SufA – a novel subtilisin-like serine proteinase of *Finegoldia magna*

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*Finegoldia magna* is an anaerobic Gram-positive bacterium and commensal, which is also associated with clinically important conditions such as skin and soft tissue infections. This study describes a novel subtilisin-like extracellular serine proteinase of *F. magna*, denoted SufA (subtilase of *Finegoldia magna*), which is believed to be the first subtilase described among Gram-positive anaerobic cocci. SufA is associated with the bacterial cell surface, but is also released in substantial amounts during bacterial growth. Papain was used to release SufA from the surface of *F. magna* and the enzyme was purified by ion-exchange chromatography and gel filtration. A protein band on SDS-PAGE corresponding to the dominating proteolytic activity on gelatin zymography was analysed by MS/MS. Based on the peptide sequences obtained, the *sufA* gene was sequenced. The gene comprises 3466 bp corresponding to a preprotein of 127 kDa. Like other members of the subtilase family, SufA contains the catalytic triad of aspartic acid, histidine and serine with surrounding conserved residues. A SufA homologue was identified in 33 of 34 investigated isolates of *F. magna*, as revealed by PCR and immunoprinting. The enzyme forms dimers, which are more proteolytically active than the monomeric protein. SufA was found to efficiently cleave and inactivate the antibacterial peptide LL-37 and the CXC chemokine MIG/CXCL9, indicating that the enzyme promotes *F. magna* survival and colonization.

**INTRODUCTION**

*Finegoldia magna* (formerly *Peptostreptococcus magnus*) is part of the anaerobic commensal microflora present in the mouth, the upper respiratory and gastrointestinal tracts, the female genitourinary system and in the skin. *F. magna* is also the most common Gram-positive anaerobic coccus isolated from clinical specimens (Murdoch, 1998). Soft tissue abscesses, wound infections, bone and joint infections, and vaginoses are the most common infections caused by *F. magna* (Bowler & Davies, 1999; Hansson et al., 1995; Murdoch, 1998; Stephens et al., 2003). Due to their anaerobic nature and relatively slow growth, Gram-positive anaerobic cocci have been little studied and their potential as human pathogens is often overlooked in the clinical setting (Murdoch, 1998).

Proteolysis is believed to contribute to bacterial virulence. Several bacterial species secrete proteinases, causing matrix destruction and inactivation of host defence responses. Degradation of host tissues and induction of inflammation provide bacteria with nutrients (Potempa et al., 2000; Rasmussen & Björck, 2002; Travis et al., 1995). Microbial proteinases can also inactivate important host immune-defence molecules, such as immunoglobulins (von Pawel-Rammingen & Björck, 2003), proteins of the complement system (Chen & Cleary, 1990) and antimicrobial peptides (Schmidtchen et al., 2002; Sieprowska-Lupa et al., 2004; Thwaite et al., 2006). Moreover, several species of oral streptococci produce extracellular proteinases capable of degrading albumin (Lo & Hughes, 1996), immunoglobulin A (Plaut et al., 1974) and salivary proteins (Choih et al., 1979). Many bacteria-derived proteinases cleave human kininogens, resulting in the release of kinins, potent proinflammatory peptides (Herwald et al., 1996; Imamura et al., 2004; Scott et al., 1993; for a review see Imamura et al., 2004). A streptococcal proteinase was also shown to degrade CXC chemokines, thereby blocking the clearance of bacteria from infected tissues (Hidalgo-Grass et al., 2006).

Despite the fact that *F. magna* is one of the most commonly isolated anaerobic cocci from clinical specimens, the
pathogenic significance of proteolytic activity of this bacterium is largely unknown. However, the production of gelatinases and collagenases by some \textit{F. magna} isolates has been reported (Harrington, 1996; Steffen & Hentges, 1981), and \textit{F. magna} strains isolated from diabetic wound infections and non-puerperal breast abscesses were reported to have collagenase, gelatinase and hippurate hydrolase activity (Krepl et al., 1992), suggesting that enzymic activity plays a role in the developement of \textit{F. magna} soft tissue infections. Moreover, \textit{F. magna} supernatants were reported to inhibit fibroblast and keratinocyte proliferation and wound healing, indicating that these supernatants contain factors that contribute to the pathogenesis of chronic wounds (Stephens et al., 2003).

The present investigation was undertaken to study proteolytic activity among \textit{F. magna} strains. The results show that \textit{F. magna} expresses a subtilisin-like proteinase, denoted SufA (subtilase of \textit{Finegoldia magna}), that degrades antibacterial peptides of the human innate immune system. The implications of this proteolytic activity for these processes in human colonization and infection are discussed.

**METHODS**

**Bacterial strains and growth conditions.** \textit{Finegoldia magna} strains were isolated at Lund University Hospital, Lund, Sweden. Bacteria were grown under strict anaerobic conditions (Anaerobic Workstation, Elektrotek) in Todd–Hewitt (TH) broth (Difco) supplemented with 0.5 % Tween 80. Cultures were harvested by centrifugation at 5000 g for 30 min at 4 °C. Spent culture medium was sterilized by filtration through 0.22 μm membranes. The bacterial cell pellet was washed with 20 mM Tris/HCl, pH 7.5, and the bacterial concentration was adjusted to 10 % (2 x 10^9 cells ml^-1).

