Cloning and Sequence Analysis of the Muramidase-2 Gene from *Enterococcus hirae*

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Extracellular muramidase-2 of *Enterococcus hirae* ATCC 9790 was purified to homogeneity by substrate binding, guanidine-HCl extraction, and reversed-phase chromatography. A monoclonal antibody, 2F8, which specifically recognizes muramidase-2, was used to screen a genomic library of *E. hirae* ATCC 9790 DNA in bacteriophage λ gt11. A positive phage clone containing a 4.5-kb DNA insert was isolated and analyzed. The *Eco*RI-digested 4.5-kb fragment was cut into 2.3-, 1.0-, and 1.5-kb pieces by using restriction enzymes *Kpn*I, *Sau*3AI, and *Pst*I, and each fragment was subcloned into plasmid pJDC9 or pUC19. The nucleotide sequence of each subclone was determined. The sequence data indicated an open reading frame encoding a polypeptide of 666 amino acid residues, with a calculated molecular mass of 70,678 Da. The first 24 N-terminal amino acids of purified extracellular muramidase-2 were in very good agreement with the deduced amino acid sequence after a 49-amino-acid putative signal sequence. Analysis of the deduced amino acid sequence showed the presence at the C-terminal region of the protein of six highly homologous repeat units separated by nonhomologous intervening sequences that are highly enriched in serine and threonine. The overall sequence showed a high degree of homology with a recently cloned *Streptococcus faecalis* autolysin.

Two muramidases, muramidase-1 and muramidase-2, have been found in *Enterococcus hirae* ATCC 9790 (formerly *Streptococcus faecium*) (15). Muramidase-1 is a glycoprotein (16) which can hydrolyze the re-N-acetylated peptidoglycan and the sodium dodecyl sulfate (SDS)-treated cell walls of *E. hirae* ATCC 9790 (15). Muramidase-1 is present in a latent form (130 kDa) that can be converted into an active form (87 kDa) via the action of any of a variety of proteinases, including trypsin (16). Muramidase-1 is unusual in that it possesses about 12 5-mercaptouridine monophosphate residues covalently linked to the protein (6).

Muramidase-2 is also an unusual enzyme that appears to be a gene product different from muramidase-1 (23). It differs from muramidase-1 in substrate specificity and rapidly hydrolyzes the nonacetylated peptidoglycan fraction of *E. hirae* and walls of *Micrococcus luteus* (15), both of which are resistant to the action of muramidase-1. When extracted from intact or disrupted bacteria, highly purified preparations of muramidase-2, upon SDS-polyacrylamide gel electrophoresis (PAGE), showed the presence of two polypeptides of about 125 and 74 kDa (5), both of which possess peptidoglycan hydrolase activity. Substantial amounts of the smaller, 74-kDa form have been found in and isolated from the culture medium (14, 15).

In this paper, we report the cloning and complete nucleotide sequence of the extracellular muramidase-2 gene of *E. hirae* ATCC 9790 that codes for a peptide 666 amino acids long with a calculated molecular mass of 70,678 Da.

MATERIALS AND METHODS

Bacteria and media. *Escherichia coli* DH5 α and Y1090 were purchased from Bethesda Research Laboratories, Inc. (Gaithersburg, Md.) and Promega (Madison, Wis.), respectively. *E. hirae* ATCC 9790 was grown in S broth (4); the *E.*

coli strains were grown in L broth containing (per liter) 10 g of tryptone, 5 g of yeast extract, and 5 g of sodium chloride.

Enzymes and chemicals. Restriction enzymes were all purchased from Promega and used in accordance with the specifications of the manufacturer. The Sequenase DNA sequencing kit was purchased from U.S. Biochemical Corp. (Cleveland, Ohio). [α -³²P]dCTP and [α -³²P]dATP were purchased from ICN Radiochemicals, Inc. (Irvine, Calif.). The isopropyl- β -D-thiogalactopyranoside (IPTG) and 5-bromo-4-chloro-3-indolyl- β -D-galactoside (X-Gal) were purchased from Bethesda Research Laboratories.

