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A novel secreted metzincin metalloproteinase from *Bacillus intermedius*

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ABSTRACT

The *mprBi* gene from *Bacillus intermedius* 3–19 encoding a novel secreted metalloproteinase was identified. The *mpriBi* gene was expressed in an extracellular proteinase-deficient *Bacillus subtilis* BG 2036 strain and the corresponding protein was characterized biochemically. The 19 kDa MprBi protein was purified to homogeneity and sequenced by mass spectroscopy and Edman degradation methods. Amino acid sequence analysis of MprBi identified an active site motif HEYGHNFGLPHD and a conserved structural component Met-turn, both of which are unique features of the metzincin clan. Furthermore, MprBi harbors a number of distinct sequence elements characteristic of protein-ase domains in eukaryotic adamalysins. We conclude that MprBi and similar proteins from other *Bacillus* species form a novel group of metzincin metalloproteinases in prokaryotes.

1. Introduction

Proteinases, which hydrolyze proteins into short peptides or amino acids, represent one of the most commercially important groups of industrial enzymes and comprise the majority of the to-tal enzyme sales worldwide. *Bacillus* species synthesize several groups of extracellular proteinases, such as serine and metal pro-teinases, which are classified based on their mechanism of action and catalytic amino acids [1].

Zinc-dependent endo-metalloproteinases are a subset of metalloproteinases found in all kingdoms of life. These enzymes are characterized by a consensus amino acid sequence HExxH, where the histidines are zinc ligands and the glutamic acid functions as a catalytic base. A third zinc ligand is provided by the side-chain of His, Glu or Asp, usually located downstream of this motif [2]. In the MEROPS proteinase database, the HExxH motif-containing enzymes are grouped in the MA clan (http://merops.snger.ac.uk).

The metzincin subclan of zinc-dependent endo-metalloprotein-ases includes several extracellular and membrane-bound protein-ase families containing an extended consensus sequence,

HExx<u>H</u>xxGxx<u>H</u>x, which comprises three zinc ligands (underlined) and the general base glutamic acid [3,4]. Many typical metzincins display very limited amino acid conservation overall and often share less than 20% sequence identity. Despite this fact, the tertiary structure of their catalytic domains and their active site sequences are remarkably conserved [5–7]. The name of this subclan origi-nates from a conserved methionine located in a 1,4-β-turn (so called Met-turn) of the protein. Metzincins have been recognized as key players in a variety of biological systems, where they regu-late the activity of other biological molecules (cytokines, growth factors, other proteinases) by limited proteolysis [4,6].

The metzincin subclan contains several proteinase families, including astacins (BMP-1, tolloids, meprins) and adamalysins or ADAMs (a disintegrin and metalloproteinase-like). Over 120 astac-ins have been described in a variety of organisms from bacteria to mammals, but interestingly not in plants, fungi or in Bacilli. Astac-ins are produced as zymogenes, with a propeptide and a signal peptide at the N-terminus of the mature form, and typically re-quire activation via proteolytic processing, in many cases after a basic residue (autocatalytically in the case of astacin) [7–10]. The ADAMs (adamalysins/reprolysins) are multi-domain proteins found in mammalian reproductive tissues and in snake venom. The ADAMs are secreted as pro-enzymes with the N-terminal signal sequence and propeptide and are activated upon propeptide removal either by other proteinases or autocatalytically [11,12].

Bacillus intermedius 3–19 is a species of soil bacterium, which secretes a number of extracellular enzymes, including a glutamyl-endopeptidase and a subtilisin-like proteinase [13–19]. Here we report the cloning, purification and sequence analysis of a novel extracellular metalloproteinase from *B. intermedius*, dubbed MprBi. Our data indicate that *mprBi* encodes an unusual metalloproteinase of the metzincin subclan, the first characterized enzyme of this type in the genus *Bacillus*.

