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N-Acetylmuramyl-L-Alanine Amidase of *Bacillus licheniformis* and Its L-Form

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A cell wall lytic enzyme has been demonstrated to be a component of the membrane of *Bacillus licheniformis* NCTC 6346 and an L-form derived from it. The lytic enzyme, characterized as an *N*-acetylmuramyl-L-alanine amidase, is solubilized from membranes by nonionic detergents. Ionic detergents inactivate the enzyme. In the bacterium the specific activities of amidase and D-alanine carboxypeptidase in mesosomes are approximately 65% of those in membranes. Selective transfer of lytic enzyme from nongrowing L-forms, L-form membranes, and protoplasts to added walls occurred after mixing, and 31 to 77% of the enzyme lost from L-form membranes was recovered on the walls. Membranes isolated from L-forms growing in the presence of added walls contained as little as 13% of the amidase found in membranes of a control culture. These results have been interpreted as showing that in vivo the amidase is "bound" to the surface of the bacterial cell membrane in such a location that it can be readily accessible to the cell wall.

Lytic enzymes of gram-positive bacteria have been isolated in the culture fluid as extracellular products (2, 19, 30) and recognized as intracellular activities. In this latter case the enzymes have usually been recovered on walls prepared from the bacterial cells (36, 42), although in other cases variable amounts have been recovered from the disrupted cell supernatant fluid (8, 19, 38).

In *Bacillus subtilis* 168 (42) and *Bacillus licheniformis* (15, 20), the major lytic enzyme present in the cell is an *N*-acetylmuramyl-L-alanine amidase. Partial purification of this enzyme as a complex with teichoic acid has been accomplished (5) and, independently, Fan (12) has probably separated the enzyme-wall complex by treatment at 0 C with 3 M LiCl, a procedure previously used in the separation of the lytic enzyme from autolytic walls of *Streptococcus faecalis* (27). The amidase has been implicated in the turnover of peptidoglycan in the cell wall of growing cells (7, 25) and the separation of dividing cells (15).

Examination of the lytic enzyme present in an L-form of *B. licheniformis* now reveals this enzyme as an *N*-acetylmuramyl-L-alanine amidase present exclusively on the cytoplasmic membrane of the cell. Lytic activity is also present in protoplast and mesosome membranes of the parent bacteria. When intact L-forms or protoplasts are mixed with walls, a large portion of the lytic activity in their membranes is transferred to the walls. These results are discussed in relation to current concepts of the physiological role of the lytic enzyme.

MATERIALS AND METHODS

Chemicals and enzymes. All chemicals used were analytical reagent grade unless otherwise stated. Sodium methicillin was purchased from Beecham Research Laboratories Ltd., Surrey, England. Sodium lauryl sulfate, specially purified (SLS), and polyethylene glycol 600 (PEG) were obtained from BDH Chemicals Ltd., Poole, Dorset, England. Sarkosyl NL97 was from Geigy (UK) Ltd., Manchester, England. Triton X-100 (Triton), sodium deoxycholate (DOC), Brij 58, 2,6-dichlorophenolindophenol, and phenazine methosulfate were obtained from Sigma Chemical Co. Ltd., London, England. ^{14}C-L-aspartic acid (specific activity 208 mCi/mmole, uniformly labeled) and ^{3}H-L-tryptophan (specific activity >2,000 mCi/mmole, uniformly labeled) were purchased from The Radiochemical Centre, Amersham, UK. Deoxyribonuclease type 1 (EC 3.1.4.5) was purchased from the Worthington Co. Ltd., and lysozyme (EC 3.2.1.17) from Armor Pharmaceutical Co. Ltd., Eastbourne, Sussex, England.

Organisms. The bacterial strains used in these experiments were: *B. licheniformis* NCTC 6346, *B. licheniformis* NCTC 6346 *his*, *B. subtilis* 168 *trpC2*, and *Micrococcus lysodeikticus* NCTC 2665. The L-form used was derived from *Bacillus licheniformis* NCTC 6346 (14).

Media and cultural conditions. The bacilli were maintained as spores in water at 4 C; *M. lysodeikticus* was maintained on nutrient agar slopes. The bacilli were grown in either medium A, casein hydrolysate-salts medium containing 0.5% glucose (29), or medium B, Spizizen's minimal medium (37) supplemented with 0.3% sodium glutamate. L-Histidine (final concentration 100 μg/ml) and L-tryptophan (final concentration 20 μg/ml) were added when necessary. *M. lysodeikticus* was grown on a casein hydrolysate-yeast extract-glucose medium (20). All cultures were grown at 35 C with agitation.

The L-form cultures were maintained by weekly subculture in DP medium (22) containing sodium methicillin (200 μg/ml), but without agar. After growth at 35 C for 24 hr the culture was maintained at room temperature. To obtain grown cultures, Erlenmeyer flasks (250 or 500 ml) containing one-fifth their volumes of liquid DP medium were inoculated with 24-hr stationary cultures (10%, v/v) and shaken very slowly for 18 to 24 hr. If included, ^{3}H-L-tryptophan was added to give 0.1 mCi/100 ml of medium, and cell walls in water, sterilized by autoclaving and diluted in 2 × DP medium, were added to give final concentrations of 28 mg of unlabeled walls and 2 mg of ^{14}C-labeled walls per 100 ml medium.