Purification of extracellular muramidase-2. Cultures of *E. hirae* were grown to early stationary phase (1.3 mg [dry weight] per ml) in 600 ml of S broth (14). The culture was rapidly chilled to 0°C and centrifuged (20,000 $\times$ g, 10 min, 4°C). Sonicated *E. hirae* peptidoglycan (50 mg in 5 ml of 10 mM sodium phosphate [pH 7.0]) was added to the supernatant culture medium, and the mixture was allowed to stand at 0°C for 30 min with occasional mixing. After binding, the peptidoglycan-enzyme complex was pelleted (20,000 $\times$ g, 20 min, 4°C); the pellet was suspended in 1 ml of 10 mM sodium phosphate, pH 7.0, transferred into a microcentrifuge tube, centrifuged (14,000 $\times$ g, 15 min, 4°C), resuspended, and then washed in 1.5 ml of 10 mM sodium phosphate, pH 7.0. The pellet was extracted twice with 500 μ l of 6 M guanidine-HCl, and the extracts were combined and then stored at -70°C. For further purification, defrosted 6 M guanidine-HCl extract was injected into a Pro RPC HR 5/10 (reversed-phase), fast-performance liquid chromatography (FPLC) system (Pharmacia, Inc., Piscataway, N.J.) in 0.1% trifluoroacetic acid (TFA) and fractionated by using a 0 to 60% gradient of acetonitrile in 0.1% TFA. The column was monitored on the basis of A₂₈₀, and 1.0-ml fractions were collected. Portions of each fraction, or pooled fractions, were evaporated to dryness in a vacuum concentrator (Savant Instruments, Inc., Hicksville, N.Y.). The samples (dried in a microcentrifuge tube) were dissolved in 6 M guanidine-HCl and used for

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FIG. 1. Reversed-phase chromatography of the 6 M guanidine-HCl extract of the peptidoglycan-muramidase-2 complex obtained from supernatant culture medium. Symbols: —, A_{280} of the column eluate; ---, acetonitrile gradient; and ●, muramidase-2 activity of each 1-ml fraction. The guanidine-HCl extract (720 μ g of protein, 596 U of muramidase-2 activity) was injected into a Pro RPC HR 5/10 column by using the Pharmacia FPLC system equilibrated with 0.1% TFA. The column was then eluted with a linear gradient of acetonitrile in 0.1% TFA, and elution was monitored on the basis of A_{280} . Portions (900 μ l of each 1 ml) of fractions 8 to 17 were evaporated to dryness and dissolved in 100 μ l of 6 M guanidine-HCl, and 5- to 15- μ l samples were assayed for muramidase-2 activity. In general, 50 to 70% of the muramidase-2 activity and about 40 to 50% of the protein applied to the column were recovered in fractions 9 to 13.

muramidase-2 activity and protein assay. Muramidase-2 activity was assayed by using *M. luteus* cell wall as the substrate (14). Protein content was determined as described elsewhere (14).

DNA manipulation. Chromosomal DNA from *E. hirae* ATCC 9790 was purified as described previously (19). Large-scale isolation of plasmid DNA was performed by cesium chloride gradient centrifugation (18). Plasmid DNA was isolated on a small scale by the alkaline lysis procedure (3). Phage DNA was isolated by the method of Maniatis et al. (18).

Construction of the genomic library. Chromosomal DNA of *E. hirae* ATCC 9790 was partially digested with *Eco*RI, and the digested DNA was ligated into the *Eco*RI site of bacteriophage λ gt11. The recombinant λ gt11 DNA was packaged in vitro and used to infect the host strain *E. coli* Y1090. The titer of bacteriophage was determined, and the library was stored at 4°C for further screening.

Screening phage plaques for muramidase-2. λ gt11 recombinant phage were plated on a lawn of *E. coli* Y1090 and incubated at 42°C for 3 to 4 h. An 82-mm-diameter nitrocellulose filter, previously saturated with 10 mM IPTG, was overlaid on the plate. The plates were then incubated at 37°C overnight. The filter was removed and incubated in PBST (50 mM sodium phosphate [pH 7.4], 150 mM sodium chloride, 0.05% Tween 20) plus 5% nonfat dry milk for 4 h at room temperature. The filter was washed three times in PBST for 20 min each time at room temperature. The filter was then incubated in PBST plus an anti-muramidase-2 monoclonal antibody, 2F8 (14), overnight at 4°C and again washed in PBST three times (each time for 20 min at room temperature). Finally, the filter was incubated with anti-mouse immunoglobulins G and M conjugated with alkaline phosphatase (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, Md.) for 90 min at room temperature. After being washed with PBST, the filter was developed with 0.005% 5-bromo-4-chloro-3-indolylphosphate disodium salt, and

FIG. 2. SDS-PAGE (minigel) analysis of reversed-phase-chromatography-purified extracellular muramidase-2. (A) Coomassie blue-stained gel. (B) Western blot with monoclonal antibody 2F8. Extracellular muramidase-2 was isolated and purified by reversed-phase FPLC chromatography as described in the legend to Fig. 1. Fractions 9 to 13 (total of 5 ml), containing muramidase-2 activity (Fig. 1), were pooled, and a 1-ml sample was evaporated to dryness. Portions of this sample, dissolved in the SDS sample buffer, were applied to each lane. Lane A1 contained 20 μ g of protein, lane B1 contained 1.25 μ g, and lane B2 contained 156 ng. Lane M contained the following molecular mass standards: phosphorylase b, 94 kDa; albumin, 67 kDa; catalase, 60 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 30 kDa; and trypsin soybean inhibitor, 20 kDa.