2. Materials and methods

2.1. Bacterial strains and plasmids

Streptomycin-resistant *B. intermedius* strain 3–19, obtained from the All-Russia Collection of Microorganisms (B-3833), was used for genomic DNA library construction. The shuttle vector pCB22 [20] was used for cloning of proteinase gene. The extracellular proteinase-deficient *Bacillus subtilis BG 2036* strain was kindly supplied by E. Ferrari (Genencor Int. Inc. USA) and used as a recombinant plasmid host.

2.2. Cloning and identification of mprBi gene

Genomic DNA of *B. intermedius* was isolated as described previously [21]. Total DNA (100 μg) was digested with *Sau*3A (4 U) at 37 °C for 60 min. The partially digested DNA was run on 0.8% agarose gel. Fragments in the range of 6 kb were electroeluted, ligated into *Bgl*II digested pCB22 plasmid and transformed into extracellular proteinase-deficient *B. subtilis BG 2036* strain as described by Anagnostopolous and Spizizen (1961). This *B. subtilis* strain lacks both known secreted proteinases (a neutral proteinase nprE522 and an alkaline proteinase apr-684) and is therefore completely free of any extracellular proteinase activity, but is otherwise unaffected in its growth, morphology or sporulation [22]. Transformants were plated on an SG-skim milk plate containing 10 μg/ml of erythromycin. One colony surrounded by the biggest halo was selected for plasmid isolation. The 6 kb genomic DNA insert from the isolated plasmid was sequenced and analyzed using the NCBI BLAST server and Open Reading Frame Finder (ORF Finder) (http://www.ncbi.nlm.nih.gov) [23]. Promoter regions were identified using the BPROM program (http://www.softberry.com). Signal peptide was identified using the SignalP 3.0 server (http://www.cbs.dtu.dk/services/SignalP). The coding region of the identified *mprBi* gene and its regulatory sequences were subcloned into the empty pCB22 vector and the entire construct was re-introduced into the extracellular proteinase-deficient *B. subtilis BG 2036* strain for further analysis.

2.3. Determination of proteolytic activity

Proteolytic activity was determined by the azocasein cleavage assay. 100 μl of 1% azocasein solution in 100 mM Tris–HCl buffer containing 5 mM $CaCl_2$, pH 7.1 was incubated with 50 μl of the culture supernatant for 15 min at 37 °C. The reaction was stopped by the addition of 200 μl of 10% trichloracetic acid. After centrifugation at $9500 \times g$ for 5 min, 250 μl of the supernatant was mixed with 50 μl of 5 M NaOH and the optical density was measured at 450 nm on a Model 2550 Microplate Reader (BioRad, USA). One unit of activity was defined as the amount of enzyme necessary to change the absorbance by 1 optical density unit per min.

2.4. Protein localization

Subcellular proteinase localization was determined by the azocasein cleavage assay using proteins isolated from different subcellular fractions and from the culture medium. Briefly, after 24, 30 and 36 h of cultivation cells were sedimented by centrifugation ($10\,000 \times g$, 5 min) and washed using 0.85% NaCl. The resulting cell suspension was incubated with lyzosyme (1 mg/ml) in 10 mM Tris–HCl buffer pH 8.5 in the presence of 20% sucrose for 25 min at room temperature. Protoplast formation was monitored by microscopy. Protoplasts were purified by centrifugation ($10\,000 \times g$, 15 min), while cell wall proteins remained in the supernatant. Membrane-bound proteins were solubilized by detergent treatment with 0.1% Triton X-100 in 0.1 M Tris–HCl buffer pH 8.0 with 50 mM NaCl and 20% sucrose for 20 min at room temperature, followed by centrifugation ($13\,000 \times g$, 20 min). To isolate intracellular proteins, protoplasts were lysed by osmotic shock with the addition of 5 mM Tris–HCl buffer pH 7.8 at 4 °C. Protoplast extracts were digested with DNase I (1 mg/ml) for 30 min at room temperature and centrifuged ($15\,000 \times g$, 30 min) to obtain the fraction of intracellular proteins. To inhibit intracellular serine proteinases, possibly present in *B. subtilis* protein extracts, proteolytic activity against azocasein in all fractions was determined in the presence of 5 mM PMSF, a specific serine proteinase inhibitor. Proteinase activity in all fractions was defined as unit/mg of biomass.