Preparation of cell walls, O-acetylated cell walls and Procion-conjugated cell-agar plates. Cell walls were prepared from late exponential-phase cultures of *B. licheniformis* 6346 grown on medium A and *B. subtilis* 168 grown on medium B. The harvested cells were suspended in 4% SLS for 1 hr and centrifuged, and the cell pellet was resuspended in water. The cells were disrupted in a Braun tissue disintegrator fitted with a CO_2 cooling device. *M. lysodeikticus* cells were not treated with SLS before disruption. The walls were separated from the solubilized material by centrifugation at 30,000 × *g* for 20 min, washed once with 4% SLS, three times with 1 M NaCl, four or five times with water, and then lyophilized. The *B. licheniformis* walls were incubated in 0.1 M NH_4OH at 35 C for 2 hr to remove the ester-linked alanine and subsequently washed in water and lyophilized (34). Radioactive ^{14}C-cell walls (93,000 dpm/mg) were prepared as outlined above from *B. licheniformis* 6346 cells which had been grown in a synthetic medium (29) containing added casein hydrolysate (0.02%), L-aspartic acid (0.3 mg/ml), and ^{14}C-L-aspartic acid (0.6 μCi/ml).

O-Acetylated cell walls of *B. licheniformis* were prepared by the method of Brumfitt et al. (6).

Procion-conjugated cell walls in sandwich plates made with DP agar medium containing 0.8% agar were prepared by the procedures outlined by Forsberg and Rogers (15).

Preparation of crude N-acetylmuramyl-L-alanine amidase. Cell walls containing amidase (20) were prepared from logarithmic-phase cells of *B. licheniformis* 6346 grown on medium B. The cell walls (10 mg/ml) were allowed to lyse in sodium carbonate buffer [ionic strength (I) 0.05, *p*H 9.5]; after centrifugation the supernatant fraction was dialyzed against water and finally frozen.

Preparation of membranes and mesosomes. L-form membranes were prepared as follows. Deoxyribonuclease (final concentration, 1 μg/ml) was added to growing L-form cultures, and the incubation was continued for 15 min before centrifuging the cultures at 17,300 × *g* for 20 min at 2 C. Unless otherwise stated, all subsequent operations were carried out at 0 to 4 C. The cells were lysed by suspension in 0.01 M tris(hydroxymethyl)aminomethane (Tris)hydrochloride, *p*H 8.0, containing 0.01 M $MgSO_4$ (Tris-Mg^{2+} buffer) and added deoxyribonuclease (5 μg/ml) at 30 C for 5 min. Membranes were recovered by centrifuging them at 35,000 × *g* for 20 min, washed twice in buffer without deoxyribonuclease, and resuspended at a protein concentration of 5 to 10 mg per ml.

Protoplast membranes and mesosomes were prepared essentially as described by Reaveley and Rogers (29) from bacterial cells in the logarithmic phase of growth. Cells were harvested by centrifugation at 8,000 × *g* for 10 min and washed once in 0.05 M sodium maleate buffer (*p*H 6.5) containing 0.01 M $MgSO_4$ and 20% (v/v) PEG. The washed cells were resuspended to a concentration of 10 mg (dry weight) per ml in the same buffer containing lysozyme (40 μg/ml) and incubated with gentle shaking at 35 C. Formation of protoplasts was followed microscopically, and when it was judged to be complete (usually 1 to 2 hr) the protoplasts were harvested by centrifugation at 30,000 × *g* for 30 min. The protoplasts were either washed three times in the same medium without lysozyme, or lysed immediately by centrifuging and suspension in Tris-Mg^{2+} buffer containing deoxyribonuclease (5 μg/ml). After incubation at 30 C for 5 min, membranes were recovered by centrifugation at 30,000 × *g* for 20 min and washed twice in the same buffer without deoxyribonuclease before being resuspended at a protein concentration of 10 to 15 mg/ml.

Mesosomes were recovered from the initial protoplast supernatant fluid by centrifuging at 200,000 × *g* for 60 min. They were washed twice with Tris-Mg^{2+} buffer before being resuspended at a protein concentration of 10 to 15 mg/ml.

For the preparation of membranes from mechanically rather than enzymically disrupted cells, bacteria were washed in Tris-Mg^{2+} buffer and resuspended in the same buffer at 10 to 20 mg (dry weight of cells) per ml. Cells (10 ml) plus Ballotini beads (7 ml) were shaken for 10 min in a Mickle disintegrator at 4 C. The glass beads were removed by filtration. The membranes and walls were treated with deoxyribonuclease, collected, and washed by the procedure outlined for disrupted protoplasts. The walls were subsequently separated from the membranes by centrifugation on a sucrose gradient.

All membrane and mesosome preparations were purified on sucrose density gradients. Linear gradients of 40 to 70% sucrose (15 ml total volume) in Tris-Mg^{2+} buffer were prepared over pads (1 ml) of 70% sucrose in the same buffer. Membrane and mesosome suspensions (2 to 3 ml) in 20% sucrose were layered on the upper surfaces of the gradients and centrifuged at 139,000 × *g* for 5 hr at 4 C. After this treatment the mesosomes and membranes were banded in the gradients, and the walls were at the bottom of the tubes.

