. Rogers, unpublished data).

The superlytic mutants have a very large amount of amidase compared with the parent organism, sufficient to cause lysis even during exponential growth, although the enzyme activities associated with the membranes are normal. The wall chemistry of these mutants differs quantitatively from that of the parent in the non-mucopeptide components and in the ratio of these to the mucopeptide. These differences, however, may be trivial and may be explained by the constant lysis that is occurring. The ratios of mucopeptide to nonmucopeptide components differ in fractions from enzymic hydrolysates of walls (15). It is then possible that the preparations of the exceedingly lytic walls have suffered hydrolytic loss, during growth, of parts of the mucopeptide with differing amounts of teichuronic and teichoic acids attached. One of the mutants (MH-2) would appear to be a protease-negative organism, and its properties appeared, at least in part, to be due to an increased β-*N*-acetylglucosaminidase content. Protease-negative bacilli are known to contain high levels of this enzyme, presumably owing to its great sensitivity to proteolytic attack (3).

The superlytic mutants illustrate the importance of looking for intracellular enzymes in the culture supernatant of microorganisms suspected of secreting exocellular enzymes as was originally suggested by Pollock (19). In the absence of this test, these organisms would have been said to be secretors of the amidase. In fact, it is likely to be present in the culture supernatant solely as a result of cell lysis even early in the exponential phase of growth.

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