2.5. The effect of inhibitors

Fifty microliters of supernatant or homogeneous enzyme solution in 50 mM Tris–HCl buffer (pH 7.1) containing 0.2 mM $CaCl_2$ and either 10 mM 1,10-phenanthroline, or 10 mM ethylenediaminetetraacetic acid (EDTA), or 10 mM phenylmethyl sulfonyl fluoride (PMSF) was incubated at 25 °C for 1 h. Residual proteolytic activity was determined by hydrolysis of 1% azocasein in 50 mM Tris–HCl buffer containing 1 mM $CaCl_2$, pH 7.1, as described above.

2.6. Purification of MprBi proteinase

Bacterial cells were grown in the culture medium (0.01 g/l $CaCl_2$, 0.01 g/l $MgSO_2$, 0.3 g/l NaCl, 0.01 g/l $MnSO_4$, 0.01 g/l NH_4Cl, 1 g/l yeast extract, 1.7 g/l peptone) and harvested by centrifugation ($4500 \times g$, 60 min, 4 °C). Extracellular proteins were precipitated with ammonium sulfate (20–70% saturation interval). The precipitate was dissolved in 50 mM Tris–HCl buffer pH 7.3 containing 5 mM $CaCl_2$, dialyzed, and loaded onto a Bacitracin-sylochrom column, equilibrated with the same buffer. Protein was eluted with 50 mM Tris–HCl buffer pH 7.3 containing 1 M NaCl and 7% isopropanol. Elution fractions were analyzed for total protein concentration by Bradford assay and proteolytic activity was measured with the azocasein assay. Fractions with high proteolytic activity were combined, dialyzed against 50 mM Tris–HCl buffer pH 7.3 containing 35% ammonium sulfate and placed on a Octyl-Sepharose hydrophobic column «HiTrap» ("Pharmacia"), equilibrated with the same buffer. The protein was eluted using a linear ammonium sulfate gradient (35–0%) in the same buffer at a flow rate of 1 ml/min. The proteolytic activity and protein amount were determined in the collected fractions as described above. The concentrated protein samples were applied to a 12.5% preparative SDS–polyacrylamide gel, followed by Coomassie brilliant blue staining and Imidazol/ZnCl2 negative staining as described (http://www.molbiol.ru/protocol/17_03.html).

2.7. MALDI-TOF and N-terminal sequencing

MprBi proteinase was digested by trypsin according to the method described at http://www.sigmaaldrich.com/etc/medialib/docs/Sigma/General_Information/maldi_tof_ms_analysis.Par.0001.File.tmp/maldi_tof_ms_analysis.pdf and analyzed using Ultraflex II BRUKER mass spectrometer (Justus Liebig University, Germany).

The theoretical peptide mass for the predicted amino acid sequence of *B. intermedius* metalloproteinase MprBi was calculated using the Peptide Mass Fingerprint program (http://www.expasy.net/tools/dna.html). Mass spectrometry data for the peptide weights were matched with peptide sequence from protein databases in NCBI using the MASCOT search program (www.matrixscience.com). N-terminal amino acid sequence was determined by automated Edman degradation method using a Model 816 Protein Sequences (Germany) equipped with a Model 120A PTH Analyzer (Applied Biosystems, USA).

2.8. Nucleotide sequence accession number

Nucleotide sequence of the cloned 6 kb genomic DNA from *B. intermedius*, containing the *mprBi* gene, was deposited into GenBank, accession number EU678894.2. MprBi protein accession number is ACE75740.

