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Extracellular and Cellular Distribution of Muramidase-2 and Muramidase-1 of *Enterococcus hirae* ATCC 9790

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A substantial portion of the second peptidoglycan hydrolase (muramidase-2) activity of *Enterococcus hirae* ATCC 9790 (formerly *Streptococcus faecium*) is present in the supernatant culture medium. In contrast, nearly all muramidase-1 activity is associated with cells in the latent, proteinase-activatable form. Muramidase-2 activity is produced and secreted throughout growth, with maximal levels attained at or near the end of exponential growth in a rich organic medium. Muramidase-2 activity in the culture medium remained high even during overnight incubations in the absence of proteinase inhibitors. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of supernatant culture medium concentrated by 60% saturated ammonium sulfate precipitation showed the presence of several Coomassie blue-staining bands. One intensely staining protein band, at about 71 kDa, selectively adsorbed to the insoluble peptidoglycan fraction of cell walls of *E. hirae*, retained muramidase-2 activity, and reacted in Western immunoblots with monoclonal antibodies to muramidase-2. The mobility of extracellular muramidase-2 in sodium dodecyl sulfate-polyacrylamide gel electrophoresis was indistinguishable from that of muramidase-2 extracted with 6 M guanidine hydrochloride from intact bacteria. Muramidase-2 appears to have only a limited number of binding sites on the peptidoglycan of *E. hirae* cell walls but binds with high affinity. Although high levels of muramidase-2 activity were present in supernatants of stationary-phase cultures, the bacteria were resistant to autolysis. Thus it appears that the peptidoglycan in walls of intact cells of *E. hirae* is somehow protected from the hydrolytic action of extracellular muramidase-2.

Enterococcus hirae ATCC 9790 (formerly *Streptococcus faecium*) was shown to possess two separate and distinct peptidoglycan hydrolase activities; both enzymes are *N*-acetylmuramoylhydrolases (muramidases) (18, 29). Both enzymes are high-molecular-weight, complex proteins that possess a number of rather unusual properties, especially in contrast to avian and bacteriophage lysozymes that hydrolyze the same bond in the cell wall peptidoglycan. Muramidase-1 (M-1) was isolated and purified to homogeneity and was shown to occur in a latent, 130-kDa form that can be proteolytically hydrolyzed to an active, 87-kDa form (17, 24, 30). M-1 was also shown to be a glycoenzyme containing covalently attached monomeric and oligomeric glucose (17) and to possess approximately 12 phosphodiester-linked monomeric 5-mercaptouridine monophosphate residues (8). In addition, M-1 was shown to processively hydrolyze linear, soluble, un-cross-linked peptidoglycan chains (1).

Muramidase-2 (M-2) was partially purified from the supernatant culture medium and was shown to be a 70- to 75-kDa protein by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (18). M-2 was also purified to apparent homogeneity from the insoluble pellet of disrupted *E. hirae* (7). Two polypeptide bands, one of about 125 kDa and the other of about 71 kDa, were shown to possess M-2 activity. Both polypeptides bind radioactive penicillin with low affinity, and evidence was obtained that the 71-kDa form was derived from the 125-kDa form (7). M-2 differs from M-1 in several properties, including substrate specificity. In contrast to M-1, it rapidly dissolves cell walls of *Micrococcus luteus* and dissolves the peptidoglycan fraction of *E. hirae* cell walls. However, M-2 has little ability to dissolve *E. hirae* cell walls, which are a good substrate for purified M-1 activity (18). Both enzyme activities dissolve the re-*N*-acetylated peptidoglycan of *E. hirae* (18). Furthermore, again in contrast to M-1, M-2 fails to bind to concanavalin A-Sepharose and fails to stain with the periodate-Schiff reagent, suggesting that it is not glycosylated (18).

The inability of extracts of a thermosensitive mutant of *E. hirae*, Lyt-14, to dissolve walls of *M. luteus* when grown at the nonpermissive temperature (6) is consistent with the view that M-1 and M-2 are separate gene products, although it remains possible that M-1 results from posttranslational modifications (e.g., glucosylation and nucleotidylation) of M-2.