0.01% Nitro Blue Tetrazolium (Sigma Chemical Co., St. Louis, Mo.).

Gel electrophoresis. Protein samples were analyzed by SDS-PAGE (17). Dried samples were dissolved in SDS sample buffer (0.0625 M Tris-HCl [pH 6.8], 2% SDS, 10% glycerol, 5% 2-mercaptoethanol) and exposed to 100°C for 5 min. The stacking and separating gels used were 4 and 10% polyacrylamide at pH 6.8 and 8.8, respectively. Gels (minigel) were subjected to electrophoresis at 155 V of constant voltage for approximately 2 h under a continuous SDS-Tris glycine buffer system (pH 8.3). Gels were stained with Coomassie blue G-250 as described previously (5). Immunodetection by Western blotting (immunoblotting) was performed as described elsewhere (14).

DNA was analyzed after electrophoresis on horizontal 0.7% agarose gels with TAE buffer (40 mM Tris-acetate [pH 8.5], 2 mM EDTA). The gels were stained with ethidium bromide (18).

Southern blotting and hybridization. DNA samples were electrophoresed through agarose gels and transferred to nitrocellulose paper (Nitroplus; Micron Separations, Inc., Westborough, Mass.) by using a vacuum blotter (Trans-Vac TE 80; Hoefer Scientific Instruments, San Francisco, Calif.). The probe was labelled with [α - 32 P]dCTP by using the Prime-a-gene system from Promega. Hybridization was done by the method of Southern (24). The final washing was done under stringent conditions (15 mM sodium chloride, 1.5 mM sodium citrate, 0.1% SDS, 50°C). Radioactive bands were detected by using XAR-5 film (Eastman Kodak Co., Rochester, N.Y.) with intensifying screens at -70°C.

Transformation of *E. coli* cells. Plasmids and λ gt11 DNA containing the insert were cleaved with the appropriate restriction enzyme, ligated, and transformed into *E. coli* DH5 α cells (18). Transformants were selected on L agar

FIG. 3. Southern blot analysis of DNA from *E. hirae* ATCC 9790 and a positive phage clone. Lane 1, phage DNA containing the 4.5-kb insert; lane 2, *EcoRI*-digested chromosomal DNA of *E. hirae* ATCC 9790. The 4.5-kb fragment was labeled with [α - 32 P]dCTP and used as a probe for hybridization.

plates containing 100 mM IPTG, 0.02% X-Gal, and the appropriate antibiotic (ampicillin [50 μ g/ml] for plasmid pUC19 or erythromycin [500 μ g/ml] for plasmid pJDC9).

Amino acid sequencing. Automated microsequences were determined on an Applied Biosystems (Foster City, Calif.) 477A pulsed liquid sequenator with on-line analysis of the amino acid phenylthiohydantoin derivatives by using a 120-A analyzer. Two separate preparations of reversed-phase-chromatography-purified, dried extracellular muramidase-2 were dissolved in 10% TFA, and about 50 μ g of protein samples was analyzed to determine amino acid sequences in two different laboratories (Fels Institute for Cancer Research and Molecular Biology, Temple University School of Medicine, Philadelphia, Penn., and Laboratorium voor Microbiologie en Microbiële Genetica, Rijksuniversiteit-Gent, Ghent, Belgium).

DNA sequencing. The plasmid DNA used for sequencing was purified by using CsCl gradient centrifugation. Sequencing was performed by using the dideoxy chain-termination method of Sanger et al. (22), with a modified T7 DNA polymerase supplied with the Sequenase kit from U.S. Biochemical Corp. The sequence was confirmed with overlapping clones, and different oligonucleotide primers (17-mer) were synthesized to sequence the entire gene in both orientations.

FIG. 4. Restriction map of DNA in the vicinity of the muramidase-2 gene. (A) The 4.5-kb DNA insert. (B) The 2.3-kb fragment. (C) The 1.0-kb fragment. (D) The 1.5-kb fragment. The location and orientation of the muramidase-2 gene are indicated. Restriction enzyme sites are designated as follows: E, *EcoRI*; K, *KpnI*; P, *PstI*; S, *Sau3AI*.

Nucleotide sequence accession number. The GenBank accession number for the muramidase-2 sequence is M77639.

