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A novel member of the thermolysin family, cloning and biochemical characterization of metalloprotease from *Staphylococcus pseudintermedius*★❀☆

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Thermolysins constitute a family of secreted bacterial metalloproteases expressed, among others, by several pathogens. Strains of *Staphylococcus pseudintermedius* isolated from diseased dogs and judged as protease-positive, by skim milk agar plate culture, were investigated for protease content. No proteolytic activity was detected when the bacteria were grown in regular liquid media. Unexpectedly, supplementation of the medium with calcium ions resulted in expression of a metalloprotease and profound changes in the profile of extracellular proteins. On the basis of homology to other staphylococcal metalloproteases, the nucleotide sequence of the gene encoding this protease (Pst) and its flanking regions was determined. The full-length *pst* codes for a protein with an open reading frame of 505 amino acids. The internal region contains the HEXXH catalytic domain that is conserved in members of the thermolysin family. Regardless of the presence of calcium in the medium, the expression of the protease gene was of the same intensity. This suggests that regulation of the metalloprotease production by calcium ions is at a post-transcriptional level. Isolates of *S. pseudintermedius* exhibit a proteolytic phenotype due to the metalloprotease expression, however only in presence of calcium ions, which most probably stabilize the structure of the protease.

Keywords: staphylococcus, metalloprotease, pathogen, calcium

INTRODUCTION

Staphylococcus pseudintermedius has been described as a new coagulase-positive species of animal pathogens (Devriese *et al.*, 2005). However, the first case of the bacterium infection in a human was communicated recently (Van Hoovels *et al.*, 2006), indicating that investigation of staphylococcal species of animal origin may also have medical application. The biochemical properties of *S. pseudintermedius* are similar to those of *S. aureus*, *S. delphini* and *S. intermedius* (Devriese *et al.*, 2005, Sasaki

et al., 2007), which readily leads to misidentification of the species by commercial identification systems not including *S. pseudintermedius* in their databases. Therefore, proper identification of the species has to be supported with carefully designed molecular analysis (Lautz *et al.*, 2006). This is particularly true if an inconsistent phenotypical identification pattern is observed, such as unexpectedly high or low enzymatic activity.

Among others, production of proteases is regarded as an important virulence factor in staphylococcal infections (Shaw *et al.*, 2004, Imamura *et al.*, 2005). *S. aureus*, the most virulent and important pathogen among staphylococci, produces a range of proteases belonging to three catalytic classes. A metalloprotease (aureolysin) plays a crucial role in developing *S. aureus* proteolytic activity because it is responsible for activation of glutamyl endopeptidase (Drapeau, 1978), which in turn processes staphopain B zymogen (Massimi *et al.*, 2002). Moreover, *in vitro* aureolysin inactivates human blood serpins, α-1-antichymotrypsin and α-1-antitrypsin, liberating neutrophile-specific chemoattractant peptides from the former (Potempa *et al.*, 1991), and activates prothrombin showing a pseudocoagulase activity (Wegrzynowicz *et al.*, 1980). The enzyme has also been suggested to contribute to the resistance of *S. aureus* to the innate immune system by degrading human antibacterial peptide LL-37 (Sieprawska-Lupa *et al.*, 2004) and inhibiting immunoglobulin production by lymphocytes (Prokesova *et al.*, 1991). A gene coding for aureolysin occurs in two conserved allelic groups and its presence was confirmed in all community- and hospital-acquired strains investigated (Sabat *et al.*, 2000, Moore & Lindsay, 2001), indicating a crucial role of the gene product in *S. aureus* survival. Apart of the metalloprotease, all strains of *S. aureus* produce two cysteine proteases — staphopain A and B, and a serine protease, also referred to as V8 protease. Moreover, in the majority of strains, a unique operon (*spl*) coding for six serine protease-like proteins was identified. The encoded Spl proteins share 44 to 94% amino-acid sequence identity with each other and 33 to 36% sequence identity with V8 protease (for review see: Dubin, 2002, Wladyka & Pustelny, 2008).