Attempts to quantify M-1 and M-2 levels in cells and supernatant culture medium proved to be more difficult than initially anticipated. The presence in *E. hirae* of two apparently distinct muramidases, both of which appeared to be present in unfolded or partially folded forms in addition to active forms (7, 8, 17, 18, 29), resulted in the need to efficiently extract and concentrate both activities, either together or separately, and to consider the stability, yield, and cellular locations of each enzyme.

Here we report quantification of the levels of M-1 and M-2 that are produced and secreted during growth in a complex culture medium. In addition, a single polypeptide band that migrates in SDS-PAGE with a molecular mass of about 71 kDa was definitively identified as the polypeptide that possesses M-2 activity, reacts with monoclonal antibodies, and selectively binds to an apparently very limited number of binding sites on the cell wall peptidoglycan but with very high affinity.

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MATERIALS AND METHODS

Bacterial growth. *E. hirae* was grown in a complex medium (S broth [5]). Growth of cultures was monitored turbidimetrically at 675 nm with a Milton Roy (Rochester, N.Y.) model 501 spectrophotometer. One adjusted optical density unit (34) (absorbance adjusted to agree with Beer's law) is equivalent to 0.29 μg cellular dry weight per ml of culture. Cultures were inoculated into prewarmed S broth and grown for selected intervals to several growth phases. The cultures were rapidly chilled in an ice bath, proteinase inhibitors (2 mM EDTA and 0.1 mM phenylmethylsulfonyl fluoride, final concentrations) were added, and cultures were centrifuged (20,000 × *g*, 10 min, 4°C). The bacterial pellets were washed twice with ice-cold distilled water, transferred to one or several microcentrifuge tubes (each containing at most 100 mg cellular dry weight), centrifuged (14,000 × *g*, 10 min, 4°C), and then either stored at −70°C or used immediately for extraction of muramidases. Ammonium sulfate (grade 1; Sigma Chemical Co., St. Louis, Mo.) was gradually added to the decanted supernatant culture medium to 60% saturation. Precipitates were allowed to form for 30 min at 0°C, removed by centrifugation (20,000 × *g*, 30 min, 4°C), immediately dissolved in 10 mM sodium phosphate (pH 7), and stored at 0°C.

Preparation of cell wall matrices. Large-scale cultures of *E. hirae* grown in a complex medium (7) were kindly provided by E. I. du Pont de Nemours & Co., Inc. (Wilmington, Del.). Lyophilized *M. luteus* cells were purchased from Sigma. *E. hirae* and *M. luteus* cells were disrupted as described previously (7, 16). SDS-washed cell walls of *E. hirae* and *M. luteus* and acid-treated and re-*N*-acetylated peptidoglycan of *E. hirae* were prepared as described previously (16). For binding and elution experiments, acid-treated and re-*N*-acetylated peptidoglycan of *E. hirae* was further washed twice with 6 M guanidine-HCl (GuHCl).

Assay of enzymatic activities. M-1 activity was assayed by its ability to dissolve *E. hirae* cell walls (17). Total M-1 activity (latent plus active) was measured in the presence of 0.4 μg of trypsin per ml. M-2 activity was assayed by its ability to dissolve *M. luteus* cell walls or the *E. hirae* peptidoglycan fraction (18). The standard assay system consisted of 0.33 mg of either *E. hirae* or *M. luteus* cell walls per ml or 0.17 mg of *E. hirae* peptidoglycan fraction per ml plus the enzyme preparation in a final volume of 1.5 ml of 10 mM sodium phosphate (pH 7). Routinely, 100-mg (cell dry weight) samples of intact bacteria were incubated with 1 ml of 6 M GuHCl or another extractant at 0°C for 10 min and then centrifuged (14,000 × *g*, 10 min, 4°C). Two successive extractions were performed. Combined extracts (15 μl) were diluted 1 to 100 into the assay buffer (1.5 ml). Ammonium sulfate precipitates of either 100 or 200 ml of supernatant culture medium were dissolved in 4 ml of 10 mM sodium phosphate (pH 7), and 50 μl was added to each enzyme assay tube. Dissolution of substrate was monitored by decrease in A_{450}. One unit of muramidase activity is defined as the amount of enzyme that results in a decrease of 0.001 A_{450} unit per min.