**Proteinase isolation and FPLC.** \textit{F. magna} strain ALB8 was treated with 0.1 mg papain (Sigma) and 3 mg L-cysteine (Sigma) per 1 ml 10 % bacteria in 20 mM Tris/HCl, pH 7.5, and incubated at 37 °C for 1 h. Papain was inactivated by adding E64 (Sigma) to a final concentration of 1 μM. The supernatant was collected by centrifugation at 8000 g and dialysed against 20 mM Tris/HCl, pH 7.5, and concentrated on a Centricon 6 spin column (Amicon). Extracellular proteins in sterile culture medium from strain ALB8 were fractionated by sequential ammonium sulphate precipitation at 30 % and 80 % saturation. The precipitated proteins were collected by centrifugation at 5000 g for 30 min at 4 °C, dissolved in 20 mM Tris/HCl, pH 7.5, and dialysed against the same buffer. The proteins from ammonium-sulphate-precipitated (30–80%) spent culture medium or from papain-treated bacterial cells were applied to a Mono Q 5/50 GL ion-exchange column (GE Healthcare) equilibrated with 20 mM Tris/HCl, pH 7.5. Proteins were eluted with a linear gradient of NaCl (0–1 M) and collected in 0.5 ml fractions. The enzymic activity in the fractions was analysed by gelatin zymography (see below). Fractions containing gelatinase activity (0.28–0.40 M NaCl) were combined and concentrated by centrifugation filtration (Ultra-15, Amicon), followed by separation on a Superose 12 10/300 GL gel filtration column (GE Healthcare) equilibrated with 0.15 M NaCl, 20 mM Tris/HCl, pH 7.5.

**Antibodies.** The non-ambiguous residues of the internal peptide 3, EFSSWGLTPDLR (EFS12) (see Table 1) were synthesized and used for production of a custom peptide polyclonal antiserum (Innovagen). Recombinant SufA [zymogen form devoid of glutathione S-transferase (GST)] was separated by SDS-PAGE. The gel was stained with Coomassie R-250 and the band corresponding to SufA (155 kDa)

### Table 1. Amino acid sequences and masses of SufA tryptic peptides obtained by tandem mass spectrometry

To confirm the MS/MS identification SufA was in silico trypsin digested. The masses of the computed peptides were compared to the masses of the 23 de novo peptides. Of the 23 de novo peptides, 17 were found to match with a mass accuracy of 0.5 Da. The sequences of the de novo peptides were compared to the matched SufA sequences. Identical amino acid residues are indicated in bold/underlined.

<table>
<thead>
<tr>
<th>Peptide no.</th>
<th>Experimental mass</th>
<th>Theoretical mass</th>
<th>Δ (Da)</th>
<th>Sequence position</th>
<th>De novo peptide sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2126.16</td>
<td>2126.35</td>
<td>0.19</td>
<td>1018 : 1036</td>
<td>MTSADL</td>
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<tr>
<td>2</td>
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<td>2004.26</td>
<td>0.15</td>
<td>944 : 960</td>
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</tr>
<tr>
<td>3</td>
<td>1737.90</td>
<td>1737.94*</td>
<td>0.04</td>
<td>536 : 550</td>
<td>VDEFSSWGLTPDLR</td>
</tr>
<tr>
<td>4</td>
<td>1631.85</td>
<td>1631.79</td>
<td>−0.06</td>
<td>459 : 508</td>
<td>VYSVF</td>
</tr>
<tr>
<td>6</td>
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<td>1516.61</td>
<td>−0.16</td>
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</tr>
<tr>
<td>7</td>
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<td>1503.66</td>
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</tr>
<tr>
<td>13</td>
<td>1335.70</td>
<td>1335.47</td>
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<td>EGVSPT</td>
</tr>
<tr>
<td>15</td>
<td>1290.74</td>
<td>1290.47*</td>
<td>−0.28</td>
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<tr>
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<td>EGVSPT</td>
</tr>
<tr>
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<td>1199.47</td>
<td>−0.19</td>
<td>268 : 279</td>
<td>GVP</td>
</tr>
<tr>
<td>18</td>
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<td>−0.25</td>
<td>879 : 888</td>
<td>NLF</td>
</tr>
<tr>
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<td>YL</td>
</tr>
<tr>
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<td>903.03</td>
<td>−0.46</td>
<td>193 : 200</td>
<td>LSP</td>
</tr>
</tbody>
</table>

The theoretical mass was deduced considering the following modifications: *pyroglutamic modification when Gln was at N terminus; †C-terminal Ser stripped.
was cut out and used for production of a polyclonal rabbit antiserum (Antibody AB, Sweden). For purification of anti-SufA IgG, antiserum against recombinant SufA was applied to a protein G-Sepharose column (GE Healthcare Bio-Sciences). The column was extensively washed with PBS and bound IgG was eluted with 0.1 M glycine-HCl, pH 2.0. The pH was raised to 7.0 with 1 M Tris solution. Production of rabbit antibodies against a peptide of high-molecular-mass kininogen (NAT26) has been described (Frick et al., 2006).

