Modular design of the *Enterococcus hirae* muramidase-2 and *Streptococcus faecalis* autolysin

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1. SUMMARY

The mature forms of the extracellular muramidase-2 of *Enterococcus hirae* and *Streptococcus faecalis* autolysin have very similar primary structures. Each consists of an active-site-containing N-terminal domain fused to a multiple-repeat C-terminal domain. Polypeptide segments occurring at equivalent places in these two bacterial wall lytic enzymes have homologues in two phage lysozymes and in three functionally unrelated proteins, illustrating the principle that protein molecules frequently are constructed from modules that are linked in a single polypeptide chain.

*Enterococcus hirae* ATCC 9790 gene that codes for the extracellular muramidase-2 (Genbank accession number: M77639)\(^{[2,26]}\) have been cloned and sequenced, giving access to primary structures of high similarity. Research aimed at identifying proteins which would be structurally related to the *S. faecalis* and *E. hirae* enzymes led to the conclusion that a significant extent of similarity exists, at least at the level of defined polypeptide segments, with the *Bacillus subtilis* \(\phi PZA\) lysozyme [3], the homologous *Bacillus* gene 15 \(\phi 29\) lysozyme [4], the *Salmonella typhimurium* FlgJ flagellar protein [5], the *Listeria monocytogenes* pathogenicity-associated P60 protein [6] and *Staphylococcus aureus* protein A [7].

2. INTRODUCTION

The *Streptococcus faecalis* gene that codes for an autolysin of unknown specificity [1] and the

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3. METHODS

Comparison of the primary structures of the *S. faecalis* and *E. hirae* enzymes with the protein sequence database was made using the FASTA and TFASTA algorithm of the software package of the Genetics Computer Group (GCG) (University of Wisconsin Biotechnology Centre, Madi-
son, WI) [8]. Sequences similar to a query sequence were identified using a word size of one. The proteins were compared by using the DIAGON plot procedure [9] and hydrophobic cluster analysis [10]. Homologies between aligned amino acids correspond to a threshold of 0.8 according to Gribskov and Burgess [11]. Hydrophobic cluster analysis rests upon a representation of the protein sequences on an a-helical two-dimensional pattern in which hydrophobic residues tend to form clusters that usually correspond to secondary structural elements. Clusters of similar shapes, sizes and relative positions express similarity in the polypeptide folding of the proteins.

4. RESULTS

4.1. Molecular organization of the E. hirae muramidase and S. faecalis autolysin

Intersequence comparisons show that: i) the E. hirae muramidase (Fig. 1a) comprises an approx. 250-amino acid N-terminal domain and an approx. 400-amino acid C-terminal domain that consists of six 45-amino acid repeats connected by five non-homologous (S and T rich) intervening sequences; ii) the S. faecalis autolysin (Fig. 1b) comprises an approx. 350 amino acid N-terminal domain and an approx. 250-amino acid C-terminal domain that consists of four 68 amino acid contiguous repeats; iii) the two proteins (Fig. 1c) have similar primary structures except for the presence of an approx. 114-amino acid stretch (E52-A166) in the N-terminal domain of the S. faecalis autolysin, that is not present in the E. hirae muramidase. Providing that this E52-A166 segment is excluded from the analysis, the N-terminal domains of the E. hirae muramidase and S. faecalis autolysin show almost complete identity in the shape and distribution of the hydrophobic and hydrophilic clusters along the amino acid sequences (Fig. 2a,b). The same conclusion applies to the carboxy-terminal domains of the proteins (Fig. 3a,b) except that, when compared to the S. faecalis autolysin, the E. hirae muramidase has two additional repeats. The alignments derived from the above analysis, yield 141 identities for approx. 250 aligned amino acids (55%) for the amino-terminal domains of the E. hirae muramidase and S. faecalis autolysin (Fig. 4); 26 identities for 45 aligned amino acids (54%) for the six repeats of the E. hirae muramidase (Fig. 5a); 57 identities for 68 aligned amino acids (84%) for the four repeats of the S. faecalis autolysin (Fig. 5b); and 11 identities for 45 aligned amino acids (25%) for all the repeats of the E. hirae muramidase and the S. faecalis autolysin (as derived from the data shown in Figs. 5a,b).