Modulation of virulence determinant expression is mainly controlled by accessory gene regulator (*agr*) that functions in a growth-phase dependent manner. In the post-exponential phase it represses the synthesis of cell-wall-associated proteins and activates expression of extracellular proteins, including proteases (Novick *et al.*, 1993, Shaw *et al.*, 2004). Therefore, it was suggested that the latter play a role in the transition of *S. aureus* cells from an adhesive to an invasive phenotype by degrading bacterial cell surface proteins, such as fibronectin binding pro-

tein and protein A (Karlsson *et al.*, 2001). Moreover, proteomics studies have revealed that the proteases are responsible for the qualitative and quantitative changes in extracellular protein pattern (Kawano *et al.*, 2001, Nakano *et al.*, 2002) which may be related to staphylococcal virulence.

In this study, we report gene cloning and biochemical characterization of an unusually expressed metalloprotease (Pst) from *S. pseudintermedius* strains isolated from diseased dogs. The enzyme alters the profile of extracellular proteins, however, its production was observed only in the presence of calcium ions which may restrict its action only to particular environmental conditions.

MATERIALS AND METHODS

Strains source and growth conditions. Ten *S. pseudintermedius* isolates were analyzed in this study. The isolates were recovered from skin abscess lesions in dogs, among these, five were a generous gift from Dr. A. Kasprowicz from the Dr. Jan Bobr Center of Microbiological Research and Autovaccines Ltd. (Krakow, Poland), and five were provided by Dr. M. Binek from Warsaw Agricultural University (Warszawa, Poland). All isolates were epidemiologically unrelated and all came from different dogs. The reference *S. pseudintermedius* strain CCUG 49543 from the Culture Collection University of Göteborg, *S. delphini* strain ATCC 49171 from the American Type Culture Collection (ATCC), and *S. intermedius* strain ATCC 29663 were included for comparison. The bacteria were grown on tryptic soy agar (TSA) plates (overnight, 37ºC) and stored at 4ºC. Liquid cultures were grown in tryptic soy broth (TSB) medium (overnight, 37ºC), supplemented with 5% (v/v) skim milk or 5 mM $CaCl_2$, when mentioned.

Total DNA extraction. DNA was extracted with the Genomic DNA Prep Plus kit (A&A Biotechnology, Gdynia, Poland) as previously described (Sabat *et al.*, 2003).

DNA sequencing. The PCR products were purified by using Clean Up Kit (A&A Biotechnology). Sequencing reactions were prepared by using a Big Dye terminator (version 1.1) cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer`s instruction. The sequencing products were purified by using the ExTerminator Spin Columns (A&A Biotechnology). Both strands were sequenced on a 310 Genetic Analyzer (Applied Biosystems).

Genetic identification of staphylococcal species. (i) Partial sequence analysis of the *16SrRNA* and *rpoB* genes. PCR products were amplified according to the protocols described by Becker *et al.*, (2004), and Drancourt and Raoult (2002), respective-

ly. The sequences for partial *rpoB* gene were deposited in the EMBL database under accession numbers: AM921786, AM921787 and AM921788 for the strains CCUG, 2INT and 3p, respectively.

(ii) PCR-restriction fragment length polymorphism (PCR-RFLP) of the *gap* gene. PCR product was generated by the protocol described by Yugueros *et al.* (2000). RFLP analysis was performed by digestion of PCR products with restriction enzyme *AluI* (MBI Fermentas, Vilnius, Lithuania) and by electrophoresis in 4% (w/v) Micropor GAMMA agarose (Prona Agarose, Madrid, Spain) using 50 bp DNA ladder (Amersham Biosciences, Uppsala, Sweden).