Protein assay. Protein was determined by the bicinchoninic acid method (32) (Pierce Chemical Co., Rockford, Ill.). Since GuHCl interfered with absorbance measurements, standard curves were obtained with bovine serum albumin (Sigma) dissolved in 6 M GuHCl. It was difficult to obtain true levels of secreted proteins in the supernatant culture medium because of the apparent protein content of 60% ammonium sulfate precipitates of S broth (that is, the background content was high). Thus, equivalent volumes of culture media were used to compare the levels of secreted proteins in S broth.

Preparation of samples for SDS-PAGE. The different samples required different treatments before SDS-PAGE analysis, as follows. Proteins in samples (400 μl) of ammonium sulfate precipitates of 100-ml samples of supernatant culture medium were dissolved in 4 ml of 10 mM sodium phosphate (pH 7), precipitated by adding 20 μl of 100% trichloroacetic acid (TCA; final concentration, 5%) for 10 min at 0°C, and then centrifuged (14,000 × *g*, 15 min, 4°C). The TCA precipitates were gently washed with cold (−20°C) acetone, air dried, dissolved in 100 μl of SDS sample buffer (0.0625 M Tris-HCl [pH 6.8], 2% SDS, 10% glycerol, 5% 2-mercaptoethanol), and then boiled (100°C) for 5 min. GuHCl (6 M) extracts of intact bacteria representing cells from 100 ml of culture medium, or of peptidoglycan-enzyme complex derived from the binding of enzyme present in 100 ml of supernatant culture medium, were dialyzed four times against 1.0 liter of sodium phosphate (pH 7) containing 0.1 mM phenylmethylsulfonyl fluoride; then portions (100 μl of 1-ml samples) were precipitated with 5% TCA, and the precipitates were washed in cold acetone as described above. Thus, each 100-μl sample in SDS sample buffer contained protein (from intact cells or supernatant culture medium) produced by bacteria grown in 10 ml of culture medium.

SDS-PAGE. The electrophoresis method used was similar to that of Laemmli (19). The stacking and separating gels were 4.5 and 7.5% polyacrylamide at pH 6.8 and 8.8, respectively. Gels were subjected to electrophoresis and stained with Coomassie blue G-250 as previously described (7).

Production of MAbs against M-2. Monoclonal antibodies (MAbs) against M-2 were generated by S. Drummond and D. L. Dolinger. M-2 purified from the insoluble pellet of disrupted *E. hirae* (25 μg of protein per ml of saline; fraction 7 [7]) was used to immunize female BALB/c mice (at least 8 weeks of age) intraperitoneally (0.2 ml of a 1:1 emulsion of the antigen and Freund incomplete adjuvant; Difco Laboratories, Detroit, Mich.). Seven days later the mice were again injected intraperitoneally with 0.1 ml of the antigen. Additional injections (0.1 ml) were performed 2, 4, and 5 weeks later. Three days after that, fusions were carried out essentially as described previously (13). For detection of anti-M-2 antibodies, enzyme-linked immunosorbent assays were performed with crude alkaline-extracted M-2 bound to the peptidoglycan fraction of *E. hirae* (0.2 μg of protein per well) as the antigen. Supernatants from positive hybridoma cell cultures were further characterized by Western immunoblots. MAbs 2F8, 4B2 and 2D4, directed against M-2, were identified as isotype immunoglobulin G1 by double gel diffusion in 1% agarose (13).

Western blotting and immunodetection. After SDS-PAGE, proteins were transferred (35) to polyvinylidene difluoride (Immobilon-P) membranes (Millipore, Bedford, Mass.) by using an electroblotting apparatus (Hoefer Scientific Instruments, San Francisco, Calif.) for 16 h at 130 mA in a transfer buffer consisting of 25 mM ethanolamine–25 mM glycine–0.0375% SDS in 20% (vol/vol) methanol (pH 9.8). The immunodetection protocol consisted of blocking with 5% nonfat dry milk in 50 mM sodium phosphate (pH 7.4)–150 mM NaCl containing 0.05% (vol/vol) Tween 20 (PBST) and then incubation with an appropriate dilution of the primary antibody (MAb), produced as described above, for 16 to 20 h at 4°C with gentle shaking. After extensive washing in

PBST, a second antibody consisting of affinity-purified alkaline phosphatase-conjugated goat anti-mouse immunoglobulins G and M (heavy and light chains), supplied by Kirkegaard & Perry Laboratories, Inc. (Gaithersburg, Md.), was used. This was followed by washing in PBST and visualization with nitroblue tetrazolium (100 μg/ml) and 5-bromo-4-chloro-3-indolyl phosphate (50 μg/ml) in 100 mM Tris-HCl (pH 9.5) containing 4 mM $MgCl_2$ as the substrates.