Fractions (1 ml) were taken successively from the bottom of each gradient with a modified automatic syringe, taking care not to disturb any sedimented material present. The E_{280} value (extinction at 280 nm) and refractive index of each fraction were recorded. Densities were calculated from the refractive index measurements (10). Radioactivity in cell walls (^{14}C) and protein (^{3}H) was located in gradient fractions by counting 0.05-ml samples in 0.5 ml of water plus 10 ml of dioxane scintillation fluid (4). The radioactivity in all samples was counted in a Packard 3000 Tri-Carb liquid scintillation spectrometer. The differential counting efficiency for ^{14}C was 44.2% and that for ^{3}H was 7.9%.

Membranes were recovered from the appropriate sucrose gradient fractions by diluting them in four volumes of 1.0 M NaCl and 0.01 M $MgSO_4$ and centrifuging them at 30,000 × *g* for 20 min. Mesosomes were treated in a similar manner and centrifuged at 200,000 × *g* for 60 min. The samples were washed one or two times in the same salts solution and suspended in 0.02 M sodium pyrophosphate (*p*H 8.5) at a concentration of up to 10 mg (protein) per ml.

The membrane and mesosomal preparations were examined for purity by electron microscopic examination of negatively stained preparations by I. D. J. Burdett of this Institute.

Treatment of the membrane preparations by various detergents and inorganic salts to effect release of lytic activity was carried out at the concentrations described at 0 C for 15 min. In all cases insoluble material was sedimented by centrifuging at 30,000 × *g* for 30 min, although with many of the detergent treatments the amount of sedimentable material was negligible.

Enzyme assays: (i) N-acetylmuramyl-L-alanine amidase. The sample was mixed with 0.4% Triton in 0.5 ml of 0.02 M sodium pyrophosphate (*p*H 8.5) on ice for 5 to 10 min. *B. licheniformis* cell walls (2.5 mg) and sodium carbonate buffer (I = 0.05, final concentration) were added to produce a final volume of 4 ml in matched round cuvettes. The tubes were transferred to 35 C and the decrease in optical density at 450 nm was measured at intervals in a Unicam SP-600 spectrophotometer.

(ii) Lysozyme activity. This was assayed as in (i) except that *M. lysodeikticus* walls were used as substrate and sodium phosphate (*p*H 6.5) as the buffer (I = 0.05, final concentration) (26). For both assays, 1 unit of activity was defined as that amount of enzyme giving a linear decrease of 0.001 optical density units per min. Specific activity was defined as units per milligram of protein.

(iii) Succinic dehydrogenase. Succinic dehydrogenase (EC 1.3.99.1) was assayed by a modification of the procedure of Arrigoni and Singer (1). The reaction mixture contained 50 μmoles of potassium phosphate buffer (*p*H 7.0), 1 μmole of KCN, 4 nmoles of 2,6-dichlorophenolindophenol, 0.3 μmoles of phenazine methosulfate, 20 nmoles of sodium succinate, and enzyme in a total volume of 1.0 ml. The reaction was followed at 25 C by the decrease in optical density at 600 nm in a Unicam SP-500 spectrophotometer. Specific activity was expressed as micromoles of 2,6-dichlorophenolindophenol reduced per minute per milligram of protein at 25 C. An E_{600} (*p*H 7.0) for 2,6-dichlorophenolindophenol of 19,100 was used (18).

(iv) D-Alanine carboxypeptidase. D-Alanine carboxypeptidase was assayed by the release of D-alanine from uridine diphosphate (UDP) muramyl-L-alanyl-D-isoglutamyl-*meso*-diaminopimelic acid ^{14}C-D-alanyl-^{14}C-D-alanine prepared by the method of Ito et al. (21). The reaction mixture at 25 C contained 6 μmoles of potassium acetate (*p*H 6.0), 175 nmoles of zinc acetate, 10 nmoles of UDP-muramylpentapeptide (0.5 μCi per μmole of D-alanine), and enzyme in a total volume of 40 μliters. The reaction was stopped by heating at 100 C for 2 min, and free alanine was converted to the dinitrophenol (DNP) derivative by addition of 60 μliters of sodium bicarbonate (1%, w/v), 25 μliters of sodium tetraborate (5%, w/v), and 12.5 μliters of 1-fluoro-2,4-dinitrobenzene (130 μliters in 10 ml of ethanol), followed by heating at 60 C for 30 min. The reaction mixture was acidified with 200 μmoles of HCl and DNP amino acids were extracted with ether. DNP amino acids were separated by thin-layer chromatography in silica gel G, using chloroform-methanol-glacial acetic acid (95:5:1, v/v) as the solvent. Spots corresponding to standard DNP-alanine were eluted with methanol-1 M NH_4OH (1:1, v/v), taken to dryness, redissolved in 0.2 ml of water and counted with 10 ml of dioxane scintillation fluid (4).

Specific activity is defined as nanomoles of D-alanine released per hour per milligram of protein at 25 C.