Sequencing of the metalloprotease gene. PCR was conducted with a degenerate primer pair coaF-1 and coaR (Table 1), and *Taq* DNA polymerase (Fermentas). Amplification conditions were 30 cycles of 94°C for 30 s, 42°C for 30 s, and 72°C for 20 s. For use in inverse PCR, genomic DNA was digested with a restriction enzyme according to the manufacturer's instructions and subsequently purified with Clean-Up (A&A Biotechnology). Then, 50 ng of the digested DNA was circularized in a 10 μl reaction volume containing T4 DNA ligase (Fermentas). The reaction mixture was incubated overnight at room temperature. Aliquots of the ligation mixture were used as template in the inverse PCR with primers (InvF56, InvR57, InvR59, InvF61, InvR62, InvF67, and InvR68) pointing outwards (Table 1) using the Expand High Fidelity PCR System (Roche Molecular Biochemicals, Basel, Switzerland) and the following

cycling conditions: 30 s at 94°C, 30 s at 50°C, and 5 min at 72°C, 30 times.

PCR-RFLP analysis and nucleotide sequencing of complete *aur-like* gene in *S. pseudintermedius* strains. To obtain the complete coding sequence of the *aur*-like gene, primers RFLP-F and RFLP-R (Table 1) were designed. Amplification products were treated with the restriction endonucleases *AluI*, *Csp*6I, *Hpy*F3I, *Eco*130I, and *Taq*I, according to the manufacturer's instructions. Digestion products were analyzed by electrophoresis in 2% (w/v) agarose SeaKem LE gels (FMC Bioproducts, Philadelphia, PA, USA). For all selected isolates, the *aur*-like gene was sequenced by using pair of primers F1-RFLP and R1-RFLP, F2-RFLP and R2-RFLP, F3-RFLP and R3-RFLP, and F4-RFLP and R4-RFLP (Table 1).

Reverse transcription (RT) and quantitative PCR. Total RNA (from bacteria in the late exponential or stationary phase of growth) was isolated using Trireagent (Sigma-Aldrich, St. Louis, MO, USA). Trace amounts of DNA were removed by DNase (Qiagen, Hilden, Germany) digestion followed by RNA repurification using RNeasy Mini Kit (Qiagen). Reverse transcription was performed with random hexamers and M-MLV reverse transcriptase (both from Promega, Madison, WI, USA) according to manufacturer's instructions. RT-PCR was conducted with primer pair AurLRT and AurLRTR (Table 1), and *Taq* DNA polymerase (Fermentas). For quantitative PCR, DyNAmo HS SYBR Green qPCR Kit (Finnzymes, Espoo, Finland), pair of primers AurLq and AurLRTR for target gene, and 16SrRNAF and 16SrRNAR for reference gene (*16SrRNA*) were used (Eleaume & Jabbouri, 2004, Sabersheikh & Saunders, 2004). Serial dilutions (10, 50, 250, and 1250 times) of cDNA were used as a template. The reaction was set up in triplicates. Amplification conditions were 32 cycles of 94°C for 30 s, 53°C for 30 s, and 72°C for 30 s. Threshold cycle (C_T) values for target and reference were calculated from the corresponding standard curves (constructed by plotting C_T against the logarithm of the template dilution). The normalized amount of target was calculated by dividing the amount of target by the amount of reference. The relative expression level of the target gene in samples (growth medium supplemented with calcium ions) was determined by dividing the normalized target amount by the value of the calibrator (growth medium without additives).