4.2. Homologue of the N-terminal domain

The only protein which showed similarity, at least in part, with the N-terminal domain of the E. hirae and S. faecalis enzymes, is the 316-amino acid FlgJ flagellar protein of S. typhimurium. This protein is of unknown function; it has no repeats and it may be cytoplasmic [5]. As shown in Fig. 2c, the shape and distribution of the hydrophobic and hydrophilic clusters along the carboxy-terminal half of FlgJ (from S152 to F316) closely resemble those exhibited by the T62-G230 segment of the E. hirae muramidase (Fig. 2b) and the P180-P350 segment of the S. faecalis autolysin (Fig. 2a). Alignment of the three peptide segments (Fig. 4) yields 59 identities/homologies for 160 aligned amino acids (34%).

4.3. Homologies of the C-terminal domain

Four proteins have polypeptide segments that show significant similarity with the repeats of the

Fig. 1. Comparison matrices. Intersequence comparisons of the E. hirae muramidase (a), the S. faecalis autolysin (b), the PZA lysozyme (d), the L. monocytogenes P60 protein (e) and the S. aureus protein A (f) and between the E. hirae muramidase and the S. faecalis autolysin (e). In all cases, the span length is fixed to 11 amino acids and the selected threshold score is such that the probability that similarity between pairs of segments occurs by chance is lower than 1 in 1000. R1—R6: repeats. The arrow in (f) indicates the peptide segment which has similarity with the repeats of the E. hirae and S. faecalis enzymes.
E. hirae muramidase and S. faecalis autolysin: i) the 258-amino acid ΦPZA lysozyme, and its homologue, Φ29 lysozyme, have two almost contiguous repeats in the carboxy-terminal region of the protein, from K164 to 1204 and from K215 to S258, respectively (Fig. 1d). The two repeats share 26 identities for 41 aligned amino acids (63%) (Fig. 5c); ii) the L. monocytogenes 432-amino acid P60 protein has two repeats in the amino-terminal half of the protein from V30 to N72 and from A204 to K246, respectively (Fig. 1c). These two repeats share 16 identities for 38 aligned amino acids (42%) (Fig. 5d) iii); the S. aureus 510 amino acid protein A consists of 5 contiguous large repeats spanning from D40 to D330, 11 contiguous small repeats spanning from N331 to N420 and, downstream from this cluster, a non-repeated sequence that extends from K420 to H509 (Fig. 1f, arrow). As shown in Fig. 5, the six repeats of the E. hirae muramidase, the four repeats of the S. faecalis autolysin, the two repeats of the PZA lysozyme, the two repeats of the L. monocytogenes P60 protein and the non-repeated V432-D474 stretch of the S. aureus protein A align well, yielding a consensus of 16 identities/homologies for 42 aligned amino acids (38%).

5. DISCUSSION

The E. hirae muramidase and S. faecalis autolysin have very similar primary structures except that the S. faecalis enzyme has an additional 114-amino acid insert in the N-terminal domain. This insert is of unknown function but could

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**Fig. 2.** Hydrophobic and hydrophilic cluster analysis of the amino-terminal domains of the S. faecalis autolysin (from M1 to P348) (a) and the E. hirae muramidase (from M1 to G230) (b), and of the carboxy-terminal region of the S. typhimurium FigJ protein (from T140 to F316) (c). The hydrophobic residues are encircled and the hydrophobic clusters are also delineated. The hydrophobic residues and clusters conserved in the three sequences are in bold. Those common only to the S. faecalis and E. hirae proteins are shaded. The hydrophilic residues and clusters conserved in the three sequences are marked by scattered points. The one-letter code is used except for Pro: *; Gly: ♦; Cys: ®; Ser: □ and Thr: □.
Fig. 3. Hydrophobic- and hydrophilic-cluster analysis of the carboxy-terminal repeats of the \textit{S. faecalis} autolysin (a) and the \textit{E. hirae} muramidase (b). The hydrophobic residues are encircled and the hydrophobic clusters are also delineated. The hydrophobic residues and clusters conserved in the second sequences are in bold. Those occurring in the repeats 5 and 6 of the \textit{E. hirae} muramidase are shaded. The hydrophilic clusters conserved in the two sequences are marked by scattered points. Pro: *; Gly: ◦;