**SDS-PAGE, immunoblot and immunoprecipitation.** SDS-PAGE was performed as described by Laemmli (1970) using a total polyacrylamide concentration of 8% or 10%, and 3.3% cross-linking. Gels were stained with Coomassie R-250 or separated proteins were transferred onto an Immobilon-P membrane (Millipore) by using the Mini Trans-Blot system (Bio-Rad). SufA was detected with EFS12-antibodies (1:10000) or with rabbit antibodies raised against recombinant SufA (1:1000). Bound antibodies were detected by using horseradish-peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad) and Supersignal West Pico chemiluminescent substrate (Pierce). Coomassie R-250 stained bands of interest were excised from SDS-PAGE gels and sent to Swegian Proteomics Facility (Lund, Sweden) for MALDI-TOF mass spectroscopy. Analysis of antibacterial peptide degradation was performed with Tris-Tricine peptide gels (Schägger & von Jagow, 1987) using a total concentration of 16.5% polyacrylamide, and 3.3% cross-linking. SufA preparations were immunoprecipitated with polyclonal antibodies against SufA or specific polyclonal NAT26 antibodies and protein G-Sepharose at 4°C for 16 h. Following centrifugation the supernatants were removed and analysed by gelatin zymography or used for cleavage of antibacterial peptides.

**Gelatin zymography.** Protein samples were dissolved in non-reducing sample buffer and incubated for 5 min at room temperature. Proteins were separated by SDS-PAGE using 8% or 10% polyacrylamide gels containing 0.1% porcine gelatin (Bio-Rad). After electrophoresis, gels were incubated in 2.5% Triton X-100 for 30 min, washed in 50 mM Tris/HCl, pH 7.5, 0.2 M NaCl and 5 mM CaCl2 and then incubated for 18 h in the same buffer. Areas of proteolytic activity were detected by Coomassie R-250 staining of the gel (Liotta & Stetler-Stevenson, 1990). The following proteinase inhibitors were used for blocking of proteolytic activity: trans-epoxysuccinyl-l-leucylamido(4-guanidino)butane (E-64), for blocking cysteine proteinases; PMSF, for blocking serine proteinases; EDTA, for blocking metalloproteinases (all from Sigma).

**Mass spectrometry and peptide de novo sequencing.** The protein band on SDS-PAGE above 250 kDa (see Fig. 2b, lane 2) corresponding to the highest gelatinase activity in Mono Q fraction 21 was excised and subjected to in-gel trypsinisation. The peptide samples were analysed on a Qtof Ultima API (ESI-MS/MS from Waters) coupled to a CapLC (Waters). The auto-sampler injected 5 µl sample and the peptides were trapped on a pre-column (C18, 300 µm x 5 mm, 5 µm, 100 Å, LC-Packings), and separated on a reversed-phase analytical column (Atlantis, C18, 75 µm x 150 mm, 3 µm, 100 Å, Waters). The flow through the column was 200 nL min⁻¹. Solvent A consisted of 2% acetonitrile, 98% water with 0.1% formic acid; solvent B consisted of 90% acetonitrile, 10% water and 0.1% formic acid. The HPLC method started with 5% B for 3 min., then progressed from 5 to 60% B in 42 min, from 60 to 80% B in 5 min, kept at 80% B for 25 min, 80 to 5% B in 1 min and 5% B for 15 min. Total run time 90 min. The mass spectrometer analysis was made by Data Directed Analysis (DDA). The mass range m/z was 400–1600 for MS and 50–1800 for MS/MS. Only spectra from ions with charge states 2 and 3 were acquired.

Interpretation of the tandem mass spectra using the ProteinLynx Global Server 2.2 software returned 23 peptide sequences, each with up to five interpretations of the mass/charge spectra (no distinction between the amino acids L and I due to their identical masses). The MS/MS analysis was performed by SciBiu-Swegenve Centre for Integrative Biology at Lund University, Sweden. The non-ambiguous residues of the individual peptide sequence interpretations were used for BLAST search in the NCBI non-redundant database and for subsequent construction of degenerate primers (see below).

After the cloning of sufA (see below), the MS/MS identification was confirmed. The ORF was in silico digested with trypsin in the MS-Digest program (http://prospector.ucsf.edu/prospector/4/0.8.5/html/mdsigest.htm) with one allowed missed cleavage site and with considered modification peptide N-terminal Glu to pyroGlu and stripping of one C-terminal residue. The computed mean molecular mass of the tryptic peptides was compared to the measured mass of the de novo peptides. A difference between computed and measured masses less than 0.5 Da was considered as a matched peptide. The amino acid sequence identity was then compared between the matched in silico tryptic peptides and the obtained sequence interpretations of the spectrum.

**Cloning and DNA sequencing.** Chromosomal DNA from the F. magna ALB8 strain was extracted using the Genta systems DNA isolation kit and used as template for PCR. Degenerate PCR primers were designed from the 23 MS/MS peptide non-ambiguous sequences using F. magna codon usage table [Countcodon program (Nakamura et al., 2000), http://www.kazusa.or.jp/codon/countcodon.html] based on published and unpublished sequences. To reduce degeneracy, an inosine base was used in the primers when any of the four bases was required (Knoth et al., 1988).