Analysis of nucleotide and amino-acid sequence data. Nucleotide sequences were aligned using ClustalW (www.ebi.ac.uk/clustalw) software. The proportion of synonymous (silent, *ds*) and nonsynonymous (amino acid-changing, *dn*) substitution rates was calculated using the software SNAP (www.hiv.lanl.gov). The amino-acid sequences were

Table 1. Oligonucleotide primers used in this study

Primer	Sequence
16SrRNAF	5'-AACTGAGACACGGTCCAGACTCC-3'
16SrRNAR	5'-GGCTGCTGGCACGTAGTTAGC-3'
AurLq	5'-CAATGGAACCCGATGTGTC-3'
AurLRT	5'-TTCTAACGAAACGGCATACC-3'
AurLRTR	5'-GCCATTACTGCAGAACCTG-3'
coaF-1	5'-GCNCAYGARATHACNCAYG-3'
coaR	5'-TRTGNACNCCNCCRTTRTC-3'
InvF56	5'-AGTATGTCTGACACATTTG-3'
InvR57	5'-GTTCAAAGCACCTGGTT-3'
InvR59	5'-CACCATCAATAGAGTTGAT-3'
InvF61	5'-CATTGTAAAAGCGGTTTGT-3'
InvR62	5'-CCGAAACAACATCAACC-3'
InvF67	5'-TTCTAATGGGATATGTTGG-3'
InvR68	5'-TCGTTCAACGATGCGAT-3'
RFLP-F	5'-AGTTAAGGAGTTGTTTAAATG-3'
RFLP-R	5'-CATGATTAAGGCAAGCAAT-3'
F1-RFLP	5'-AAGTTATTTTACACTTAGGCA-3'
R1-RFLP	5'-CTTCCCCGTTAATGAGT-3'
F2-RFLP	5'-AGAGGTAAAAGTCCACG-3'
R2-RFLP	5'-TGTCTGGGTCTGTAATTAA-3'
F3-RFLP	5'-GAAGATGTGACAGGTTC-3'
R3-RFLP	5'-ACATCTTCACCGATTAAAG-3'
F4-RFLP	5'-AGTATGTCTGACACATTTG-3'
R4-RFLP	5'-TCATGATTAAGGCAAGCAA-3'

edited in Genedoc software (version 2.6.02) (Nicholas *et al.*, 1997).

Protein purification. All steps were done at 4°C, unless mentioned otherwise. The overnight culture fluid (1 l, bacteria grown in TSB medium supplemented with 5% (v/v) milk) was clarified by centrifugation and concentrated to 50 ml by ultrafiltration with a 30 kDa-cut off disc. The concentrated supernatant was dialyzed overnight against 5 l of buffer A (20 mM bis-Tris/HCl, pH 6.0, 5 mM CaCl$_2$), applied to a Q-Sepharose FF column (Amersham Biosciences) equilibrated in buffer A and washed with the buffer. Then, gradient (0–0.3 M) elution with NaCl in buffer A was performed. The fractions containing the protease activity were pooled and concentrated using VivaSpin centrifuge concentrator (Sartorius AG, Goettingen, Germany) with a 3 kDa molecular mass cut off membrane. The enzyme solution was incubated for 2 h at 37°C and loaded onto a Superdex 75 column equilibrated with 20 mM bis-Tris/HCl, pH 6.0, 150 mM NaCl, 5 mM CaCl$_2$. Fractions containing the protease were pooled, concentrated, aliquoted and stored at –20° C for further analysis.