3. Results and discussion

3.1. Cloning, identification and sequence analysis of B. intermedius mprBi gene

B. intermedius is a rich source of extracellular enzymes. Among other hydrolases, we have previously isolated and characterized two secreted serine proteinases from *B. intermedius* [13–19]. During the course of these earlier studies, we detected an additional weak proteolytic activity present in *B. intermedius* culture medium, and preliminary experiments indicated that this enzyme may represent a previously uncharacterized metalloproteinase. However, detailed biochemical characterization of this activity was stymied by the presence of two highly abundant serine proteinases. In an effort to identify this potentially novel enzyme, we constructed a genomic library of *B. intermedius* and introduced it into an extracellular proteinase-deficient *B. subtilis BG 2036* strain on pCB22

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301  TTTTTGTTTTTGTAAGACCCAGACCAAAAGCGCTATTTTCCGATGAAATG
351  TGTGCTGAAATATCCGAAAAATTAGTGAATATGACGTATTTCGACTTTTT
                                      -35
401  TTTTGTGTATCCAATTCCCTGTTTTTTGACTGAATAGAAGGAAAATGGTGA
        -10              +1          RBS              M
451  ATTTTCAGTGAATATATAAACCTTGTGAAAATCAAAGGAGGGATAGGAATG
        K  K  R  S  V  F  L  S  F  L  L  V  G  S  L  L  P
502  AAAAAGCGTTCAGTCTTTTTGTCATTTTTATTGGTAGGTAGTTTGTTACCG
        G  V  S  S  A  S  A  P  V  A  S  A  G  H  G  H  D
553  GGAGTAAGTTCAGCTTCAGCACCAGTCGCGAGTGCTGGTCATGGGCATGAT
        H  G  H  A  P  F  E  T  H  I  S  E  G  L  P  K  A
604  CATGGTCATGCGCCGTTCGAAACACATATTAGTGAGGGGTTGCCAAAGGCA
        N  D  F  K  D  L  T  K  A  P  P  I  E  R  D  V  K
655  AACGATTTTAAAGATTTGACGAAAGCACCTCCAATTGAACGAGATGTGAAA
        T  K  V  L  D  E  S  G  K  Q  V  G  S  R  T  F  K
706  ACAAAGGTATTAGATGAGTCTGGTAAGCAAGTAGGCTCTAGAACCTTTAAG
        A  N  T  G  D  S  I  S  T  K  A  S  T  G  S  Q  K
757  GCGAATACAGGAGATTCTATTTCAACAAAGGCAAGCACAGGCAGTCAAAAA
        V  T  V  Y  A  V  A  D  A  Q  Y  R  A  K  Y  S  D
808  GTAACGGTTTATGCTGTAGCAGATGCGCAGTATCGTGCGAAATATAGTGAC
        W  Q  T  R  I  V  S  I  I  E  Q  A  D  V  T  F  N
859  TGGCAGACGCGGATTGTCAGCATCATTGAGCAAGCGGACGTGACCTTTAAC
        R  D  H  D  V  D  F  V  V  Q  A  V  G  S  W  T  S
910  CGTGATCATGATGTGGACTTTGTCGTACAAGCCGTAGGATCTTGGACGTCT
        S  G  S  N  A  E  Q  I  L  S  N  L  S  R  S  F  D
961  TCAGGATCAAATGCAGAGCAAATTTTATCTAACCTTTCGCGCAGCTTTGAT
        G  R  G  Y  D  F  V  T  G  F  T  A  N  P  N  F  D
1012 GGCAGAGGATACGATTTTGTCACTGGATTTACAGCAAATCCAAACTTTGAT
        A  G  G  I  A  Y  V  Y  N  S  A  P  S  G  S  A  F
1063 GCGGGCGGAATCGCTTATGTATACAATAGTGCACCGAGCGGAAGTGCATTC
        A  V  N  L  D  Q  G  T  A  N  T  A  K  A  A  T  H
1114 GCCGTTAACCTTGATCAAGGAACAGCGAACACCGCAAAAGCGGCTACGCAT
        E  Y  G  H  N  F  G  L  P  H  D  P  Q  G  S  G  I
1165 GAATACGGTCATAACTTTGGCTTACCGCATGACCCTCAAGGCAGCGGCATT
        V  C  L  M  N  Y  D  Y  S  Y  T  V  D  F  F  D  A
1216 GTCTGCTTAATGAACTATGATTATTCCTACACAGTCGATTTCTTTGATGCG
        A  H  K  N  Q  V  N  R  N  K  A  W  Y  R  *
1267 GCTCATAAAAATCAAGTGAACCGTAACAAAGCGTGTGTACAGATAAAATAAG
1318 AGACAAAAGGACAAACACCTATGTGCTTGTCCTTTTTTATGCTTACAGGTC
1369 GCTGATGCGTTTTCCTGCTACGGCATAGTGTTCTTTTTTTCATTTCTTCAAT
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Fig. 1. Sequence of the *B. intermedius mprBi* gene. Nucleotide and amino acid sequence of MprBi are shown. Putative −35 and −10 regions of the promoter and ribosome binding site (RBS) are marked with grey color. The transcription initiation codon GTG is underlined, and the translation initiation codon ATG is shown in bold. Asterisk indicates a stop codon. Potential transcription terminators are indicated by single lines after the stop codon. Signal peptide sequence is shown in italics, propeptide sequence is underlined. N-terminal ten amino acids, determined by the Edman degradation method, are shown in bold. Active site and Met-turn motifs are shown in boxes.