Analytical methods. Amino acids and amino sugars were determined with a Beckman-Spinco automatic amino acid analyzer after hydrolysis of the samples in 6 N HCl for 16 hr at 105 C for amino acids, and 4 N HCl for 4 hr at 100 C for amino sugars. Diaminopimelic acid (DAP) was measured quantitatively in wall hydrolysates by the method of Work (41). For qualitative identification of DAP, samples were run with a standard sequentially in three solvent systems: isobutyric-1 M-ammonium hydroxide (5:3, v/v); butan-1-ol-pyridine-water (6:4:3, v/v); methanol-water-pyridine-HCl (32:7:4:1, v/v). The spots were detected with ninhydrin. Protein was determined by the method of Lowry et al. (24) with bovine plasma albumin as a standard. Deoxyribonucleic acid was estimated by the method of Giles and Myers (17). Reducing groups were measured by the method of Thompson and Shockman (39), with glucose as the standard. The release of free amino groups after lysis of purified cell walls was measured essentially as described above for the assay of D-alanine carboxypeptidase. Samples containing cell wall materials (1.33 mg) were reacted with fluorodinitrobenzene in potassium borate as described. Dinitrophenylated fragments were hydrolyzed in 4 N HCl at 105 C for 16 hr. DNP alanine was separated from the hydrolysate by thin-layer chromatography, the spots were eluted with methanol-1 M NH_4OH (1:1, v/v), and the extinction was read at 360 nm; E_{360} of DNP-alanine was taken as 15,000 (16). To establish the configuration of the alanine released, the dinitrophenyl derivative, purified as described above, was dissolved in 0.125 M Na-

HCO_3 and its optical rotatory dispersion (ORD) was measured in a Polarmatic 62 apparatus (Bellingham and Stanley, London, N15, UK) fitted with a Hewlett-Packard 7005B X-Y recorder.

RESULTS

Detection and solubilization of the lytic activity in L-forms. Autolysin activity in colonies of bacilli has previously been detected by formation of clear haloes on agar containing Procion Red dye-conjugated cell walls (15). When *B. licheniformis* L-forms were plated out on an osmotically supported cell wall agar, similar clarification of the medium was observed, suggesting the presence of a cell wall lytic enzyme. However, lytic activity was barely detectable when L-form cells were osmotically disrupted and incubated with a suspension of cell walls. Attempts to increase the yield of lytic activity by sonic treatment and freezing and thawing the cell suspension were not successful. However, addition of the non-ionic detergent, Triton, to the incubation mixture brought about solubilization of the wall suspension (Table 1, A). The detergent alone was without effect.

Washing membranes in 0.01 M Tris-chloride buffer (*p*H 8.0) resulted in a gradual loss of protein without significant release of lytic activity in an active form. Addition of $MgSO_4$ (0.01 M) to the washing solution prevented the loss of protein but decreased the amount of lytic activity solubilized by subsequent treatment with Triton. Membranes were more completely solubilized and more lytic activity was released by treatment in the presence of NaCl (Table 1, B). Since NaCl aided in the solubilization process, the membranes were routinely washed in a 1.0 M NaCl-0.01 M $MgSO_4$ solution (*p*H 6.0) and suspended in 0.02 M pyrophosphate (*p*H 8.5) for detergent treatment. Lytic activity was not released from the membranes by washing them in either 1.5 M NaCl or 3.0 M LiCl. The ionic detergents SLS (0.4%) and Sarkosyl (0.4%) caused complete inactivation of the solubilized lytic enzyme, whereas DOC (0.4%) resulted in only partial inactivation. Brij 58 (0.4%) was as effective as Triton in the release of lytic activity.

With Tris-chloride-washed membranes, detergent concentrations in excess of that necessary to release maximum lytic activity had little inhibitory effect (Table 2).

Localization of the lytic activity in L-forms. When lytic activity was measured at points during growth of L-forms in liquid culture, the activity was found to be proportional to cell mass (as measured by optical density) of the culture.

Lytic enzyme was located (93%) in the membrane fraction of L-forms, which accounted for 23.2% of the cellular protein and contained 42.1% protein by weight. No nucleic acid was detected. Amino acid analysis and paper chromatography showed the absence of muramic acid and DAP from acid hydrolysates of the membranes.

Specificities of the lytic enzymes from L-form membranes and autolytic cell walls. Lysis of cell walls by solubilized L-form membrane preparations was accompanied by the release of *N*-terminal L-alanine, established by ORD of the dinitrophenyl derivative (Fig. 1); there was no detectable increase in other *N*-terminal amino acids or in the reducing power of the lysate. Thus, the only demonstrable lytic enzyme at *p*H 9.5 is an *N*-acetylmuramyl-L-alanine amidase. Activity of the L-form and bacillary enzymes were compared, with *B. licheniformis*, *B. subtilis*, *M. lysodeikticus*, and *O*-acetylated *B. licheniformis* cell walls used as substrates (Table 3). *O*-Acetylation has previously been shown to be responsible for a reduction in lytic activity of egg white lysozyme on *M. lysodeikticus* cell walls (6). The specificities of the amidase preparations were very similar with the exception of minor differences in their activities on *B. subtilis* walls (Table 3).

TABLE 1. *Effect of method of treating L-form membranes on their lytic activity*

Washing procedure	Treatment	Lytic activity (units)
A. Washed in 0.01 M Tris-chloride (*p*H 8.0) and resuspended in 0.005 M Tris-chloride (*p*H 8.0)	Untreated	0.17
	Sonically disrupted	0.30
	Freezing and thawing	0.20
	0.1% Triton	1.30
B. Washed in 0.01 M Tris-chloride (*p*H 8.0) + 0.01 M $MgSO_4$ and resuspended in water	0.2% Triton	0.96
	0.4% Triton	0.96
	0.4% Triton + 0.5 M NaCl	1.41
	0.4% Triton + 2.0 M NaCl	1.16

TABLE 2. *Effect of Triton on the release of lytic activity from L-form membranes*[a]

Triton (%)	Lytic activity (units)
0	0.4
0.005	0.5
0.025	0.9
0.050	1.0
0.10	1.7
0.20	1.9
0.50	1.6
1.00	1.5

[a] Membranes were from a 500-ml culture in 4.5 ml of 0.005 M Tris (*p*H 8.0) (protein concentration 1.6 mg/ml). Either water or detergent was added to samples; after centrifuging, the supernatant fractions were assayed.