The initial partial amplification of the sufA gene was performed with PCR using the degenerate primers originating from peptide 17 (GVAPNAAGALLGMK) forward (5'-GCDCCDAAYGCDCAAHTITHTGGHTATG-3') and peptide 3 (EFSSWGLTPDLR) reverse (5'-TCHGGHGT1ADDCCCSWDWSDWARYTYC-3'). The 750 bp product designated 17Fwd/3Rev was gel-purified and sequenced. To obtain the flanking downstream DNA sequence of the gene, a forward PCR primer Inv1 (5'-CAACACTCATCCTCAGTACATTCTG-3'), based on the 17Fwd/3Rev sequence, was used together with degenerate reverse primer 8Rev (5'-GCRTHGHGRTTYYCTYTRTCYT-3') based on MS/MS peptide 8 (YEDKENPDAF). The PCR generated a 1950 bp product. The PCR product (Inv1Fwd/8Rev) was gel-purified and sequenced. Later on, the MS/MS peptide 8 was not found to match any of the SufA in silico tryptic peptides. Reverse primer binding for the Inv1Fwd/8Rev product was probably due to a sequence corresponding to the degenerate primer sequence.

To obtain the flanking upstream and downstream DNA sequence of the gene, non-specific nested suppression PCR (Lardelli, 2002) was performed. The first PCR (non-specifically primed PCR using a single primer) was performed with primer NT1 (5'-CCITTCTCTACT-GTAACTGTAGAC-3') directed upstream and primer CT1.5 (5'-TTACCTGGTACCAACTAAC-3') directed downstream. Cycling conditions were as follows: denaturation 95 °C 30 s, annealing 40 °C 1 min and extension 72 °C 2 min, repeated 35 cycles using Taq polymerase. The amplified PCR reaction was diluted 1:1000 and used as template for the reamplification PCR. The extended primers in the second PCR were ENT1 (5'-CITCCCTCTACT-GTAACTGTAGAC-3') directed upstream and ECT1 (5'-GACCTGGTACCAACTAAC-3') directed downstream. Cycling conditions were as follows: denaturation 95 °C 30 s, annealing 57 °C 1 min and extension 72 °C 4 min, repeated 35 cycles using Pfu polymerase. The PCRs resulted in a 1500 bp upstream fragment (ENT1) and an 800 bp downstream fragment (ECT1). The products were gel-purified and sequenced. BigDye terminal DNA sequencing (Applied Biosystems) was performed according to the manufacturer’s protocol. The sequence fragments were assembled using Invitrogen ContigExpress software generating a 4362 bp contig.
Recombinant expression of SufA. The regions of the sufA gene encoding the zymogen protein (amino acids 29–1154) and the mature protein (amino acids 148–1154) were produced by High Fidelity PCR (Fermentas) using F. magna ALB8 chromosomal DNA as template. For the NH₂-terminal GST-tagged constructs, we designed the following PCR primers: sufA pre-forward (5'-GATTCGA-CAAAAGATGACACTATAT-3'), sufA mature forward (5'-GATTCGAAGGATCCTGCAGTGGC-3') and sufA reverse (5'-AGCGGGCGCTTTAACACTTTGAAATTTCAAGTG-3'). The forward primers incorporated the Suf restriction sites and the reverse included a NotI site (underlined). The reverse primer also included two stop codons, TGA and TAA, directly after the coding segment. The amplified PCR products and the expression vector pGEX-6P-1 (GE Healthcare) were ligated with T4 DNA ligase (Fermentas) and transformed into BL21 competent cells (Novagen) for protein expression. BL21 transformants carrying the expression plasmids were grown in 2YT medium (1% tryptone (Difco) and 1% yeast extract (Oxoid), 0.5% NaCl) adjusted to a concentration of 1% (2× YT medium) and lysed by sonication, according to the manufacturer's protocol. The lysates were subjected to glutathione-Sepharose affinity chromatography (GE Healthcare). The GST-tag were removed with PreScission Protease (GE Healthcare).

PCR analysis of genomic DNA for identification of SufA. To analyse the presence of the sufA gene in different F. magna isolates, PCR template DNA was extracted using a Gentra Systems DNA isolation kit. The used PCR primers were SigSeq Fwd (5'-TTTTTCTCATGATGGCATTACC-3'), His247 Fwd (5'-GATCAACCGAAAGTGCGACGGATC-3') and Ser578 Rev (5'-GCCATTGATGTACCACTCAT-3'). PCR products were analysed by agarose (1%) gel electrophoresis.

Cleavage of antibacterial peptides. The LL-37 peptide (LLG-CGGCGGCGTCGAGATCTGC-3') was chosen for further analysis and proteinase identification. When grown in liquid culture, strain ALB8 reaches stationary phase at approximately 89 h of cultivation (Fig. 1b); growth medium from different time points was adjusted to a concentration of 1% (2× YT medium) and incubated with 1 mM PMSF at 37°C for 3 h or 16 h, and the reactions were carried out for 1 h. Serial dilutions of the mixtures were plated on TH agar in duplicates and incubated for 3 days under strict anaerobic conditions. The number of c.f.u. was then counted. All dilutions were performed in 10 mM Tris/HCl, 5 mM glucose, pH 7.5.