\begin{align*}
\text{E.h.} & \quad \text{MKKSMRSIE RRKQAQQFKT PVQWKKSTTL PSSALIVSV GTPVALLPVT} \quad 50 \\
\text{S.f.} & \quad \text{MENIARKE RRRLNETKRF KKV.KRSAAL VTGAMVSCS AAPLI.QPVQ} \quad 42
\end{align*}

\begin{align*}
\text{E.h.} & \quad \text{VDAQTPTQF GARIN} \quad 61 \\
\text{S.f.} & \quad \text{A----} \quad \text{inser} \quad \text{T-------------}
\end{align*}

\begin{align*}
\text{E.h.} & \quad \text{TAAFAELIAT YAQPQVAKND LYASMVIMQA VVESGWOSSA L...SOQPY} \quad 108 \\
\text{S.f.} & \quad \text{PSFIIAQAR CAQPQVAKND LYASMVIMQA IVESGWOAST L...SKAPNY} \quad 226 \\
\text{Flg.J} & \quad \text{SKDFLARLGL PRLASEQGQ VPHILLILAQA AESGWOQQR ILRENGEP} \quad 201 \\
\text{Consensus} & \quad \text{---FIA-I---A--------L---L-AQA---LESGWQ---I-------E-P} \\
\text{E.h.} & \quad \text{L L} \\
\text{S.f.} & \quad \text{V V} \\
\text{Flg.J} & \quad \text{V L}
\end{align*}

\begin{align*}
\text{E.h.} & \quad \text{NLFGIK.GS YQQTVYMDT LEILNNKWS KKEPFPVQPS FAESPMDMA} \quad 156 \\
\text{S.f.} & \quad \text{NLFGIK.GS YNGQSVYMD TWEIENKLYV KKEPFPVQPS YMESPDMA} \quad 274 \\
\text{Flg.J} & \quad \text{NVFGKVATAS WKGVTEITTT TFEYENEA DKVAKRFVSS YLEALSDV} \quad 251 \\
\text{Consensus} & \quad \text{NLFGIK---S W-G-----T -EY-N-----K--FR-Y-S P-E-F-D-----} \\
\text{E.h.} & \quad \text{V V} \\
\text{S.f.} & \quad \text{Y L}
\end{align*}

\begin{align*}
\text{E.h.} & \quad \text{VLRNTSFNG .YVAYAGWKS NTSEYTDATA CLTGRYATDP GYAGKLNN} \quad 205 \\
\text{S.f.} & \quad \text{VLSKTSFQAG VYVYAGAWKS NTSSYRDATA WLTGRYATDP SYNKLNN} \quad 324 \\
\text{Flg.J} & \quad \text{LRTINP....RYAVATTA AATA.QGAV LQNYATPDY NYARKLTM} \quad 294 \\
\text{Consensus} & \quad \text{L----------Y--------A-A-----ATDP---Y----KL---I} \\
\text{E.h.} & \quad \text{V V} \\
\text{S.f.} & \quad \text{Y L}
\end{align*}

\begin{align*}
\text{E.h.} & \quad \text{TTYGLVKYDPT PSSGANGGGV TIGNGTNQG TSNSGSGMN SGGS} \quad 358 \\
\text{S.f.} & \quad \text{TYNLLQTVYGD PSSGANGGGV TV.NPGTGG NNQSG} \\
\text{Flg.J} & \quad \text{QQ....} \quad \text{LLLL} \\
\text{Consensus} & \quad \text{---L---} \quad \text{---} \quad \text{---G---} \quad \text{---} \quad \text{---} \quad \text{---} \quad \text{---} \quad \text{---} \quad \text{---}
\end{align*}

Fig. 4. Amino acid sequence alignment of the amino terminal domain of the \textit{E. hirae} muramidase (E.h), the amino-terminal domain of the \textit{S. faecalis} autolysin (S.f) and the carboxy-terminal region of the \textit{S. typhimurium} FlgJ protein. The insert E52-A166 of the \textit{S. faecalis} autolysin is excluded from the alignment. Identities occurring along the sequences of the \textit{E. hirae} and \textit{S. faecalis} proteins are marked by black circles (●). The consensus shows the identities and homologies occurring along the sequences of all three proteins.
possibly be a pre-pro-peptide. Given the high extent of similarity in the structure, one may safely conclude that the E. hirae and S. faecalis peptidoglycan hydrolases have similar specificities and catalytic mechanisms. In all likelihood, their active centre is located in the N-terminal domain and results from the folding of that approx. 200-amino acid polypeptide stretch which shows high similarity in the two enzymes.