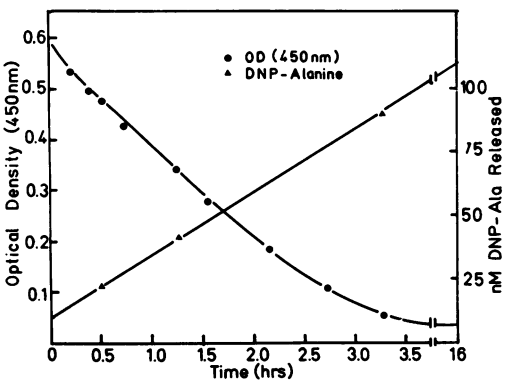

FIG. 1. *Decrease in optical density and increase in N-terminal L-alanine during the lysis of B. licheniformis cell walls by bound L-form lytic enzyme.*

Test for lysozyme contamination. To extend the amidase localization study from L-forms to the parent bacterium it was necessary to develop a method to test membranes for contamination by lysozyme which may have become absorbed during the course of preparation. Examination of the lysis of *B. licheniformis* and *M lysodeikticus* cell walls by amidase and lysozyme at their *p*H optima revealed several differences in their properties (Table 4). At *p*H 6.5 *M. lysodeikticus* walls were nearly insensitive to amidase but very susceptible to the activity of lysozyme. Walls of *B. licheniformis* are relatively insensitive to lysozyme, a difference accentuated at *p*H 9.5. Therefore, by assaying for lysozyme at *p*H 6.5 on *M. lysodeikticus* walls and for amidase at *p*H 9.5 on *B. licheniformis* walls, a relatively specific estimation of each enzyme was possible. A mixture of lysozyme and amidase assayed on *B. licheniformis* walls at *p*H 9.5 showed a 41% increase in activity over that expected had their activities been additive. Similarly, at *p*H 6.5 the same enzyme mixture assayed on *M. lysodeikticus* walls produced a 15% synergistic effect. After correcting for these synergistic effects, the maximum levels of contamination of the membranes by lysozyme were 0.013 to 0.29 units of amidase activity per mg of protein.

Preparation of protoplast membrane and mesosome fractions and localization of enzyme activities. Protoplasts were prepared and membranes and mesosomes were isolated and washed. The protoplast membranes and mesosomes were further purified by centrifuging them through a linear sucrose gradient (Fig. 2A, B). Under these conditions the membrane preparation had a density of 1.21 g/cm^3 whereas that of the mesosomes was 1.17. In addition, the membrane gradients usually contained a small pellet of aggregated phase-light vesicles which was discarded. Examination of

TABLE 3. *Lysis of cell walls by lytic enzymes from L-form membranes and a cell wall amidase preparation of Bacillus licheniformis*

Walls	Rate of wall lysis[a]	
	L-form membrane amidase	Cell wall amidase
B. licheniformis	100 (100)[b]	100 (100)
Micrococcus lysodeikticus	9.6	8.1
B. subtilis 168	19.2	4.2
O-Acetylated *B. licheniformis*	(3.5)	(2.5)

[a] The amounts of L-form and cell wall amidase used in each assay were 4.8 and 7.5 units, respectively. The activity of these preparations on *B. licheniformis* walls was taken as 100.

[b] Results in parentheses are from assays conducted in 0.05 M phosphate buffer (*p*H 8.0); the other assays were as outlined in the procedure.

TABLE 4. *Lysis of Bacillus licheniformis and Micrococcus lysodeikticus cell walls by amidase and lysozyme*

Walls	*p*H	Rate of wall lysis[a]	
		Amidase	Lysozyme
B. licheniformis	9.5	100.0	4.3
	6.5	18.0	8.6
M. lysodeikticus	9.5	8.2	32.0
	6.5	8.2	100.0

[a] The amounts of cell wall amidase and lysozyme used in each assay were 19.6 and 25.0 units, respectively. The sample in each series with the maximum rate of wall lysis was taken as 100.

the mesosome preparations by electron microscopy, using a negative staining technique, revealed the presence of tubules and vesicles but no large membranous sheets; there was also a slight contamination with flagella. The material in the membrane band consisted almost entirely of protoplast ghosts. Table 5 shows the distribution of enzymatic activities between protoplast membranes and mesosomes. Succinic dehydrogenase was chosen as a control enzyme, because its distribution between similar preparations has previously been studied in detail (13, 29). The mesosomes contained on average 10.5% of the specific activity found in the protoplast membranes, and values ranged from 8.5 to 13.0%. Some variation in the absolute specific activities of succinic dehydrogenase in the membranes was found in the course of these and subsequent experiments. However, in a given experiment, fractions were always assayed simultaneously under identical conditions. There was considerably less difference between the *N*-acetylmuramyl-L-alanine amidase and D-alanine carboxypeptidase activities in membranes and mesosomes. The mesosomes consistently contained slightly lower amounts of both. The activities and distribution of enzymes were similar in both the wild type and the histidine auxotroph of *B. licheniformis*.