Table 1
Specific proteinase activity in the *B. subtilis* culture medium and in subcellular fractions of recombinant and untransformed cells.

Fraction	Specific endopeptidase activity, U/mg of cells $\times 10^3$					
	24 h		30 h		36 h	
	Recombinant cells	Untransformed cells	Recombinant cells	Untransformed cells	Recombinant cells	Untransformed cells
Culture medium	715.00	3.20	1324.00	3.90	910.00	4.50
Cell wall	4.60	3.10	5.00	3.40	6.00	5.50
Membrane	7.80	8.20	8.70	8.10	9.20	9.00
Cytoplasm	3.40	3.70	5.00	4.80	6.90	7.00

Table 2
The effects of group-specific proteinase inhibitors on the MprBi enzymatic activity in *B. subtilis* culture medium.

Inhibitor (10 mM)	Residual proteolytic activity (%)
PMSF	99.20
EDTA	3.90
1,10-Phenanthroline	0.10

plasmid. Following transformation, erythromycin-resistant *B. subtilis* colonies were screened on SG-skim milk plates for the presence of recombinant extracellular proteinase from *B. intermedius*. One colony producing the biggest halo was selected for further analysis.

The isolated plasmid harbored a 5896 bp insertion of *B. intermedius* genomic DNA. G/C ratio was determined to be 43%, which is typical for bacillar genomes. Preliminary sequence analysis of the cloned *B. intermedius* genomic DNA indicated that it contained several possible open reading frames (ORFs), one of which appeared to encode a metalloproteinase. This ORF was designated *mprBi*

(Fig. 1). SignalP analysis of MprBi protein sequence revealed a potential propeptide cleavage site (ASA), indicating that this protein is likely to be extracellular. Thus, *mprBi* gene was selected for further analysis. The putative promoter sequence contains the typical −35 and −10 regions and the Shine–Dalgarno sequence (SD-site), located 7 bp upstream of translation start site ATG. The complete ORF consists of 810 bp. The putative signal peptide contains 30 amino acids.

3.2. mprBi gene encodes a secreted metalloproteinase

We PCR-amplified *mprBi* gene from the pCB22 plasmid and reintroduced it into the extracellular proteinase-deficient *B. subtilis* BG 2036 strain for further analysis. Different cellular fractions of *B. subtilis*, as well as its culture medium, were tested for the presence of high levels of proteinase activity. As expected for an extracellular proteinase, the peak of activity was found in the culture medium (Table 1). Only trace amounts of proteolytic activity were detected in various cellular fractions as well as in the culture medium of untransformed *B. subtilis* cells, which served as a negative

Table 3
Purification of MprBi from the *B. subtilis* culture medium.