RESULTS

Extraction of muramidases from intact bacteria. Previously we reported the extraction of muramidase activity from intact cells of *E. hirae* (and from the insoluble fraction of disrupted bacteria) with very high (e.g., 0.5 M) salt concentrations (23) and later with dilute (0.005 to 0.02 N) sodium hydroxide (5, 17, 18). Although useful for M-1, both treatments were unsuitable for M-2 activity (7, 18). Thus, we recognized the need for alternate procedures for the quantification of both muramidases. Intact bacteria (and the insoluble fraction of disrupted cells) were extracted with a variety of concentrations of an assortment of salts, including the chaotropic salts GuHCl, guanidine thiocyanate, sodium thiocyanate, and lithium thiocyanate. Both muramidase activities were recovered in these extracts, in some cases in amounts two- to threefold higher than that extracted with sodium hydroxide (pH 12), and at comparable specific activities. Experiments were designed so that the concentrations and conditions used would permit refolding and renaturation of proteins in extracts diluted 1 to 100 or more directly into the assay buffer. GuHCl (8.0 M solution, sequanal grade; Pierce Chemical Co.) at concentrations of 2 to 8 M appeared to extract both muramidase activities efficiently. Consistently, the amounts of enzyme activity extracted with 6 M GuHCl per mg of dry bacteria were about 1.5 to 2 times the amounts extracted with sodium hydroxide (pH 12) in parallel extracts. For example, extraction with 6 M GuHCl yielded about 1.5 U of M-1 and 3 U of M-2 per mg (dry weight) of late-exponential-phase bacterial cells (0.89 mg cellular dry weight per ml) extracted. About 0.9 U of M-1 and 1.5 U of M-2 were extracted with sodium hydroxide (pH 12). Extraction with 6 M GuHCl at 0°C appeared to be very rapid, if not instantaneous. Successive extractions with 6 M GuHCl yielded 70 to 85% of total M-1 and M-2 activity in the first extract with the remaining 15 to 30% recovered in a second extract. Thus, two successive GuHCl extracts were routinely combined. The requirement to dilute out the GuHCl to permit renaturation of the enzyme activities limited our ability to estimate the true levels of either native enzyme in the intact bacteria, since the exact level of renaturation for either enzyme was not known. In addition, because the enzyme assay used was indirect and not based on enzymatic cleavage of susceptible bonds, levels of activity appeared to vary with the batch of substrate used. Thus, all comparisons were made from the results of single experiments. Also, the values for enzyme activity given below for cellular content of M-1 and M-2 are for native plus at least a portion of denatured (unfolded) or partially denatured enzymes.

Extracellular and cell-associated muramidase activities. Problems similar to those mentioned above were encountered in detecting and estimating the true levels of the two muramidases in the supernatant culture medium. For example, concentration by filtration through a PTGC membrane (polysulfone filter plates) with the Minitan-S ultrafiltration system (Millipore) proved to be inefficient, probably because of adherence of extracellular proteins to the membrane.