RESULTS

Purification of extracellular muramidase-2. Previously employed methods to purify muramidase-2 from the insoluble pellet of disrupted cells of *E. hirae* (5) or from supernatant culture medium (15) used extractions at high pH (e.g., 0.005 to 0.02 N sodium hydroxide). The resulting preparations tended to precipitate from the solution, with concomitant loss of enzyme activity that was extremely difficult to recover, resulting in low yields. Recent observations showed that muramidase-2 activity present in supernatant culture medium was stable, even at 37°C in the absence of proteinase inhibitors (14). The new simple and efficient purification procedure resulted in stable preparations. The two important steps are (i) directly binding the activity present in culture supernatant to the insoluble peptidoglycan fraction of *E. hirae*, and (ii) extraction of muramidase-2 from the peptidoglycan-enzyme complex with 6 M guanidine-HCl (14), resulting in concentrated preparations that are stable for periods of up to 1 year at -70°C and that can be injected directly into the reversed-phase chromatography column. The results of purification of muramidase-2 by reversed-phase chromatography are shown in Fig. 1. The peak of enzyme activity (eluted at 20% acetonitrile in 0.1% TFA) corresponds to the major peak at A_{280} and contained only one detectable Coomassie blue-staining band (Fig. 2A, lane 1) at about 70 kDa which reacts with monoclonal antibody 2F8. This monoclonal antibody recognized as little as 156 ng of muramidase-2 (Fig. 2B, lane 2).

Cloning and subcloning of the muramidase-2 gene. A genomic library of *E. hirae* ATCC 9790 was constructed by using bacteriophage λ gt11. The library was screened with monoclonal antibody 2F8 against muramidase-2 (14). Of 60,000 screened, one plaque consistently gave a positive reaction with monoclonal antibody 2F8. The DNA of the positive phage clone was isolated (18), digested with *EcoRI*,

AAGGTTACAGTCTGATTACAATAAGCAGTCACACACTTAAATACTTTTAATAAAGATT
 -35 -10 S.D.
 AGAGAAAGTTATAATTATTAATAGTATAGAAATGTAGATAGAAGTTGGAGGATAGAAG
 ATGGAAAAATTGCAAGAAAAAGACGTAGACGTTTAAATGAAACCAACGTTTTCGTAAA
 M E N I A R K E R R R L N E T K R F R K
 GTAAAAAGTAGTGTGCACTTGTGGTACGGCGATGGTGGCTGTTCTGTGGCAGCGCCA
 V K R S A A L V G T A M V G C S V A A P
 TTGATTGACCGTGTACAAGTAGATGCTGATCAAAACCCACACAATTGGTGCCAGAATC
 L I Q P V Q V D A D Q T P T Q F G A R I
 AATACAGCAGCGTTTATTGCTGAGATTGCTACGTATGCACCAACCAATTGCTCAAGCCAAC
 N T A A F I A E I A T Y A Q P I A Q A N
 GATTATATGCCTCAGTCATGATTGCTCAAGCAGTCGCGAAAGTGGTGGGAAGTAGT
 D L Y A S V M I A Q A V V E S G W G S S
 GCCTGTGCAAGCACCTTACTATAATCTATTGGGATTAAGGAAGTTATCAAGGTCAA
 A L S Q A P Y N L F G I K G S Y Q G Q
 ACTGTTTATATGGTACACTCGAATATCTTAAACAATAATGGGTATCTAAAAAGAACCT
 T V Y M D T L E Y L N N K W V S K K E P
 TTCCTGAGTACCCCTTCTTTGAGAGTCATTAAATGACAATGCCTATGTTTAAAGAAAT
 F R Q Y P S F A E I A T Y A Q P I A Q A N
 ACCTCTTTGGTAATGGCTATTATTATGACAGTACCTGGAAGCAATACTAAATCTTAT
 T S F G N G Y Y Y A Y T W K S N T K S Y
 ACTGATGCTACTGCTTGTGACAGGTCGTTATGCAATGATCCAGGATGCTGGAATA
 T D A T A C L I S H S F N D T P G Y A T D
 CTAAACAATATTATTACAACCTATGGATTGACCAAAATACGATACACAGTACGCGGAAC
 L N N I I T T Y G L T K Y D T P A S G N
 GCTGGAGCGGTGTAAAGATTGGAATGGCGGAATACGGAATACATCAATCTGGA
 A G G G V T I G N G N T S N S G
 AGTACTAGTGGAACTCAGCGGTTCTGCAACTACGACTGGTACGACTTATACCGTCAAA
 S T S G N S G G S A T T T G T T Y T V K
 TCAGGAGACTCCGTTTGGGAACTCTCTCATTATTGGAATCAGGATGGCGCAGTTAATC
 S G D S V W G I S H S F N D T P G Y A T D
 GAATGGAATAATATCAAAATAATTTTATCTATCCAGGACAAAATTAACGATCAAGGT
 E W N N I K N N F I Y P G O K L T I K G
 GGCAATCTGCAAGTCTCTCAACCACTAACTGGTAATGCTTCTCTGGAATACT
 G Q S G S S T T N T G N N A S S G N T
 TCTGGCAACGAAATACGTCGCGAGTACGGGACAAGCAACTGGTGCAAAATATACGGTT
 S G N T N T S G S T G Q A T G A K Y T V
 AAATCAGGCGATTCTGTTTGGAAAAATGCGAATGATCATGGGATTTCGATGAACCACTG
 K S G D S V W K I A N N K N T A S S G N T
 ATCGAATGAATAACATCAAGAATAACTTGTGTTATCCAGGTCAACAGTTGGTTGTTCA
 I E W N N I K N N F V Y P G O Q L V V S
 AAAGGTAGTTCTTTCAGCAAGCGGTTCTACTTCTAATACATCGACAGGTAATACATCTAGT
 G S S S A S S T S T G N T A S S G N T
 AATACGGCAATACAGGAAGTACTACTTCTGGAAGTACTTATCTGTCAAAGCAGGCGAA
 N T A N T G S T T S G S T Y T V K A G E
 TCGGTTTGGAGTGTTCGAATAAATTTGGAATTTCCATGAACCAACTGATTCAATGGAAC
 S V W S V S N Q L I E W N N I K N N
 AATATTAATAAATTTTATTTTACCAGGCAAAATTTGATTGTCAAAGTGGTTTCATCT
 N I K N N F I Y P G O K L I V K G G S S
 TCAAGTAATCGCTCCACTTCGACAGCTAATAACAAGAACACCGCATCAAGCAATACCAGC
 S S N A S T S T A S N N K N T A S S N T
 TCAACTGCGACAGGCAAGCGACTTATCTGTCAAGGCGAGCAATCAGTATGGGGTGT
 S T A T G Q A T Y T V K A G E S V W G V
 GCAATAAAAAATGGGATTTCCATGAACAGTGTGATTGAATGAACAATATTAAAGATAAC
 A N K N G I S M N Q L I E W N N I K N N
 TTCATTTATCCAGGACAGAAATGATCGTCAAGGTGGAAGCAGTAAGCAAGCGCAACA
 F I Y P G O K L I V K G G S S K A S A T
 GCAACAATCAACCAACAGCTTCAACGCCAGCTTCTCAACTCCAACGGCTTCTTCAAGC
 A T I K P T A S T T P T A S S T
 GGTGATACGAATATACTGTCAAAGCAGGCGAATCGGTGTGGGCGTTGCCAATAAACAC
 G D T K Y T V K A G E S V W G V A N K H
 CATATTACGATGGATCAGTTGATTGAATGGAACAACATCAAAACAATTTTATTATCCA
 H I T M D Q L I E W N N I K N N F I Y P
 GGACAAGAAGTCATCGTTAAAAAGGCACTGCACAATCAACGCCAGGCAATCAGATGAA
 G O E V I V K K G T A Q S T P A K S D E
 AAAACCTATACCGTCAAAGCAGGCGAATCAGTATGGGCGTTGCCGATAGTCATGGAATC
 K T Y T A V K A G E S V W G V A D S H G I
 ACCATGAATCAATTGATCGAATGGAATAATCAAGAATAAATTTATTATCCGGGACAA
 T M N Q L I E W N N I K N N F I Y P G Q