Purification steps	V (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Purification fold	Yield (%)
Culture medium	480.00	7260.00	392.00	0.05	1.00	100
Ammonium sulfate fraction*	18.00	269.00	222.00	0.83	15.70	57
Bacitracin-sylochrom	64.00	22.80	49.00	2.10	39.60	12.5
Octyl-sepharose	6.00	5.14	31.40	6.10	115.00	8

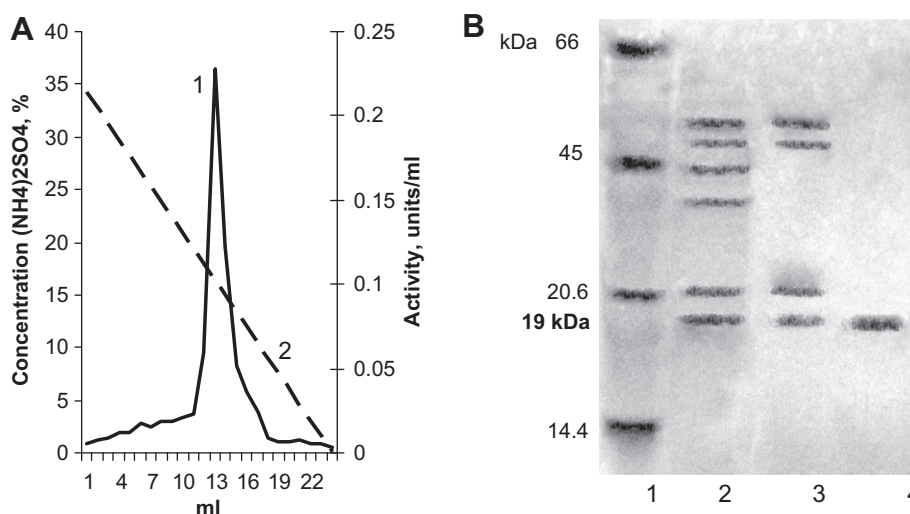* Values shown for dialyzed samples.

Fig. 2. Purification of MprBi proteinase. (A) Chromatography of MprBi on Octyl-sepharose column. 1 – endopeptidase activity, 2 – ammonium sulfate, %. (B) SDS–PAGE of purified MprBi. Lane 1, protein markers; lane 2, protein fraction after ammonium sulfate precipitation; lane 3, protein fraction after bacitracin column chromatography; lane 4, MprBi after Octyl-sepharose column chromatography. Protein markers: bovine albumine (66 kDa), ovalbumin (45 kDa), papain (20.6 kDa), lysozyme (14.4 kDa).

control. The presence of the proteolytic activity predominantly in B. subtilis culture medium indicates that MprBi is a secreted proteinase.

As mentioned before, the original B. intermedius strain secretes a number of serine proteinases [13–15]. To analyze the nature of MprBi proteolytic activity, we tested the effects of several specific proteinase inhibitors on the enzyme activity (Table 2). The proteolytic activity in the B. subtilis culture medium was resistant to PMSF, which is a specific inhibitor of serine proteinases. In contrast, most of the proteinase activity was inhibited by a zinc-specific chelator 1,10-phenanthroline and by a general metalloproteinase inhibitor EDTA. These results indicate that MprBi is a zinc-dependent metalloproteinase.

3.3. MprBi purification and protein sequence determination

To purify MprBi from the culture medium, we employed a 3-step protocol (Table 3). First, extracellular proteins were precipitated with ammonium sulfate in 20–70% saturation interval. Specific activity of the dialyzed fraction after ammonium sulfate precipitation was increased over 15-fold, with 57% yield (Table 3). Next, the proteinase was purified over a bacitracin-sylochrom column, which resulted in 2.5-fold improvement in purification fold, with 12.5% yield (Table 3). The last purification step was protein chromatography over an Octyl-sepharose column, which allowed us to purify MprBi to homogeneity (Fig. 2A and B). The