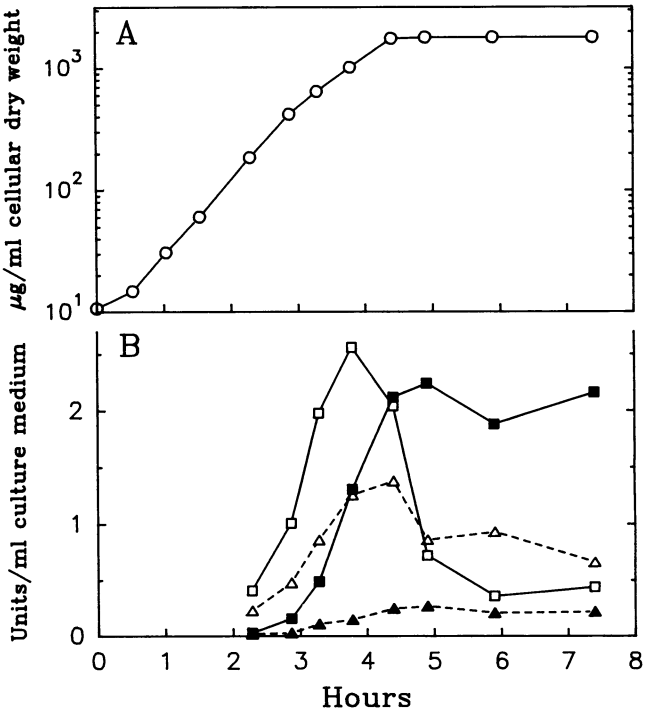

FIG. 1. Production and secretion of M-1 and M-2 during growth of *E. hirae* in S broth: growth curve (A) and total amounts (B) of cellular and extracellular M-1 and M-2 activities. Symbols: ○, cell dry weight estimated from adjusted optical density (34); □, cellular M-2 activity; ■, extracellular M-2 activity; △, cellular M-1 activity; ▲, extracellular M-1 activity. The enzyme assay was performed with *E. hirae* cell walls as a substrate for M-1 activity and *M. luteus* cell walls as a substrate for M-2 activity.

Precipitation with 5% TCA resulted in recoveries of the two enzyme activities that were erratic and, as apparent later, low, probably because of difficulties in redissolving TCA precipitates in small volumes of 6 M GuHCl. The addition of ammonium sulfate to the supernatant culture medium to 60% saturation (or higher) yielded amounts of both muramidases that were reproducible and substantially higher than the amounts recovered by previously used procedures. Dissolving 60% saturated ammonium sulfate precipitates in 6 M GuHCl and then assaying by dilution into the assay buffer resulted in the recovery of about 1.5 times more activity than that present in parallel precipitates dissolved in 10 mM sodium phosphate (pH 7). These data suggest that at least some of the M-2 activity precipitated by ammonium sulfate required unfolding and refolding to exhibit enzyme activity. Thus, we attempted to quantify the amounts and distributions of both muramidases at various stages of growth of cultures of *E. hirae* (Fig. 1); we used ammonium sulfate precipitation to concentrate culture supernatants and 6 M GuHCl to extract cells.

Nearly all of the M-1 activity was associated with the bacterial cells; the amounts of activity present in the culture supernatants were barely detectable. M-1 activity was highest in bacteria taken from exponentially growing cultures. Both the total amount (units per milliliter of culture medium; Fig. 1B) and the concentration (units per milligram of cells; data not shown) of M-1 activity decreased as cultures entered the stationary phase. About 90% of the total (latent plus active) M-1 in cells was in the proteinase-activatable

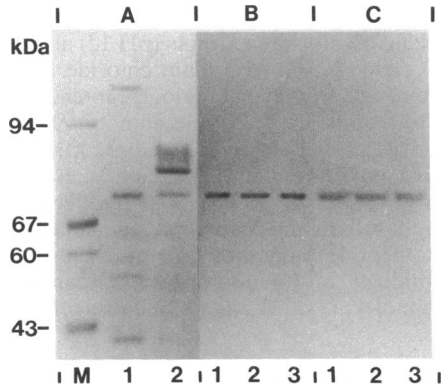

FIG. 2. SDS-PAGE profiles of 6 M GuHCl extracts of intact bacteria and of 60% ammonium sulfate precipitates of a supernatant from a late-exponential-phase culture (0.89 mg cell dry weight per ml; adjusted optical density, 3,100). Shown are a Coomassie blue-stained gel (A) and Western blots with MAb 2F8 (B) and MAb 4B2 (C). Lanes: 1, 6 M GuHCl extract of bacteria; 2, 60% ammonium sulfate precipitate of supernatant culture medium; 3, mixture of one half of the sample applied in lane 1 and one half of the sample applied in lane 2. Samples contain comparable volumes from 7.5 ml of culture for panel A and 2.5 ml for panels B and C. Molecular mass standards (lane M): phosphorylase *b*, 94 kDa; albumin, 67 kDa; catalase, 60 kDa; and ovalbumin, 43 kDa.

latent form. Decreased amounts of cell-associated M-1 were not accompanied by increased amounts of extracellular M-1 activity. Levels of extracellular M-1 activity did not increase after unfolding in 6 M GuHCl followed by refolding by dilution. Thus, M-1 does not appear to be secreted in either a nondenatured or an unfolded form.