CAGTTGATCGTCAAAAAATAAGTAATGACACATGGCAGGAAATCAGTAAATGTGTAAAT 2161
 Q L I V K K >>> 666
 AATATTGAACTGTAGCATTTATTTGTTACTATGAATCTACACTTTTGTAGTAAATAAAA 2221
 ATCAATCGCCTGAAAAGGAAGTAGTAAAAAGTCGAAAGTTTGTAGAGACACATGGTTGGT 2281
 GGAAATGTGTACGGACGCTTTTGAACCTCGCTTTGAGTTGCGCTACTGAAATCACAAAC 2341
 AGAGTAAGTAGGAGCGGTGACGACCGCTTACAACTTTGAGGCGCTTTGTGAAAAGGCAAAA 2401
 TTAGGT 2407

FIG. 5. Nucleotide sequence and deduced amino acid sequence of *E. hirae* ATCC 9790 muramidase-2. The -35 and -10 promoters and the Shine-Dalgarno (S.D.) sequences are indicated. The signal peptide cleavage site is indicated by a vertical arrow. The six repeating units are underlined. Possible transcriptional terminators are indicated by the horizontal arrows below the sequence.

and subjected to electrophoresis on a 0.7% agarose gel. This phage clone contained a 4.5-kb DNA insert.

To confirm that the 4.5-kb fragment was from the chromosome of *E. hirae* ATCC 9790, the 4.5-kb DNA band was cut out of the agarose gel, labeled with ³²P, and used as a probe for hybridization with *Eco*RI-digested chromosomal DNA of *E. hirae* ATCC 9790. The Southern blots showed that this probe consistently recognized six DNA bands at about 4.5, 5.0, 6.1, 7.9, 9.1, and 12.5 kb (Fig. 3). Thus, the cloned DNA inserts appeared to contain chromosomal DNA of *E. hirae* that had not extensively rearranged. The 4.5-kb fragment was used for all further studies.