Metzincins	Active center motif												% homology with MprBi	Met-turn motif				
	126	127	128	129	130	131	132	133	134	135	136	137		145	146	147	148	149
MprBi (B. intermedius)	H	E	Y	G	H	N	F	G	L	P	H	D	N/A	C	L	**M**	N	Y
ASTACINS																		
Astacin (crayfish)	H	E	L	M	**H**	A	I	**G**	F	Y	**H**	E	42	S	I	**M**	H	Y
α-MEP (mouse)	H	E	I	L	**H**	A	L	**G**	F	F	**H**	E	42	S	L	**M**	H	Y
β-MEP (rat)	H	E	F	L	**H**	A	L	**G**	F	W	**H**	E	42	S	V	**M**	H	Y
BMP1/procollagen C-proteinase (human)	H	E	L	G	**H**	V	V	**G**	F	W	**H**	E	50	S	T	**M**	H	Y
SPAN/BP10 (sea urchin)	H	E	I	G	**H**	A	I	**G**	F	H	**H**	E	50	S	I	**M**	H	Y
Tolloid (Dr. melanogaster)	H	E	L	G	**H**	T	I	**G**	F	H	**H**	E	50	S	I	**M**	H	Y
SERRALYSINS																		
Proteinase Serratia	H	E	I	G	**H**	A	L	**G**	L	S	**H**	P	58	S	L	**M**	S	Y
Proteinase B (E. chrysanthemi)	H	E	I	G	**H**	A	L	**G**	L	S	**H**	P	58	S	I	**M**	S	Y
MATRIXINS																		
MMP-1 (human fibroblast collagenase)	H	E	L	G	**H**	S	L	**G**	L	S	**H**	S	58	A	L	**M**	Y	P
MMP-3 (human stromelisin-1)	H	E	I	G	**H**	S	L	**G**	L	F	**H**	S	58	A	L	**M**	Y	P
ADAMALYSINS/ REPROLYSINS																		
Adamalysin II (C.adamanteus snake venom)	H	E	L	G	**H**	N	L	**G**	M	E	**H**	D	66	C	I	**M**	R	P
Atrolysin C	H	E	L	G	**H**	N	L	**G**	M	E	**H**	D	66	C	I	**M**	R	P
Trimerelysin	H	E	L	G	**H**	N	L	**G**	M	P	**H**	D	66	C	I	**M**	S	D
THERMOLYSIN	142	143			146													
Thermolysin [29] (B. thermoproteolyticus)	H	E	L	T	H	A	V	T	D	Y	T	A	25			absent		

Fig. 3. Partial amino acid alignment of active center and Met-turn motifs of MprBi and metalloproteinases from several major metzincin groups. Amino acid numbering is shown for MprBi.

Table 4
Comparison of hypothetical MprBi-like metalloproteinases in Bacillus species.

Putative adamalysins in Bacilli	Accession number	Active center motif	Met-turn motif	% homology with MprBi
MprBi (B. intermedius 3–19)	ACE75740.2	HEYGHNFGLPHD	CLMNY	N/A
Reprolysin (M12B) family zinc metalloprotease [Bacillus pumilus ATCC 7061]	ZP_03055196.1	HEYGHNFGLPHD	CLMNY	98
Hypothetical protein BPUM_3392 [Bacillus pumilus SAFR-032]	YP_001488604.1	HEYGHNFGLPHD	CLMNY	98
Hypothetical protein BL03917 [Bacillus licheniformis ATCC 14580]	YP_081058.1	HEFSHNFGLNHD	CIMNY	69
Hypothetical protein RBAM_030640 [Bacillus amyloliquefaciens FZB42]	YP_001422626	HEFSHNFGLQHD	CVMNY	62

molecular weight of MprBi, calculated based on the protein electrophoresis results, is 19 kDa (Fig. 2B).

The availability of large quantities of homogeneous recombinant MprBi allowed us to utilize MALDI-TOF and Edman degradation techniques to confirm the amino acid sequence of the mature protein (Fig. 1 and Supplementary material). The protein's first ten N-terminal amino acids are ASTGSQKVTV. The identification of alanine as the first N-terminal amino acid of the mature protein allowed us to verify the molecular organization of the *mprBi* gene. It encodes a signal peptide of 30 amino acids, a 66 amino acid propeptide sequence and 174 amino acids of the mature protein (Fig. 1).