Protein concentration and enzyme activity assays. Protein concentration was determined by the method of Bradford (1976). Proteolytic activity was detected by radial diffusion assay in a gel containing 1% (w/v) agarose, 1% (w/v) powder milk in reaction buffer R (100 mM Tris/HCl, pH 7.0, 5 mM CaCl$_2$). Samples (10 μl) were loaded into wells made in the gel and incubated at 37°C until zones of clearance appeared around the wells (Miedzobrodzki *et al.*, 2002). Elastolytic activity was detected using radial diffusion in 1% (w/v) agarose gel containing bovine ligament elastin (3 mg/ml) suspended in buffer R. The proteolytic activity against dye-attached protein substrates (azocasein, Azocoll and Hide Powder Azure) (Calbiochem, La Jolla, CA, USA) were tested in buffer R. The activity was measured as an increase in absorbance of the supernatants at 440, 520, and 595 nm, respectively, after removal of undigested substrate by centrifugation. Commercially available synthetic *p*-nitroanilide substrates (1 mM final concentration) were incubated at 37°C with 2 μg of the protease in a total volume of 200 μl of buffer R. After 16 h, absorbance at 405 nm was measured against blank samples containing all reagents except the protease. The tested substrates included the following compounds: D-VLK-pNa, AAPV-pNa, MeO-Suc-AAPV-pNa, GP-pNa, Z-GP-pNa, Suc-AAPF-pNa, Ac-K-pNa, Z-K-pNa, H-AFP-pNa, p-tosyl-GPK-pNa, L-BAPNA, BAPNA, GF-pNa, p-tosyl-GPL-pNa, Z-AP-pNa, Bz-FVR-pNa, Glutamyl-L-FA-pNa, H-AAA-pNa, ZLLE-pNa, GR-pNa, Suc-AAV-pNA, Suc-AAPK-pNa, Suc-AAVA-pNa, Suc-AAPA-pNa, Bz-FVR-pNa, Suc-AAPI-pNa, Z-AV-pNa, MeOSuc-

AFAA-pNa and Suc-GFG-pNa. Zymographic analysis of protein samples was performed according to the protocol described by Popowicz *et al.* (2006). In brief, samples were mixed with Laemmli loading buffer (4×, without β-mercaptoethanol) and incubated for 10 min at room temp. Then, the samples were loaded on 12% (w/v) polyacrylamide gel containing 0.1% (w/v) bovine β-casein (Sigma-Aldrich) and sodium dodecyl sulfate (SDS) and electrophoresed. The gels were washed in 2.5% (v/v) Triton X-100 in water (30 min) at room temp. with agitation, and incubated in 100 mM Tris/HCl (pH 7.0), 100 mM NaCl, 5 mM CaCl$_2$, for 12 h at 37°C with gentle agitation, stained with Amido black and destained with 10% (v/v) acetic acid. For the inhibition assay, 5 μg of the protease was preincubated (30 min, room temp.) with increasing amounts of human α-2-macroglobulin, α-1-antitrypsin (AT) or α-1-antichymotrypsin (ACT) (all from BioCentrum Ltd., Kraków, Poland) in buffer R. The residual activity of metalloprotease was assayed with Azocoll.

Protein profile analysis. One-dimensional SDS polyacrylamide gel electrophoresis (PAGE) was performed according to Schagger & von Jagow (1987). Two-dimensional gel electrophoresis was preformed as reported in Bonar *et al.* (2006). Briefly, isoelectric focusing was carried out after active rehydratation at 20°C on PROTEAN IEF Cell (Bio-Rad, Hercules, CA, USA). About 80 μg of protein sample was separated on 7-cm immobilized pH 3–10 gradient strips (Bio-Rad) at increasing voltage from 250 to 4000 V. Prior to SDS/PAGE, the focused proteins were equilibrated for 2×10 min in solution of 0.375 M Tris/HCl pH 8.8, 20% (v/v) glycerol, 6 M urea and 2% (w/v) SDS. In the first equilibration step 130 mM DTT (dithiothreitol) was added in order to reduce disulfide bonds. During the second step alkylation of thiol groups was carried out with 135 mM iodoacetamide. Second dimension (SDS/PAGE) was conducted with 12% (w/v) separating gels according to Laemmli buffer system (Laemmli, 1970). Gels were silver-stained following the procedure described by Shevchenko *et al.* (1996). Gel images were scanned by white light illuminator Fluor-S MultiImager. Subsequently, analyses of gel images were performed with the PD Quest 2-D Analysis Software Version 6.2 (Bio-Rad). The whole protein analysis procedure was performed three times.