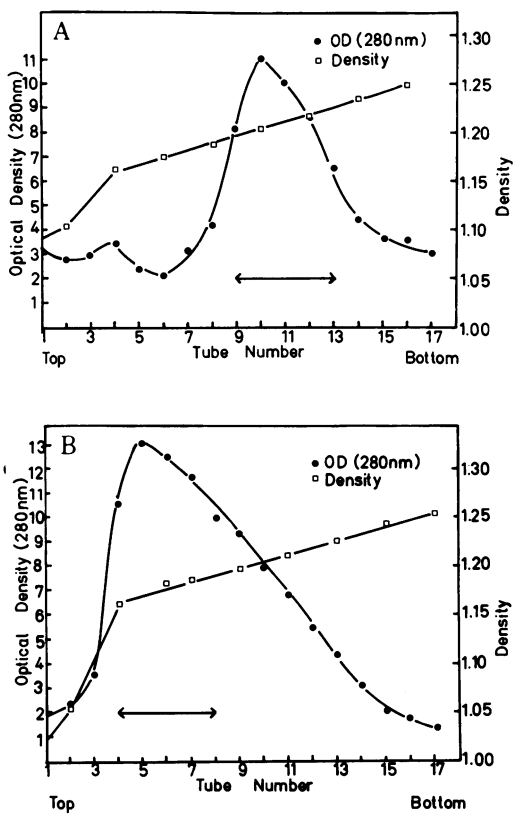

FIG. 2. *Density gradient centrifugation of (A) protoplast membranes and (B) mesosomes in sucrose gradients. Arrow markers indicate the fractions pooled for collection of either membranes or mesosomes.*

Membrane preparations assayed in the absence of Triton showed 16% of the lytic activity of those assayed in its presence. After detergent treatment, more than 75% of the activity was recovered from the fraction not sedimenting in 2 hr at 160,000 × *g*. The resuspended pellet exhibited 8% of the lytic activity, and this amount could be slightly increased by retreatment with detergent. Lysozyme contamination of the protoplast membranes and mesosomes was variable, even when they were prepared under apparently identical conditions. However, in those preparations studied, it never exceeded the equivalent of 3% of the amidase activity.

Effect of growing L-forms in the absence and presence of walls. Clive and Landman (9) have demonstrated the reversion of L-forms of *B. subtilis* to bacterial forms by addition of cell walls to the growth medium and have suggested that one possible function of the cell walls was to inhibit the action of wall-destroying enzymes. To test whether the presence of walls in growing cultures had any effect on amidase production, L-forms were grown in liquid culture in the presence and absence of *B. licheniformis* cell walls. No reversion of the L-forms was observed under

TABLE 5. *Specific enzyme activities of membranes and mesosomes from Bacillus licheniformis*

Enzyme	Specific activities	
	Membranes	Mesosomes
Amidase	18.1 [3.0][a] (15.0)[b]	11.1 (9.7)
D-Alanine-carboxypeptidase	57.1 (54.4)	46.7 (28.8)
Succinic dehydrogenase	0.62 (0.51)	0.069 (0.043)

[a] Activity without addition of Triton obtained by subtracting the turbidity due to the membranes.
[b] Results in parentheses are from a separate experiment.

these growth conditions. The L-form cells together with any residual wall material were harvested, and lytic activities were measured before and after solubilization of membranes with Triton. The amidase present in L-forms grown in the absence of walls was present in the membrane (detergent-soluble) fraction. In cells grown in the presence of walls, up to 80% of the total lytic activity was on the walls and was therefore expressed without addition of detergent to the mixture.

To ensure that the above observation was not an artifact, it was necessary to separate the added cell walls from the L-form cells or membranes prepared from them. Disrupted cells and wall mixtures were centrifuged through sucrose gradients. No differences were observed in the membrane band profiles and densities from cells grown in either the presence or absence of cell walls (Table 6). L-form membranes routinely produced two bands on density gradients, but not the small pellet found at the bottom of protoplast membrane gradients. Similar observations of multiple bands have been made in *B. licheniformis* (29) and are related to the effects of Mg^{2+} concentration on chemical composition of the membrane fractions. Figure 3 shows that added cell walls can be cleanly separated from membranous material after centrifugation at 139,000 × *g* for 5 hr. Both the light and heavy bands of membranes in each preparation contained lytic enzyme with similar specific activities, but the amount of enzyme present in membranes prepared from cells grown in the presence of walls ranged from 12.2 to 38.9% of that found when the L-forms were grown in the absence of walls.

This result suggests that, when L-forms were mixed with walls, the amidase was transferred from the L-form cells to the walls. However, this same effect would also be observed if partial inactivation of the enzyme occurred. To distinguish these possibilities, L-forms were incubated with walls; membranes were prepared from the cells and then were separated from the walls by centrifugation through a sucrose gradient. The membranes were assayed for detergent-soluble lytic enzyme and the walls for lytic activity in the absence of Triton. Table 7 shows that 38 to 58% of the amidase activity was lost from the L-form membranes. This decrease in enzyme activity also occurred when lysed L-forms were mixed with walls. Of the amidase lost from membranes, 31 to 77% was recovered with the walls. A selective transfer of the amidase had occurred since protein contamination of the recovered walls was 1 to 3%, a level insufficient to account for the active lysin associated with them if it were in a membrane-bound form. The added walls appeared to bind all free enzyme, as none was detectable in the culture supernatant fluid. A control in which cell wall amidase was mixed with walls and the lysin-containing walls were carried through the experiment showed that the activity of the bound amidase remained constant.