M-2 activity was present in the supernatant culture medium and in the bacterial cells throughout growth (Fig. 1B). The maximum amount of cell-associated M-2 was observed in cultures near the end of the exponential phase. After cells had entered the stationary phase, decreased amounts (Fig. 1B) and concentrations (data not shown) of cell-associated M-2 were accompanied by increases in extracellular M-2 until the level of extracellular M-2 was over 4 times the amount present in the cells. Overnight cultures showed about 2 times more extracellular M-2 than was found in early stationary-phase cultures (data not shown).

Comparison of SDS-PAGE profiles of polypeptides and immunoreactive band of GuHCl extracts of bacteria and ammonium sulfate precipitates of extracellular culture medium. At least four Coomassie blue-stained polypeptides, including a band at about 130 kDa and a strong band at about 71 kDa, were observed in the GuHCl extract of bacterial cells (Fig. 2A, lane 1). Concentrated culture supernatants showed the presence of three polypeptide bands (Fig. 2A, lane 2), a diffuse band at about 80 kDa, a strong band at about 75 kDa and a band at about 71 kDa, parallel to the strong band in the cell extract (Fig. 2A, lane 1). Western blots with two different anti-M-2 MAbs, 2F8 (Fig. 2B) and 4B2 (Fig. 2C), showed the presence of only one band at about 71 kDa in either sample or in an equal-volume mixture of the two samples (Fig. 2B and C, lanes 3). These data and previously obtained results that showed that a band at this location contained M-2 activity (7, 18) make it highly likely that this band represents M-2. Furthermore, the presence of only a singly immunoreactive band in the 1:1 mixture of cell extract and concentrated supernatant culture medium (Fig. 2B and C, lanes 3) indicates that the two proteins are

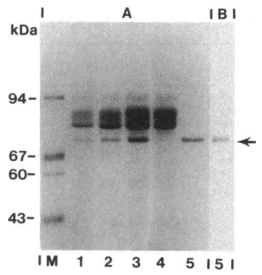

FIG. 3. Selective binding of the extracellular 71-kDa polypeptide to peptidoglycan. Shown are a Coomassie blue-stained gel (A) and a Western blot with MAb 2F8 (B). Lanes: 1 to 3, ammonium sulfate-precipitated supernatant culture medium at 0.38, 0.71, and 1.43 mg cell dry weight per ml, respectively; 4, ammonium sulfate-precipitated supernatant culture medium (1.43 mg cell dry weight per ml) after exposure of the supernatant to peptidoglycan as described in the legend to Fig. 4; 5, protein extracted with 6 M GuHCl from peptidoglycan-enzyme complex. Samples contain comparable volumes from 10 ml of culture for panel A and 2.5 ml for panel B.

indistinguishable by this criterion. The specificities of these two MAbs for M-2 are documented below. However, the 125-kDa form of M-2 (7) is not recognized by these MAbs.

Selective binding of the extracellular 71-kDa polypeptide, M-2 activity, and MAb-reactive material to *E. hirae* peptidoglycan. Consistent with the data shown in Fig. 1, the band at about 71 kDa increased in Coomassie blue staining intensity with increased culture density and increased content of M-2 activity (Fig. 3A, lanes 1 to 3). Exposure of the supernatant culture medium of a stationary-phase culture (1.43 mg cell dry weight per ml) to a sufficient quantity of *E. hirae* peptidoglycan (Fig. 4) resulted in the nearly complete and selective removal of the 71-kDa polypeptide band, leaving the rest of the Coomassie blue-stained bands in the supernatant (compare lanes 4 and 3 in Fig. 3A). This treatment resulted in the removal of about 96% of M-2 activity from the culture supernatant. Much of this activity