Amino acid sequencing. One-hundred picomole of purified protein was applied to SDS/PAGE (Schagger & von Jagow, 1987), electrotransferred onto PVDF membrane (Applied Biosystems) in 10 mM 3-(cyclohexylamino)propanesulfonic acid (Sigma-Aldrich), pH 11, 10% (v/v) methanol and lightly stained with CBB R250 (Merck, Darmstadt, Germany) (Matsudaira, 1987). The protein band was

excised with a scalpel and N-terminal protein sequence analysis was performed at the BioCentrum Ltd. facility on a Procise 491 (Applied Biosystems) automatic sequence analysis system.

Substrate specificity. Protease specificity was tested on bovine β-casein. The enzyme was mixed with β-casein in buffer R at a molar ratio of 1:150. Reaction was stopped after 30 min by mixing the sample with equal volume of 10% (v/v) trifluoroacetic acid and protein precipitate was removed by centrifugation. Subsequently, the supernatant was subjected to reverse phase (C18 column) high performance liquid chromatography separation. The peptide-containing fractions were lyophilized and subjected to MALDI–TOF mass spectrometry (Reflex IV MALDI TOF, Bruker, Bremen, Germany) analysis at the Faculty of Chemistry and Regional Laboratory, Jagiellonian University (Kraków, Poland). Assignment of masses to cleavage products was performed using web-interface of FindPept (ExPASy Proteomics Server, www.expasy.org) (Gasteiger *et al.*, 2003).

Homology modelling. Metalloprotease structure prediction was performed using automatic mode of the Swiss Model modelling program. The obtained model was based on templates 1bqbA, 1keiA, 1kjoA, 1kjpA and 1kkkA (Guex & Peitsch, 1997, Schwede *et al.*, 2003; Arnold *et al.*, 2006).

RESULTS

S. pseudintermedius identification should be based on molecular analysis but not on their biochemical properties

The bacteria isolated from skin abscess lesions in dogs were initially identified in a medical diagnostics laboratory by coagulase and Slidex Staph Plus (bioMerieux) tests as protease-secreting strains of *S. aureus*. However, production of proteases by the strains (measured as clearance zones in TSA supplemented with 5% skim milk around paper discs soaked with bacteria suspension) was significantly higher in comparison with even highly protease-secreting strains of *S. aureus*. Therefore, further analysis was performed. ID 32 STAPH (bioMerieux) test classified the bacteria as *S. intermedius* (A. Kasprowicz, unpublished). Investigation by PCR-RFLP of glyceraldehyde-3-phosphate dehydrogenase-encoding gene (Yugueros *et al.*, 2000) also showed pattern of restriction cleavage products identical to that of *S. intermedius*. Final identification was performed by sequencing of internal fragments of the 16S rRNA and *rpoB* genes. All investigated as well as reference strains had identical 16S rRNA sequences, however, comparison of the *rpoB* sequences allowed reclassification of the strains as representatives of *S. pseud-*

intermedius — a newly described coagulase-positive member of Staphylococci (Devriese *et al.*, 2005). The *S. pseudintermedius* strains investigated had identical *rpoB* sequences or differed by one or two nucleotides between each other (99–100% identity), whereas the homology to *S. delphini* and *S. intermedius* strains was lower, 95% and 97%, respectively.

Protease expression is calcium-dependent

To characterize the protease profile, bacteria were grown in liquid TSB medium. The proteolytic activity of the strains was determined by radial diffusion assay. Surprisingly, no proteolytic activity was detected. Therefore, the medium was supplemented with 5% skim milk to give a composition similar to that of the solid one, where the production of protease was manifested by clearance zones around the bacterial colonies. Indeed, in the enriched medium expression of a 32 kDa protease was observed as a single band on casein zymography gels of supernatants from overnight cultures (Fig. 1). Further variation of the medium composition showed that the agent responsible for the enzyme production is calcium ions but not the protein component. Medium supplementation with 5 mM $CaCl_2$ resulted in an identical protease profile as in the case of milk addition, whereas the addition of pure proteins: bovine milk β-casein, human serum albumin, bovine hemoglobin or proteins from milk (low molecular mass ingredients of milk were removed by dialysis), did not induce the presence of the enzyme in the medium.