TABLE 6. *Specific enzyme activities of membranes prepared from L-forms growing in the presence and absence of added cell walls*

Sample	Density	Specific activities	
		Amidase	Succinic dehydrogenase
Added walls			
Light band	≤1.14	0.53 (1.98)[a]	0.165
Heavy band	1.22	0.50 (0.89)	0.132
No walls			
Light band	≤1.14	4.33 (5.12)	0.146
Heavy band	1.22	3.90 (5.60)	0.140

[a] Results in parentheses are from a separate experiment.

Transfer of amidase from protoplasts to added walls. The transfer of amidase from L-forms to walls both during growth and under nongrowing conditions made it appear likely that a similar transfer might occur from protoplasts. Membranes prepared from a mixture of protoplasts and walls after 5 min of incubation contained only 63% of the lytic activity initially present; this amount decreased to approximately 50% on extended incubation (Table 8). Incubation with *O*-acetylated walls was less effective. Attempts to recover the added walls, to measure associated lytic enzyme, were not successful because agglutinated protoplast membranes sedimented with them. Increasing the amount of walls added, to prevent any saturation of the walls with amidase, did not significantly decrease the amidase content of the isolated membranes (Table 9). Since added walls were capable of decreasing the amidase content of protoplast membranes, membranes isolated from mechanically disrupted cells might be expected to contain less enzyme. Membranes isolated from bacteria disrupted with glass beads contained on average 65% of the amidase present in protoplast membranes, while the levels of succinic dehydrogenase in these preparations were identical.

DISCUSSION

The results described in this paper show that the cell wall lytic enzyme in an L-form of

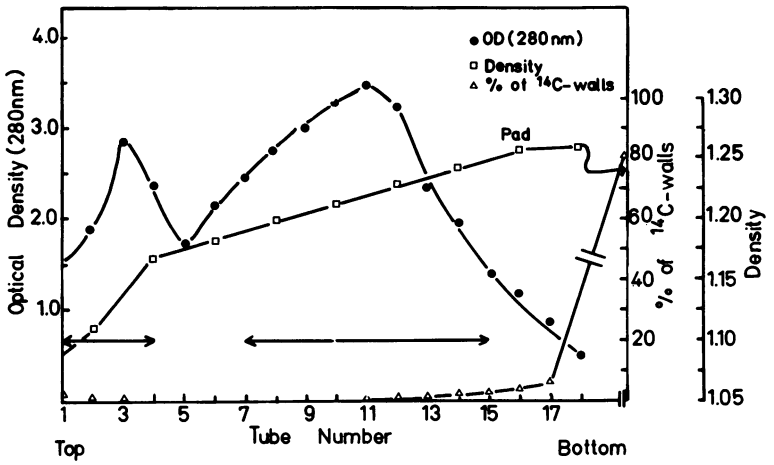

FIG. 3. *Density gradient centrifugation of L-form membranes in the presence of cell walls.*

TABLE 7. *Influence of time of incubating nongrowing L-forms with cell walls on the specific activity of the membrane-bound amidase and the distribution of amidase between the membranes and added walls*

Incubation time with walls (min)	Amidase in membranes (specific activity)	Distribution of amidase (units)[b]		Per cent recovery of amidase
		Membranes	Walls	
0 (no walls added)[a]	5.38	60.2		100
5	4.06	37.2	17.8	91
30	2.61	25.0	20.0	75
120	3.28	28.6	20.0	81
Cells disrupted, walls (30 mg) added, and mixture incubated 5 min	3.33	28.2	9.8	63

[a] L-forms (900 ml), grown in the presence of ^{3}H-tryptophan, were harvested and suspended in 90 ml of DP medium (without additions). Two 15-ml samples were removed, the cells were centrifuged, and the cell pellets were lysed in Tris-Mg^{2+} buffer. Cell walls (30 mg in 5 ml of same buffer) were added to one sample and mixed for 5 min. Cell walls (20 ml, 6 mg/ml) in DP medium were added to the remaining cells and the contents were incubated at 35 C with shaking. Samples were removed at 5, 30, and 120 min, membranes were prepared and subsequently separated from walls.

[b] Recovery of membrane in each sample was estimated from the radioactivity applied to a gradient and that recovered in the isolated membranes. The recovery of cell walls was estimated from the diaminopimelic acid in walls added to the incubation mixture and that recovered from each gradient pellet after one wash in water. These figures were used to estimate the total lytic activities in membranes and walls.

B. licheniformis is located in the cell membrane. This enzyme, when "bound" to the membrane, exhibits only 18% of the activity obtained after solubilization of the membrane with nonionic detergents. Treatment with ionic detergents, while affecting solubilization of the membrane, resulted in partial (DOC) or complete (SLS, Sarkosyl) inactivation of the lytic activity. Salts alone are without effect in releasing lytic activity from the membrane. Thus the enzyme appears to be attached to the membrane through nonionic bonds, in contrast to ionic bonding which may be the mechanism by which lytic enzymes are bound to cell walls (12, 27).