Several attempts to clone the entire 4.5-kb fragment in various plasmid vectors such as pJDC9 and pUC19 were unsuccessful. Therefore, the 4.5-kb band was cut out of the agarose gel, eluted from the agarose, and hydrolyzed with *Kpn*I. A 2.3-kb fragment (Fig. 4B) was obtained, isolated, purified, and subcloned into plasmid pJDC9 and transformed into *E. coli* DH5α. After partial sequencing of the 2.3-kb fragment, we found an open reading frame starting around 600 bp upstream from the *Kpn*I site (Fig. 4). We also located a *Sau*3AI site in the 4.5-kb fragment about 400 bp upstream from the *Kpn*I site. The 400-bp *Sau*3AI-*Kpn*I fragment was used as a probe to obtain, isolate, purify, and subclone a 1-kb *Sau*3AI-*Sau*3AI fragment (Fig. 4C) into plasmid pUC19. After sequencing the 1-kb fragment, we used the same strategy; i.e., we identified and subcloned a 1.5-kb *Pst*I-*Eco*RI fragment (Fig. 4D) into plasmid pUC19. The nucleotide sequences of the three fragments were then determined.

Nucleotide sequences of the muramidase-2 gene. The open reading frame sequence contained 1,998 bp (Fig. 5). A GGAGG consensus sequence, which is a putative ribosomal binding site, was found 8 to 12 bp upstream from a start codon (ATG, bp 122 to 124). Possible promoter sequences (10) TTAAGA (-35 region) and TATAAT (-10 region) were also identified (Fig. 5). Two potential transcriptional terminators were recognized downstream from the stop codon (TAA) (Fig. 5).

Deduced amino acid sequences of the muramidase-2 gene. The deduced amino acid sequence of the open reading frame contained 666 amino acid residues. The first 49 amino acids were recognized as a putative signal peptide. The putative signal peptide started with a region rich in basic amino acids (M1-K20), followed by a hydrophobic region (V21-L41) and a potential protease cleavage site between A49 and D50 (Fig. 5) (25, 27). The virtual identity of the determined N-terminal sequence of mature extracellular muramidase-2 (Table 1) with the amino acid sequence deduced from the nucleotide sequence clearly identifies this cleavage site between the

TABLE 1. Analysis of the amino terminus of extracellular muramidase-2

Cycle	Amino acid sequence of purified protein ^a		Deduced amino acid sequence of cloned gene
	Prepn 1 ^a	Prepn 2 ^b	
1	Asp	Val	Asp
2	Gln	Gln	Gln
3	Thr	Pro (Val)	Thr
4	Pro	Pro (Tyr)	Pro
5	Thr	Thr	Thr
6	Gln	Gln	Gln
7	Phe	Phe	Phe
8	Gly	Gly	Gly
9	Ala	Ala	Ala
10	Arg (?)	(?)	Arg
11	Ile	Ile	Ile
12	Asn	Leu	Asn
13	Thr	Thr	Thr
14	Ala	Ala	Ala
15	Ser (?)	Ile	Ala
16	Phe	Phe	Phe
17	(?)	Ile	Ile
18	Ala	(?)	Ala
19	Glu	Asn	Glu
20	Ile	Ile	Ile
21	(?)	Ala	Ala
22	Thr (?)	Asp	Thr
23	Tyr (?)	Tyr	Tyr
24	(?)	Ala	Ala

^a Sequence of the peak fraction (e.g., fraction 10 in Fig. 1) of a preparation of extracellular muramidase-2 purified by reversed-phase chromatography, determined at Temple University.

^b Sequence of the peak (e.g., fractions 9 to 13 in Fig. 1) pooled from three FPLC fractionations of extracellular muramidase-2, determined at Rijksuniversiteit-Gent, Ghent, Belgium.

signal peptide and the mature protein. No evidence for the presence of an intervening propeptide was obtained.

Repeat regions. Six 45-amino-acid-long internal repeat regions (Fig. 6) separated by intervening sequences that are highly enriched for serine and threonine (ca. 51% S+T) were found at the C-terminal end. There was more than 90% identity between repeats 3 and 4 (41 of 45 amino acids were identical). In addition, 25 of 45 amino acids (56%) were identical in all six repeats. Overall, the comparative identity of the six sequences ranged from about 69% (repeats 1, 2, and 3) to over 90% (repeats 3 and 4).