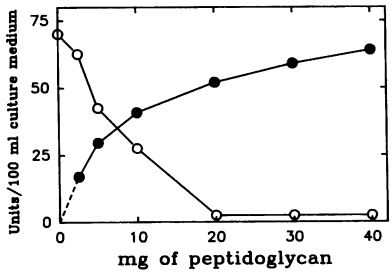

FIG. 4. Stoichiometry of binding of M-2 to acid-treated peptidoglycan of *E. hirae* cell walls. Various amounts of a suspension of acid-treated peptidoglycan fraction of *E. hirae* cell walls were added to 100-ml portions of supernatant from an early-stationary-phase culture (1.3 mg cell dry weight per ml; adjusted optical density, 4,450). After 30 min at 0°C with occasional mixing, the samples were centrifuged (20,000 × *g*, 20 min, 4°C) and the pellets were suspended in 10 mM sodium phosphate (pH 7). Assays for M-2 activity bound to the peptidoglycan (●) were performed with 1.0 mg of each of the resulting peptidoglycan complexes. M-2 activity remaining unbound in the supernatant culture medium (○) was measured by assaying 60% ammonium sulfate precipitates dissolved in 10 mM sodium phosphate with the same *E. hirae* peptidoglycan substrate. This substrate is dissolved more slowly by M-2 than are walls of *M. luteus* (18).

TABLE 1. Stability of extracellular M-2 in the culture medium

Sample	Proteinase inhibitors added[a]	Incubation		M-2 activity[b] (U/ml of medium)
		Time (h)	Temp (°C)	
1	+	0	0	2.4
2	−	0	0	2.1
3	+	17	0	2.2
4	−	17	0	2.0
5	+	17	37	2.3
6	−	17	37	4.0

[a] EDTA and phenylmethylsulfonyl fluoride (2 and 0.1 mM final concentrations, respectively).
[b] *M. luteus* cell walls were used as the substrate.

was recovered in the GuHCl extract of the material bound to the peptidoglycan. This fraction contained only one detectable Coomassie blue-stained band (Fig. 3A, lane 5), which reacted with MAb 2F8 (Fig. 3B, lane 5) in a Western blot. These data provide strong evidence that the 71-kDa polypeptide is M-2 and that the MAb 2F8 reacts with an epitope present on M-2. MAbs 4B2 and 2D4 also react with GuHCl extract of the peptidoglycan-enzyme complex (data not shown).

Stability of M-2 in the culture medium. The data presented in Fig. 1B indicate that the M-2 activity in the culture medium is rather stable. This finding was somewhat surprising, because previous studies (7, 18) showed that partially purified M-2 was unstable and tended to aggregate and precipitate out of aqueous solutions. To further examine the stability of extracellular M-2, cultures were grown to the early stationary phase and the supernatant culture medium was incubated as shown in Table 1. Regardless of the presence or absence of proteinase inhibitors, all samples (except sample 6, in which residual bacterial contamination led to an increase in turbidity and an increase in M-2 activity) contained indistinguishable levels of M-2 activity (Table 1). SDS-PAGE of the samples obtained in the experiment summarized in Table 1 showed complete conservation of a 71-kDa Coomassie blue-positive, anti-M-2 MAb 2F8-positive band without detectable generation of smaller polypeptides (data not shown). These data indicate that the protein, immunoreactivity, and enzymatic activity of extracellular M-2 are stable when it is incubated under these conditions. Consequently, proteinase inhibitors were not used in further experiments (e.g., purification to homogeneity of extracellular M-2 [3]).