3.4. MprBi is an unusual metzincin with sequence similarity to the proteinase domain of eukaryotic adamalysins

Amino acid sequence alignment of MprBi with several other metalloproteinases allowed us to identify the conserved active site motif (Figs. 1 and 3). MprBi harbors several active site histidines and a glutamate residue, indicating that this protein belongs to the class of zinc metalloproteinases. Furthermore, while most zinc metalloproteinases contain only five active site amino acids **HE**xx**H**, a subset of these proteins, called the metzincin clan, harbors an extended 12 amino acid site **HE**xx**H**xx**G**xx**H**x. A similar extended region **HE**YG**H**NF**G**LP**H**D is present in MprBi (Fig. 3), suggesting that this *B. intermedius* metalloproteinase also belongs to the metzincin clan. The other characteristic feature of all proteinases from the metzincin clan is the presence of Met-turn motif, which is typically located very close to the protein's C-terminus. As expected for a metzincin-type protein, MprBi harbors the corresponding Met-turn sequence **CLM**N**Y** (amino acids 145–149) (Fig. 3).

Several lines of evidence suggest that MprBi is most closely related to a metalloproteinase domain of one particular metzincin family – eukaryotic adamalysins. First, while both astacins and adamalysins contain a negatively charged residue in the twelfth position of the active site, **HE**xx**H**xx**G**xx**HE/D**, astacins are characterized by a conserved Glu in this position, which is involved in the formation of a salt bridge to the N-terminus after propeptide removal [9,10]. In contrast, adamalysins harbor Asp in this position, which is also present in MprBi (Fig. 3). Second, the presence of Cys 145 in the Met-turn motif of MprBi is also uniquely indicative of adamalysins (Fig. 3). Third, the overall amino acid sequence similarity of MprBi with adamalysins is 66%, while it is only 42–58% with astacins and serralysins. Finally, a unique feature of all adamalysins is a long α-helix occupying the region between β-strands sII and sIII [5]. A similar region is also present in MprBi (see Supplementary data).

While MprBi appears to be the most similar to eukaryotic adamalysins, it also harbors sequence features characteristic of astacins and serralysins, such as the presence of Tyr 149 in the fifth position of the Met-turn motif (Fig. 3). In astacins and serralysins, tyrosine in this position is used as a switch to help stabilize the transition state during substrate hydrolysis [24–27]. Interestingly, matrixins also harbor Tyr in the Met-turn motif, but in the fourth position. The unique combination of sequence features, characteristic of proteinase domains in both adamalysins and astacins/serralysins, makes MprBi a very interesting protein from a structure and function perspective.

Taken together, our biochemical and sequence analysis data strongly indicate that MprBi is most similar to the adamalysin group of metalloproteinases, and, therefore, represents the first such enzyme identified and characterized in Bacilli. In addition, a simple protein blast search indicates that similar proteins may also exist in other *Bacillus* species (Table 4). Indeed, putative genes encoding at least four MprBi-like metalloproteinases have recently

been sequenced from *Bacillus pumilis* (ZP_03055196 and YP_001488604), *Bacillus licheniformis* (YP_081058.1) and *Bacillus amyloliquefaciens* (YP_001422626.1). Similar to MprBi, all these hypothetical *Bacillus* proteinases harbor the characteristic Asp in the twelfth position of the active site, and Cys and Tyr in the 145 and 149 positions of the Met-turn motif, respectively (Table 4). Therefore, MprBi may in fact represent the founding member of a novel *Bacillus*-specific subfamily of metzincins with a unique combination of adamalysin-like and astacin/serralysin-like sequence elements.

In eukaryotes, metalloproteinases are involved in the regulation of hormonal homeostasis, signal transduction, production of bioactive peptides and modulation of protein–protein and cell-to-cell interactions [3]. Aberrant metalloproteinase functions often lead to various pathologies, including tumorigenesis, inflammation, infections, allergy and asthma [28–31]. While the exact biological function of MprBi requires further investigation, it is becoming increasingly evident that extracellular proteinases are not only involved in the digestive function, but may also participate in cellular regulatory pathways [32,33]. The identification of MprBi proteinase clearly suggests that, despite decades of extensive research, bacteria and, specifically, the genus of *Bacillus*, may still yield unexpected discoveries and provide a valuable source of novel enzymes, which could potentially be beneficial for pharmaceutical and other biotechnological industries.

Acknowledgements

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2010.09.049.

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