RESULTS

Proteolytic activity of F. magna

To visualize extracellular proteolytic activity of F. magna, supernatants from stationary-phase cultures of various isolates were analysed by zymography, using porcine gelatin as the substrate. The zymograms demonstrated that all examined strains express gelatinase activity in the molecular mass range of 130–200 kDa (Fig. 1a). Most strains also showed proteolytic activity above 250 kDa; the strain ALB8, previously used for isolation of the albumin-binding protein PAB (de Château & Björck, 1994), was chosen for further analysis and proteinase identification. When grown in liquid culture, strain ALB8 reaches stationary phase at approximately 89 h of cultivation (Fig. 1b); growth medium from different time points was adjusted to a concentration of 1% (2× YT medium) and incubated with 1 mM PMSF at 37°C for 3 h or 16 h, and the reactions were carried out for 1 h. Serial dilutions of the mixtures were plated on TH agar in duplicates and incubated for 3 days under strict anaerobic conditions. The number of c.f.u. was then counted. All dilutions were performed in 10 mM Tris/HCl, 5 mM glucose, pH 7.5.

Fig. 1. Gelatinase activity of F. magna. Proteinase activity of F. magna was analysed on polyacrylamide gels containing 8% polyacrylamide and 0.1% gelatin. Proteolytically active proteins appear as clearings in the stained gel. (a) Stationary-phase bacterial supernatants (10 μl) of indicated F. magna strains were analysed. (b) Strain ALB8 was grown under anaerobic conditions in TH medium supplemented with 0.5% Tween 80 at 37°C. At indicated time points samples were collected from the growth medium in (b). Bacteria were spun down and 10 μl samples of the resulting supernatants were analysed by zymography. Migration of molecular mass standards is indicated to the left.
analysed by zymography. Gelatinase activity could already be detected in the early growth phase (17 h), and the highest activity was observed when the bacteria were in stationary phase at 73 h and 89 h of cultivation (Fig. 1c).

To investigate whether gelatinase activity could also be detected on the cell surface of strain ALB8, papain was used to solubilize surface proteins from the bacteria, a procedure that has previously been used to release surface proteins from *F. magna* (Björck, 1988). Papain-released and secreted proteins of ALB8 displayed similar gelatinase activity, with a dominating clearing zone above 250 kDa and a weaker zone at 140 kDa (Fig. 2a, lanes 1 and 4). In both materials, the proteolytic activity was inhibited by PMSF but not by any other class of proteinase inhibitors (not shown), suggesting that the gelatinase(s) belong to the class of serine proteinases. The materials were individually subjected to ion-exchange chromatography followed by gel filtration. However, no further separation of the activity at 140 kDa and the activity above 250 kDa was obtained (Fig. 2a). Following ion-exchange chromatography, active fractions were also analysed by SDS-PAGE run under the same conditions as the zymography (non-reducing and non-heat-denaturing conditions). In the papain-solubilized material a band above 250 kDa, corresponding to the highest gelatinase activity on the zymograms, was detected. This band was cut out (Fig. 2b) and subjected to peptide de novo sequencing using tandem mass spectrometry (ESI-MS/MS). The analysis generated 23 internal peptides.

### Gene cloning and sequence analysis

In a BLAST search of the non-ambiguous sequences of the 23 peptides originating from the band above 250 kDa, peptides 3 and 17 (see Table 1) were found to align to catalytic domains of serine proteinases. PCR using degenerate primers based on these two peptide sequences yielded a 750 bp product (17Fwd3Rev) (see Fig. 3a, b). The translated amino acid sequence was homologous to serine proteinases of Gram-positive bacteria. The 3’-sequence was amplified using a forward inverted PCR primer, based on the obtained 750 bp sequence, together with a degenerate reverse primer for MS/MS peptide 8, resulting in a 1950 bp product (Inv1Fwd/8Rev). Further flanking downstream and upstream sequences were cloned using a non-specific, nested suppression PCR, yielding a 1500 bp upstream PCR product (ENT1) and a downstream 800 bp PCR product (ECT1) (Fig. 3a). The assembled nucleotide sequence was submitted to GenBank under submission number DQ679960. Homologous sequences were not found in the published bacterial artificial chromosome-end library of *F. magna* ATCC 29328 (Goto et al., 2003).

The nucleotide sequence revealed a 3466 bp ORF, which translated into a sequence of 1154 amino acid residues with a putative molecular mass of 127 kDa. A BLAST search of the ORF against the NCBI non-redundant database revealed high homology to extracellular proteinases of Gram-positive bacteria. The most similar proteins were...
lactocepin (lactococcal cell envelope-associated protei-
nases) from *Oceanobacillus iheyensis* HTE831 (accession
number NP_693854), *Exiguobacterium sibiricum* 255-15
(accession number ZP_00540359) and collagenolytic pro-
teinase from *Geobacillus* sp. MO-1 (accession number
BAF30978). The overall identity between the ORF and
similar proteins was 33–34 % and the homology 51–52 %.
The regions in the ORF showing the highest degree of
identity were residues 170–315 (55–57 % identity) and
530–600 (61–63 % identity), corresponding to regions of
the catalytic domain S8 (see below and Fig. 3a).