Given that the E. hirae enzyme (and by extension the S. faecalis enzyme) is a muramidase, one would expect that aspartic/glutamic acids are present in the active site. In fact, the N-terminal domain contains several acidic residues which are separated by 13–33 amino acids consistent with similar locations in other lysozymes. Thus E128 of the E. hirae enzyme is located 25 amino acids upstream from D153, similar to the 27-amino acid separation of these residues in the putative active site of the Chalaropsis (D6-E33) [12] and bacteriophage CPI-1 (D10-E37) muramidas [13–15]. Also, the pairs D50-E68, D125-E139, and E139-D153 in the E. hirae enzyme are spaced similarly to the pair E35-D52 in hen egg-white lysozyme [16]. However, the active site containing N-terminal domains of the E. hirae and S. faecalis enzymes lack overall sequence relatedness with other glycosidases, in particular muramidases (including the Bacillus PZ and PZ lysozymes) of known primary structure. Lysozymes exist which have a similar three-dimensional structure despite the absence of amino acid sequence similarity [17].

The E. hirae muramidase possesses several amino acid groupings (the tetrad S175NTK, the triad ASN at positions S218, S243, S317 and S320, and the triad KSG at positions K260 and K341) which, at first sight, can be regarded as equivalents of the characteristic motifs found in the serine β-lactamases and penicillin-binding proteins [18]. However, the S. faecalis autolysin lacks the SXXK active-site motif. The shape and distribution of the hydrophobic- and hydrophilic-clusters along the amino acid sequences of both E. hirae and S. faecalis enzymes differ from those found in the serine β-lactamases and penicillin-binding proteins [19,20]. Evidence has been presented that the E. hirae muramidase binds penicillin with low affinity [21]. On the basis of the data presented here, it may well be that this

Fig. 5. Amino acid sequence alignment of the carboxy terminal repeats of the E. hirae muramidase (a); the carboxy-terminal repeats of the S. faecalis autolysin (b); the carboxy-terminal repeats of the PZ lysozyme (c); the internal repeats of the L. monocytogenes P60 protein (d); and the V432-D474 segment of the carboxy-terminal region of the S. aureus protein A (e). Identities occurring in groups (a), (b), (c) and (d), respectively, are marked by black circles (●). The consensus derived from the 15 aligned peptide segments is shown in (f).
protein binds penicillin by a mechanism different from penicilloyl transfer involving an 'active' serine residue.

Based on the principle that protein molecules are frequently constructed from modules and that each of these modules folds into one domain that performs a particular function, one may hypothesize that the carboxy-terminal domain of the S. typhimurium flagellar FlgJ protein, which is similar to the active site of the E. hirae and S. faecalis enzymes, might function as a glycosidase of one kind or another, perhaps a muramidase.

Downstream from the catalytic amino-terminal domain, the E. hirae and S. faecalis enzymes have carboxy-terminal extended repeat structures. These repeats may be responsible for substrate, i.e. peptidoglycan, binding. They are similar to those of the Bacillus ϕPZA and ϕ29 lysozymes, with one segment of protein A and with the two repeats that occur at internal positions in the L. monocytogenes P60 protein. Spontaneous mutants of L. monocytogenes impaired in the synthesis of P60 lose the ability to invade phagocytic cells and the bacteria grow in long cell chains. The chains disaggregate after treatment with P60 and invasiveness of the treated mutant cells is restored [6]. Peptidoglycan hydrolase activities have been associated with cell separation in other bacterial systems (summarized in ref. 22). The repeats described here may form a particular family of ligand-binding sequences. They do not exhibit similarity to the repeats of: i) the carbohydrate-binding proteins reviewed by Wren [23]; ii) the surface proteins from Gram-positive cocci reviewed by Fischetti et al. [24]; and iii) the pneumococcal peptidoglycan amidase and CPL-1, CPL-7 and CPL-9 muramidases [25]. The repeats of the pneumococcal enzymes appear to participate in the recognition of moieties in the pneumococcal wall [15].

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