Stoichiometry of binding of M-2 to the peptidoglycan fraction of *E. hirae*. Various amounts of acid-treated peptidoglycan fraction of *E. hirae* were used to bind M-2 activity in the supernatant culture medium as described in the legend for Fig. 4. A relatively large quantity of peptidoglycan (20 mg or more per 100 ml of culture medium containing about 70 U of M-2 activity) was required to bind all or nearly all of the M-2 activity in the supernatant culture medium (Fig. 4). Re-*N*-acetylation of the peptidoglycan did not significantly decrease the amount of peptidoglycan required to bind all of the M-2 activity in the supernatant culture medium (data not shown). The affinity of M-2 (and M-1) for its insoluble peptidoglycan or wall substrates appears to be very high; very high salt concentrations (23) or high pHs (4, 17, 18) are required to dissociate these complexes. The extraction of M-2 from cells with GuHCl as described above suggested that GuHCl might also be used to dissociate M-2 from the peptidoglycan-enzyme complex. GuHCl (6 M) extracts of peptidoglycan-enzyme complex yielded about twice the M-2 activity yielded by alkaline extracts (pH 12) and 4 to 6 times the activity yielded by 8 M lithium chloride extracts. Two successive extracts from the peptidoglycan-enzyme complex yielded more than 95% of enzyme activity. Since only one visible Coomassie blue-stained band at 71 kDa was present in 6 M GuHCl extracts (see Fig. 3A, lane 5), essentially all of the protein (137 μg per 10 mg of peptidoglycan) is M-2. Calculation, based on an approximate molecular weight of a disaccharide-peptide monomer unit of 1,100 (2), indicates that the peptidoglycan fraction of *E. hirae* can bind approximately one molecule of M-2 per 4,800 disaccharide-peptide units.

DISCUSSION

Several investigators have used chaotropic salts to extract bacterial cell wall and membrane proteins (4, 10, 25, 26, 33). We found that the chaotropic salts GuHCl, guanidine-thiocyanate, sodium thiocyanate, and lithium thiocyanate could be used to extract both muramidases from the intact bacterial cells and from the insoluble residue (crude cell wall fraction) of mechanically disrupted *E. hirae*. Of the salts tested, GuHCl, which is often used to unfold and denature proteins that can then be refolded and renatured (11, 12, 20), appeared to be the most suitable. Although both muramidase activities were reproducibly recovered by direct dilution (1:100) of 6 M GuHCl extracts into the assay system, the true levels of fully active and of partially or fully unfolded enzyme present could not be precisely determined. Recently, the folding of proteins has been extensively studied (9, 14, 22, 27). Many factors, including pH, temperature, ionic conditions, cofactors, and chaperonins, appear to be involved. In addition, each protein appears to differ in renaturation processing. Therefore, we did not try to optimize conditions for refolding of M-1 and M-2. Although urea has been used to extract cell wall protein (28) and the proteins could be refolded after denaturation (20, 21), urea was not suitable for muramidase activities (data not shown).

Although substantial levels of M-2 are present in the supernatant medium of stationary-phase cultures, a portion of which appears to be in a properly folded, active form, the bacteria are resistant to autolysis. Thus, it appears that the peptidoglycan in walls of intact cells of *E. hirae* is somehow protected from the hydrolytic action of extracellular M-2. It seems possible that *E. hirae* secretes M-2 to the supernatant culture medium to prevent its potentially dangerous (to the producing cell) action. The small number of binding sites on the peptidoglycan (Fig. 4) could provide some protection of cells from its potentially lethal action. This observation is consistent with and confirms previous data indicating that intact cells of *E. hirae* failed to bind significant amounts of added autolysin (probably primarily M-1) unless or until the cell wall is damaged by the action of endogenous autolysin (15). Recently, a very limited number of binding sites for the transglycosylase on murein sacculi of *Escherichia coli* was reported (36). However, since the peptidoglycan fraction used to bind M-2 was the result of the acid treatment of walls, elucidation of this hypothesis requires further study. So far, we have fragmentary information concerning the factors that govern binding of either M-1 or M-2 to its substrate. M-1 binds to nonreducing ends of glycan chains (1) and hydrolyzes the peptidoglycan fraction of walls of *E. hirae* only very slowly unless the peptidoglycan is re-*N*-acetylated (17, 18). In contrast, it appeared that *N*-acetylation is not an important factor for M-2 binding to the

peptidoglycan or for its hydrolysis (18). It should be recalled that M-2 dissolves intact walls of *E. hirae* very slowly (18) and appears to bind to this substrate very poorly (data not shown). We previously discussed the hypothesis that extracellular M-2 functions by facilitating cell separation, consistent with one aspect of the phenotype of a thermosensitive mutant of *E. hirae*, Lyt-14 (31). However, the exact role(s) of either of the two muramidases of *E. hirae* for cell division, cell separation, cell wall expansion, and autolysis remains to be elucidated.

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