DISCUSSION

A gene that codes for muramidase-2 of *E. hirae* ATCC 9790 has been cloned in *E. coli* and sequenced. The gene codes for a 666-amino-acid polypeptide with a molecular mass of 70,678 Da (65,228 Da without the signal peptide),

- (1) TYTVKSGDSVWGISHSGFITMAQLIEWNNIKNNFIYPGQKLTIK
 : : : : :
 (2) KYTVKSGDSVWKIANDHGISNQLIEWNNIKNNFVYPGQLVVSK
 : : : : :
 (3) TYTVKAGESVWSVSNKFGISMNQLIQWNNIKNNFIYPGQKLIVK
 : : : : :
 (4) TYTVKAGESVWGVANKGISNQLIEWNNIKNNFIYPGQKLIVK
 : : : : :
 (5) KYTVKAGESVWGVANKHITMDQLIEWNNIKNNFIYPGQEVIVKK
 : : : : :
 (6) TYTVKAGESVWGVADSHGITNQLIEWNNIKNNFIYPGQKLIVK

FIG. 6. Comparison of the six repeat units in muramidase-2. The identical amino acids are indicated by colons. Similar and unrelated amino acids are indicated by dots and blank spaces, respectively.

about the size expected from SDS-PAGE studies (5, 14, 15), which indicated an approximate molecular mass of purified, mature muramidase-2 of about 74 kDa.

Comparison of the amino acid sequence deduced from the nucleotide sequence with the amino acid sequence of the first 24 N-terminal amino acids of purified extracellular muramidase-2 (Table 1) showed very good agreement. Since aspartic acid was the first amino acid of mature muramidase-2 found in the culture medium, the data indicated that the cleavage site of the putative signal peptide was between A49 and D50. The 49-amino-acid signal peptide is long compared with the signal peptides found in *E. coli* (26, 27). However, long signal peptide sequences have been found in gram-positive bacteria, especially staphylococci and streptococci (1, 12, 25). The presence of a putative signal peptide in the deduced amino acid sequence of the muramidase-2 gene was not surprising, since the enzyme is secreted into the culture medium. Secretion of extracellular proteins has been studied in gram-positive and gram-negative bacteria (20, 28). Protease secretion usually involves a signal peptide, often followed by a propeptide located between the signal peptide and the mature protein (28). The sequence of the N-terminal region of mature extracellular muramidase-2 (Table 1) demonstrated that the mature form was synthesized after cleavage of a signal sequence without an intervening propeptide. *Streptococcus faecalis* autolysin (2) might have an intervening propeptide, as shown in Fig. 7 and discussed below.

We previously reported that cell-associated muramidase-2 activity was present in a polypeptide of about 125 kDa in addition to the 74-kDa form and that the larger polypeptide could be cleaved to a smaller form, presumably by any endogenous proteolytic activity (6). The nucleotide sequence of this cloned gene demonstrates that the encoded polypeptide does not derive from a larger precursor. However, the Southern blot shown in Fig. 3 indicates that as many as five additional genes homologous to the gene cloned in this study may be present in *E. hirae* ATCC 9790 (Fig. 3). One or more of these genes may code for the larger-molecular-size form of muramidase-2.

The presence of six 45-amino-acid-long, highly homologous and conserved nucleotide and amino acid repeating units in muramidase-2 (Fig. 6) was particularly interesting. Repeat units have been found in a number of bacterial and eucaryotic proteins. In gram-positive bacteria, repeat units are found at the C terminus of staphylococcal protein A (25), streptococcal protein G (7), group A streptococcal M protein (12), staphylococcal lysostaphin (11, 21), glucan-binding protein of *Streptococcus mutans* (1), the amidase gene of *Streptococcus pneumoniae* (8), and muramidase genes of pneumococcal bacteriophages Cp-1, Cp-7, and Cp-9 (9). These repeat units have little homology with the repeats present in muramidase-2.

However, there is homology between the six repeat regions of muramidase-2 and four repeat units at the C terminus of an autolysin of unspecified specificity of *S. faecalis* that has been recently cloned and sequenced (2) (Fig. 7). The deduced amino acid sequence of this protein shows substantial areas of homology with *E. hirae* muramidase-2. These areas of homology include the putative signal peptide and much of the rest of the two polypeptides except for a 104-amino-acid insertion between amino acids 64 and 65 of *E. hirae* muramidase-2 and for the S+T-enriched sequences of muramidase-2 between the repeat sequences. The *S. faecalis* autolysin contains four 68-amino-acid-long direct repeat sequences rather than the six 45-amino-acid

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1  MENIARKERRRLNETKFRKV-KRSAALVGTAMVGCVAAPLI----- 42
   : : : : : : : : : : : : : : : : : : : : : : : : : :
3  KESMSRIERRKAQQRKKTVPQWKSTTLFSSALIVSSVGTVPVALLPVTAE 52
   : : : : : : : : : : : : : : : : : : : : : : : : : :

43  ----QPVQVDADQTPTQFGARINTAA----- 64
   : : : : : : : : : : : : : : : : : : : : : : : : : :
53  ATEEQPTNAEVAQAPTETGLVETPTTETPGITEQPTTDSSTTTESTTE 102
   : : : : : : : : : : : : : : : : : : : : : : : : : :

65  -----FIAEIATYAQPIAQANDLYA 84
   : : : : : : : : : : : : : : : : : : : : : : : : : :
153  TPDDEVKVPPEARVASAQTFSSALSPQSPSEFIAELARCAQPIAQANDLYA 202
   : : : : : : : : : : : : : : : : : : : : : : : : : :