By scanning the amino acid sequence in the MyHits (Pagni
et al., 2004) and SignalP databases (Bendtsen et al., 2004),
the sequence was found to include a 28-residue signal
peptide, an 89-residue propeptide, a 595-residue peptidase
proteinase from *Geobacillus* sp. MO-1 (accession number
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**Fig. 3.** Schematic representation of the SufA protein. (a) The PCR products ENT1, 17Fwd/3Rev, Inv1Fwd/8Rev and ECT1
used for sequencing are shown and their position in the *sufA* gene is indicated. Beginning from the 5’-end, the gene includes:
putative −35 and −10 promoter sequences followed by a ribosome-binding sequence (RBS), and an ORF coding for SufA
(1154 amino acid residues). Shown below the gene map are the putative domains found in SufA: signal peptide (S), subtilisin
N-terminal region (N), peptidase S8 domain (S8), protease associated domain (PA), and domain of unknown function
(DUF1034). The potential catalytic triad comprises Asp181, His247 and Ser578 (in scale). (b) 17 of the 23 de novo peptide
masses matched theoretical peptide masses from in silico trypsin-digested SufA. Locations of the peptides in the ORF are
indicated (in scale).

The gelatinase activity correlates with SufA
Two constructs of SufA, the pro-enzyme and the mature
SufA, were recombinantly expressed in *E. coli* using the
GST fusion system. However, both forms were proteoly-
tically inactive in zymograms. In order to obtain native and
active proteins, recombinant SufA was subjected to
denaturation with guanidinium chloride or urea followed
by refolding in different buffers using dialysis or dilution.
The refolding conditions were adopted from protocols
(http://prospector.ucsf.edu/prospector/4.0.8/html/msdigest.
htm). When comparing the experimental masses of the 23
de novo peptides with the theoretical masses of the tryptic
SufA peptides, 17 of the peptides had experimental values
in good agreement with the theoretical masses (Table 1).
Eight of these matched peptides were identical at the amino
acid level, while the others displayed high homology (40–
80 % identity). Several of the other six peptides could also
be found in the ORF but with a lower identity or with gaps.
Poor fragmentation, inaccuracies due to mass shifts caused
by drifts in instrumental parameters, and chemical
contaminants could limit the positive identification of
peptides (Ma et al., 2003). However, the result from the
MS/MS analysis of the protein band above 250 kDa is in
good agreement with reports on peptide *de novo*
sequencing of standard proteins, such as albumin (Ma et al., 2003).
The location in SufA of the matching 17 peptides is shown
in Fig. 3(b).
used for other members of the subtilase family (Hayashi et al., 1994; Nohara et al., 2000; Wang et al., 2003). However, none of the tested methods were successful, and the protein remained proteolytically inactive. This has been reported also for other recombinantly expressed subtilases (Harris et al., 2003; Kluskens et al., 2002).

SufA purified from growth medium and the cell surface of ALB8 was analysed by SDS-PAGE and Western blotting. Both preparations were heterogeneous, with dominating bands at approximately 250 kDa and weaker bands around 130 kDa (Fig. 4a, SDS-PAGE). In Western blots antibodies raised against peptide 3 (anti-EFS12) reacted with a fragment of apparently 115 kDa in both SufA preparations, corresponding to the predicted size of the mature proteinase. The antiserum also reacted with the fragment migrating just below 250 kDa in the SufA preparation purified from papain-solubilized cell-surface proteins (Fig. 4a, anti-EFS12). When antibodies against recombinant pre-SufA were used as the probe, multiple immunoreactive bands were seen in this SufA preparation, suggesting that monomeric and dimeric forms of both the pro-enzyme and the mature SufA are present in the preparation (Fig. 4a, anti-SufA). This antiserum reacted also with the high-molecular-mass band originally used for the identification of SufA. In contrast, in the SufA preparation purified from the growth medium the only band reacting with anti-SufA was the fragment below 250 kDa. To demonstrate that SufA associates with the proteolytic activity, immunoprecipitation experiments were performed. As shown in Fig. 4(b), anti-SufA antibodies depleted the SufA preparation from the ALB8 surface of all gelatinase activity, while unspecific antibodies had no such effect. Similarly, no gelatinase activity was found in the SufA preparation from the growth medium following immunoprecipitation with anti-SufA IgG (not shown). These results clearly show that SufA correlates with the enzymic activity in the preparations. Moreover, the data demonstrate that SufA is associated with the bacterial surface, but a fraction of the enzyme is also released into the medium during growth.

**SufA degrades and inactivates LL-37 and MIG/CXCL9**

It has been described that proteinases of significant human pathogens degrade the antibacterial peptide LL-37 (Schmidtchen et al., 2002; Sieprowska-Lupa et al., 2004). To examine if SufA of *F. magna* also has this activity, LL-37 was incubated with SufA purified from the ALB8 surface, followed by SDS-PAGE. As shown in Fig. 5(a), cleavage of the peptide occurs rapidly (15–30 min), and after incubation for 1–3 h LL-37 was completely degraded. The cleavage of LL-37 was efficiently blocked by PMSF. Similar results were found when LL-37 was incubated with SufA purified from the growth medium of ALB8 (not shown). The human *z*-defensin HNP-1 (human neutrophil peptide 1) and the human *β*-defensin 3 (hBD-3) were not degraded by SufA, despite incubation for 16 h (Fig. 5a). However, the ability of hBD-3 to form dimers was inhibited by SufA. Another molecule recently shown to have potent antibacterial activity is the CXC chemokine MIG/CXCL9 (Cole et al., 2001; Egesten et al., 2007). MIG/CXCL9 was also rapidly degraded, generating fragments of approximately 6–10 kDa. No further cleavage of MIG/
CXCL9 occurred as a result of prolonged incubation (Fig. 5a). Furthermore, following immunoprecipitation with anti-SufA IgG, the SufA preparation (see Fig. 4b, lane 2) had no proteolytic activity against LL-37 and MIG/CXCL9 (data not shown), while unspecific antibodies did not block the cleavage of the peptides.