The lytic enzyme in the L-form is an *N*-acetylmuramyl-L-alanine amidase of similar specificity to that of the parent bacterium (15, 20). The finding that lytic activity of the L-form is proportional to cell density throughout the growth cycle is somewhat unexpected and differs from previous findings where the potential for lysis of *B. licheniformis* (Forsberg and Rogers, *unpublished data*) was found to be greatest during exponential growth. In *B. subtilis* the amount of lytic enzyme isolated bound to cell walls also reached a maximum at this time (42). The susceptibility to lysis of purified walls, prepared throughout the growth cycle, did not significantly alter, suggesting changes in level of the enzyme rather than changes in the substrate. This subtle difference between

TABLE 8. *Influence of time of incubating protoplasts with cell walls on the specific enzyme activities of the isolated membranes*

Incubation time (min)	Specific activities	
	Amidase	Succinic dehydrogenase
0 (just prior to addition of walls)	13.2	0.43
5	8.4	0.38
15	6.0	0.36
30	6.6	0.33
60	5.6	0.29
120	6.8	0.28
O-Acetylated walls (17 hr, 4 C)	8.5	0.47

[a] Washed protoplasts (90 ml) at 10 mg (dry weight) per ml in 0.05 M maleate buffer (*p*H 6.5, 0.01 M Mg^{2+}) and 20% (v/v) PEG were mixed with an equal volume of buffer-PEG solution containing 120 mg of ^{14}C-walls (3,100 dpm/mg) or a proportional amount of *O*-acetylated walls. The mixture was incubated with slow shaking at 35 C. Samples were removed at intervals for the preparation of membranes and measurement of optical density at 600 nm. No decrease in the optical density of the suspension occurred during the course of the experiment.

the L-form and the bacteria possibly reflects an alteration in the affinity of the bacterial membrane for the enzyme rather than an increase in enzyme content or activity during exponential growth.

The amidase and D-alanine carboxypeptidase both had slightly lower activities (65%) in mesosomes than in membranes. These findings are consistent with the suggestion (32) that the mesosomes may represent areas within the cells where membrane growth occurs faster than wall extension. Differences in the levels of various enzymes found in this study and previously (29) may represent the sequential addition of these enzymes during membrane biosynthesis. However, on the basis of the experiments reported, one cannot rule out the possibility of enzyme transfer from protoplast membranes to mesosomes during extrusion of the latter on lysis of the cell. This would result in a transfer of amidase and D-alanine carboxypeptidase similar to that found between membranes and walls. The D-alanine carboxypeptidase of *B. subtilis* has previously been localized in a particulate fraction (23). The distribution of the carboxypeptidase, like that of the amidase, supports the contention that mucopeptide synthesis and turnover occur in both mesosomes and membranes; however, recent observations suggest that the action of the carboxypeptidase is not an essential step in these processes (3). These results do not preclude the possibility that wall biosynthesis in mesosomes and membranes is of a different nature, that of the mesosomes being primarily concerned with initial synthesis whereas wall extension and thickening occur essentially at sites located on the membrane.

During the growth of L-forms in the presence of walls, 87% of the amidase activity was lost from the membranes. Since no more enzyme was recovered in the walls than could be accounted for in membranes prepared from L-forms grown in the absence of walls, it was unlikely that the production of amidase was stimulated by the presence of walls in the growing L-form culture. When nongrowing L-forms or protoplasts were incubated with walls for shorter periods of time, the amidase activity of the membranes decreased by some 50%. The recovery (31 to 77%) on added walls of amidase lost from L-form membranes, without significant transfer of protein, suggests that the enzyme is located predominantly on the surface of the membrane. Transfer probably results because the cell wall, the ultimate site of enzyme action, has a greater affinity for the enzyme protein than does the membrane.

A surface location of the amidase is consistent with the extensive turnover of walls in batch-cultured bacilli (7, 25) and in either a steady state (Forsberg and Rogers, *unpublished data*) or a transitional state of growth (11) in a chemostat. Such a location has previously been postulated for the lytic enzymes responsible for turnover in *B. megaterium* (7), and a similar situation may exist for the ami-

TABLE 9. *Effect of wall concentration on the specific enzyme activities of membranes prepared from protoplasts incubated with the walls*[a]

Walls (mg)	Specific activities	
	Amidase	Succinic dehydrogenase
0	11.9	0.162
15	5.6	0.151
60	4.9	0.174

[a] Washed protoplasts (10 ml) at 10 mg (dry weight) per ml in 0.05 M maleate buffer (*p*H 6.5, 0.01 M Mg^{2+}) containing 20% (v/v) PEG were mixed with an equal volume of the same buffer-PEG solution containing the appropriate concentration of cell walls. The mixtures were incubated at 35 C with slow shaking for 45 min before membranes were prepared and isolated. No decrease in optical density of the suspension occurred during the course of the experiment.

dase in *Escherichia coli* (40). The observation that turnover does not involve newly synthesized wall (25) suggests that the amidase is not predominantly associated with the growing point. These findings contrast with those previously reported for *S. faecalis* where the lytic enzyme, present largely as a cytoplasmic proenzyme (28), has been shown to degrade newly synthesized wall first in the intact cell (35). Recently, Rogers and Forsberg (33) have shown that prolonged treatment with chloramphenicol, for a time equivalent to two cell doublings, is necessary to prevent lysis of exponentially growing *B. licheniformis* after addition of vancomycin to the culture. Lysis of *Staphylococcus aureus* H, in which the lytic enzyme is known to be predominantly intracellular (38), is prevented if chloramphenicol is added at the same time as the antibiotic inhibiting cell wall biosynthesis (31). One possible interpretation of these results is that whereas the lytic enzymes of *S. aureus* require protein synthesis to become activated or to be transported from the cytoplasm to its site of action in the wall, the bacillary amidase exists on the membrane surface in close contact with the cell wall.

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