85  SVMIAQAVVESGWSSSALSQAPYYNLFGIKGSYQGGTVMYMDTLEYLNKK 134
   : : : : : : : : : : : : : : : : : : : : : : : : : :
203  SVMMAQAIIVESGWGASTLSKAPNYNLFGIKGSYNGQSVYMDTWEYLNKK 252
   : : : : : : : : : : : : : : : : : : : : : : : : : :

135  VSKKEPFRQYPSFAESFNDNAYVLRNLSFGNG-YYYAGTWNKNTKSYTDA 183
   : : : : : : : : : : : : : : : : : : : : : : : : : :
253  LVKKEPFRKYPSYMESFQDNHVLKTTSFQAGVYYYAGAWKNTSSYRDA 302
   : : : : : : : : : : : : : : : : : : : : : : : : : :

184  TACLTRGYATDPGYAGKLNNIITTYGLTKYDTPASGNAGGGVTIGNGGNT 233
   : : : : : : : : : : : : : : : : : : : : : : : : : :
303  TAWLTGRYATDPSYNAKLNNVITAYNLTQYDTPSSGGNTGGGTV-NPGTG 351
   : : : : : : : : : : : : : : : : : : : : : : : : : :

234  GNTSNGSGTSGNSGGSATTTGTTTVKSGDSVWGISHSFGITMAQLIEWN 283
   : : : : : : : : : : : : : : : : : : : : : : : : : :
352  GSNNGSG-----TNTYYTVKSGDTLNKIAAQYGVSVANLRSWN 389
   : : : : : : : : : : : : : : : : : : : : : : : : : :

284  NIKNNFIYPGQKLTIKGGQSAGSSTTNGNASSGNTSGNTSGTSGTGA 333
   : : : : : : : : : : : : : : : : : : : : : : : : : :
390  GISGDLIFVGQKLIVKKGAS-----GNTGGSGNGGSSNNQSG----- 426
   : : : : : : : : : : : : : : : : : : : : : : : : : :

334  TGAKYTVKSGDSVWIKIANDHIGSMNQLIEWNNIKNNFVYPGQQLVSKGS 383
   : : : : : : : : : : : : : : : : : : : : : : : : : :
427  TNTYYTVKSGDTLNKIAAQYGVTVANLRSWNGISGDLIFVGQKLIVKKG 476
   : : : : : : : : : : : : : : : : : : : : : : : : : :

384  SSASGTSNTSGTSSNTANTGTTSGSTYTVKAGESVWSVSNKFGISM 433
   : : : : : : : : : : : : : : : : : : : : : : : : : :
477  SGTGGSSNGGSSNNQSGT-----NTYYTIKSGDTLNKIAAQYGVSV 518
   : : : : : : : : : : : : : : : : : : : : : : : : : :

434  NQLIQWNNIKNNFIYPGQKLIVKGGSSSSNASTTANNKNTASSNTSSTA 483
   : : : : : : : : : : : : : : : : : : : : : : : : : :
519  ANLRSWNGISGDLIFAGQKIIKKGTSNTGGSSNGGSSNNQSG----- 562
   : : : : : : : : : : : : : : : : : : : : : : : : : :
484  TQQATYTVKAGESVWGVANKGISMNQLIEWNNIKNNFIYPGQKLIVKGG 533
   : : : : : : : : : : : : : : : : : : : : : : : : : :
563  -TNTYYTIKSGDTLNKIAAQYGVSVANLRSWNGIKGDLIFAGQTIIVKKG 611
   : : : : : : : : : : : : : : : : : : : : : : : : : :

534  SSKASATATIKPTASTPASTTPTASGTGDKYTVKAGESVWGVANKHHIT 583
   : : : : : : : : : : : : : : : : : : : : : : : : : :
612  ASAGGNASSTNSASGRKHTVKSGLWGLSMQYGISIQIKQLNGLSGDT 661
   : : : : : : : : : : : : : : : : : : : : : : : : : :

584  MDQLIEWNNIKNNFIYPGQEVIVKKGTAQSTPAKSDEKTYTVKAGESVWG 633
   : : : : : : : : : : : : : : : : : : : : : : : : : :
662  IYIGQTLKVG 671
   : : : : : : : : : : : : : : : : : : : : : : : : : :

634  VADSHGITMNLIEWNNIKNNFIYPGQQLIVKK 666

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FIG. 7. Comparison of the amino acid sequences of the *S. faecalis* autolysin (bottom sequence) with muramidase-2 of *E. hirae* (top sequence). The identical amino acids are indicated by colons. Conservative amino acids are indicated by dots.

repeats with intervening S+T-rich sequences of muramidase-2. A more complete comparison of the sequence of muramidase-2 with that of this and other proteins will appear elsewhere (13).

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