Next, the bactericidal activity of the various peptides on F. magna strain 505 was examined. This strain carries the sufA gene, but at stationary phase low amounts of SufA are secreted (Fig. 1a). In these experiments the bacteria were washed and resuspended in buffer prior to the addition of the peptides. All peptides except HNP-1 dose-dependently killed the bacteria (Fig. 5b). The peptides were then preincubated with SufA followed by the testing of the antibacterial effect on strain 505. At a concentration required for 100% killing, cleavage of LL-37 and MIG/CXCL9 by SufA significantly reduced the antibacterial activity of the peptides (Fig. 5c). When SufA was blocked with PMSF, both LL-37 and MIG/CXCL9 regained their activity (Fig. 5c). The interference of SufA with hBD-3 dimerization (Fig. 5a) had no effect on the antibacterial activity of this peptide (Fig. 5c), and PMSF alone had no bactericidal effect on strain 505 (data not shown). In summary, the data demonstrate that members of the defensin family are resistant to SufA proteolysis, whereas the antibacterial peptides LL-37 and MIG/CXCL9 are cleaved and inactivated, resulting in enhanced survival of F. magna.

Distribution of the sufA gene in strains of F. magna

A collection of 34 F. magna strains isolated from clinical specimens was analysed by PCR for the presence of the sufA gene. The gene was identified using forward primers hybridizing with the signal sequence or the histidine site,
together with a reverse primer hybridizing with the serine active site. PCR products of the correct sizes were detected in 27 strains using the signal sequence forward primer, and in 33 strains using the histidine forward primer. The expression of SufA was then examined by growing the strains to stationary phase and analysing the growth media in Western blots or slot blots using the peptide antibody EFS12. Twenty-four strains were found positive for SufA expression. In fact only one strain was negative at both the gene and protein level. In Western blot analysis of growth media from the strains shown in Fig. 1(a), anti-SufA antibodies reacted with a band below 250 kDa in all strains. Apart from ELTI and 23.75, an immunoreactive band around 130 kDa was also detected in all strains (data not shown). Moreover, sequencing of the NH2-terminal amino acid residues 41–322 (see Fig. 3a) in four additional strains, including L3410, 23.75 and 505 shown in Fig. 1(a), revealed a high degree of homology (more than 80% identity). Of course it can not be excluded that, in addition to the expression of SufA homologues, the various strains in Fig. 1(a) also express other gelatinases, which could contribute to the banding pattern. Furthermore, the enzymic activity in zymograms of variants of SufA could also differ. Taken together, the data demonstrate that well-conserved homologues of SufA are present in almost all F. magna isolates.

**DISCUSSION**

Gelatinase and collagenase activities have been described previously in *F. magna* (Harrington, 1996; Krepel *et al.*, 1992; Steffen & Hentges, 1981), but the proteinase(s) responsible have not been isolated and characterized. Here we show that this anaerobic bacterium expresses a serine proteinase, denoted SufA, which is associated with the bacterial surface, but also released in substantial amounts into the growth medium. SufA is homologous to members of a family of subtilisin-like serine proteinases, also referred to as subtilases (Rawlings & Barrett, 1994; Siezen *et al.*, 1991; Siezen & Leunissen, 1997). Most subtilases of Gram-positive bacteria are secreted, although some have classical cell-wall anchoring domains (S-layer homology domains or LPXTG motifs). No such motifs were, however, found in the SufA sequence, suggesting a non-covalent association with the *F. magna* surface.

In general, subtilisins are produced as pro-enzymes and undergo autocatalytic maturation, where the pro-domain is cleaved off. Following cleavage, the propeptide generally remains non-covalently bound to the catalytic region of the subtilase, where it can act as an inhibitor (Shinde & Inouye, 1995a, b). Removal of the propeptide is also an autocatalytic process, resulting in a fully active enzyme. In some cases pro-domains have been shown to function as intramolecular chaperones involved in correct folding of the protein (Takagi & Takahashi, 2003; Zhu *et al.*, 1989). Sequence similarity suggests that SufA also has a pro-domain, which is further supported by the fact that none of the identified de novo peptides were found NH2-terminal of amino acid residue 195 in the SufA sequence (see Fig. 3). When expressed in *E. coli*, SufA showed no proteolytic activity. In order to adopt their native bioactive structure, it has been reported that *E. coli*-produced subtilisin-like proteinases require refolding (Matsubara *et al.*, 1994; Moser *et al.*, 1994; Nohara *et al.*, 2000). However, despite testing several refolding strategies, both forms of recombinant SufA remained enzymically inactive. The addition of *F. magna* cell extract or trace amounts of purified SufA also failed to restore the activity of the recombinant proteinases (data not shown). After proteolytic removal of the GST-tag, 18 amino acids from GST remain in the NH2-terminus of *E. coli*-expressed SufA. Hypothetically, these residues could interfere with the refolding, especially if the pro-domain is required for renaturation. As an alternative approach, subcloning of SufA into an expression plasmid yielding a C-terminal hexahistidine-tagged protein was attempted, but this experimental procedure was not successful.

Sequencing of SufA was based on MS/MS peptides derived from a protein migrating with an apparent molecular mass of >250 kDa and showing the highest proteolytic activity in zymography (see Fig. 2b). This band most likely corresponds to a dimer/multimer of SufA as judged by Western blot analyses, where the band reacts with antibodies raised against recombinant SufA with an apparent molecular mass of 130 kDa (see Fig. 4a). In SufA preparations from ALB8 bacteria, immunoreactive fragments compatible with the size of monomers and dimers of both the propeptide and the mature proteinase were seen. However, at the size for monomeric SufA the proteolytic activity was considerably lower, suggesting that dimer formation is required for full enzymic activity.

Antimicrobial peptides (AMPs) play a significant role in the clearance of potentially pathogenic microbes at physical barriers. Cathelicidins and defensins constitute the major AMPs (Bals & Wilson, 2003; Ganz, 2003), but other molecules such as chemokines also possess bactericidal activity (Cole *et al.*, 2001; Egesten *et al.*, 2007). During inflammation an upregulated synthesis of AMPs by epithelial cells and degranulation from neutrophils result in elevated concentrations of these peptides. Here we find that SufA rapidly cleaves and inactivates the human cathelicidin LL-37 and the chemokine MIG/CXCL9, and as a consequence growth of *F. magna* is significantly enhanced. Such proteolytic inactivation of AMPs has been reported for other bacterial proteinases (Hidalgo-Grass *et al.*, 2006; Schmidtchen *et al.*, 2002; Sieprawska-Lupa *et al.*, 2004) and could thus represent a common strategy to promote bacterial survival and colonization of epithelial surfaces. *F. magna* is associated with soft tissue infections (Murdock, 1998) and the bacterium is also isolated from patients with deep wound infections and chronic wounds (Hansson *et al.*, 1995; Stephens *et al.*, 2003). The inactivation of LL-37 and MIG/CXCL9 by SufA may contribute to the potential pathogenicity of *F. magna* by
promoting survival and spread of this commensal to sites where it is not present under normal conditions.

Among the β-defensins hBD-3 is a broad-spectrum antibacterial peptide mainly expressed in skin and tonsils (Harder et al., 2001). Only a small amount of hBD-3 is found in normal skin, but following wounding the epidermal expression of hBD-3 is greatly increased by activation of the epidermal growth factor receptor (Sørensen et al., 2006). In our in vitro assay F. magna was efficiently killed by hBD-3, but, in contrast to LL-37 and MIG/CXCL9, hBD-3 was not degraded by SufA. Interestingly, this was also the case with the α-defensin HNP-1, suggesting that cysteine-rich peptides are protected from SufA proteolysis. The observation that serine-threonine proteinases are unable to degrade the folded form of hBD-3 (Dhople et al., 2006) supports this assumption. By contrast, LL-37 is a linear α-helical peptide without cysteines (Bals & Wilson, 2003), and in MIG/CXCL9 the antibacterial activity has been localized to the C-terminal region, which has a predicted α-helical structure (Egesten et al., 2007). Thus, it seems likely that α-helical peptides in general are susceptible for proteolysis by SufA. On the other hand, SufA interfered with the ability of hBD-3 to form dimers, but this property had no impact on the bactericidal activity towards F. magna. Whether other biological functions of hBD-3, such as chemotraction (Dhople et al., 2006), are affected by SufA, remains to be investigated.

To our knowledge this study represents the first report on a subtilase produced by anaerobic Gram-positive cocci. In this group of bacteria F. magna is the most commonly isolated organism from clinical specimens, indicating a relatively higher pathogenic potential for this bacterium compared to other anaerobic Gram-positive cocci of the normal flora. At 18–24 h after inoculation at the infection site, F. magna is the most commonly isolated organism from clinical specimens, indicating a relatively higher pathogenic potential for this bacterium compared to other anaerobic Gram-positive cocci of the normal flora. At 18–24 h after inoculation at the infection site, a streptococcal C5a peptidase gene of Streptococcus pyogenes (Chen et al., 2001) was efficiently killed by hBD-3, but, in contrast to LL-37 and MIG/CXCL9, was not degraded by SufA. Interestingly, this was also the case with the α-defensin HNP-1, suggesting that cysteine-rich peptides are protected from SufA proteolysis. The observation that serine-threonine proteinases are unable to degrade the folded form of hBD-3 (Dhople et al., 2006), supports this assumption. By contrast, LL-37 is a linear α-helical peptide without cysteines (Bals & Wilson, 2003), and in MIG/CXCL9 the antibacterial activity has been localized to the C-terminal region, which has a predicted α-helical structure (Egesten et al., 2007). Thus, it seems likely that α-helical peptides in general are susceptible for proteolysis by SufA. On the other hand, SufA interfered with the ability of hBD-3 to form dimers, but this property had no impact on the bactericidal activity towards F. magna. Whether other biological functions of hBD-3, such as chemotraction (Dhople et al., 2006), are affected by SufA, remains to be investigated.

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