The strain labeled "9p" produced the highest level of the protease and, therefore, was used for further enzyme production on a preparative scale. Because calcium ions turned out to be indispensable for protease expression, 5 mM $CaCl_2$ was included in all purification buffers. Surprisingly, precipita-
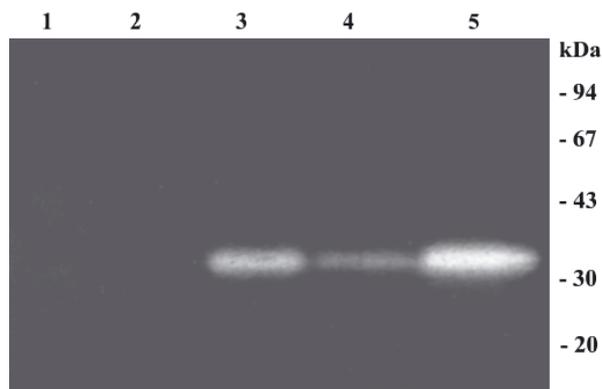
Figure 1. Protease activity of *S. pseudintermedius* visualized by casein-containing zymogram.
Bacteria were grown in TSB medium (lane 1) supplemented with 1% (w/v) casein (lane 2), 5 or 2 mM $CaCl_2$ (lane 3 and 4, respectively) and 5% (v/v) skim milk (lane 5).

tion of proteins from culture supernatant with ammonium sulfate yielded inactive enzyme. Therefore, decreasing of the culture liquid volume by ultrafiltration was applied prior to the purification steps. The protease was purified to homogeneity by combination of ion exchange and gel filtration chromatographies. The enzyme is stable in the presence of CaCl$_2$. Moreover, incubation of impure enzyme at 37°C causes proteolysis of impurities, whereas the protease remains active and uncleaved. These properties of the enzyme were used to remove proteins co-eluting with the protease from the ion exchange column prior to gel filtration. According to the procedure described above, about 1.5 mg of the protease was obtained from 1 l of culture liquid.

Biochemical properties of the protease reveal a metalloprotease

N-terminal chemical sequencing of the first twelve amino acids of the purified protease revealed the sequence AAATGTGRGVLG, highly similar to the corresponding sequence of aureolysin from *S. aureus* (Sabat *et al.*, 2000). This strongly suggested that the enzyme is a metalloprotease. Indeed, further biochemical studies showed that the protease is completely inhibited by EDTA and 1,10-phenantroline, but not by inhibitors of serine or cysteine proteases: phenylmethylsulfonyl fluoride (PMSF) and E-64 (Table 2). The protease cleaves dye-attached protein substrates (azocasein, Azocoll, Hide Powder Azure). Unlike aureolysin (Banbula *et al.*, 1998), the enzyme demonstrates also elastolytic activity. To identify a convenient substrate for measurement of the metalloprotease activity an array of synthetic chromogenic peptide substrates with *p*-nitroaniline as a leaving group were tested. None of them was efficiently hydrolyzed. The protease (Pst) is inhibited by α-2-macroglobulin but not by human blood serpins: α-1-antitrypsin (AT) and α-1-antichymotrypsin (ACT). Moreover, the enzyme cleaves those inhibitors in reactive loop regions (sequences recognized by the enzyme: AMF|LEA and LSA|LVE for AT and ACT, respectively) inactivating them.

Table 2. Effect of calcium cations and some group-specific reagents on Pst enzymatic activity

Additives	Relative activity (%)
None*	100
CaCl$_2$ (5 mM)	188
EDTA (5 mM)	0
1,10-phenantroline (5 mM)	8
E-64 (0.5 mM)	97
2-mercaptoethanol (5 mM)	93
PMSF (5 mM)	96

*Activity estimated in 100 mM buffer Tris/HCl pH 7.0.
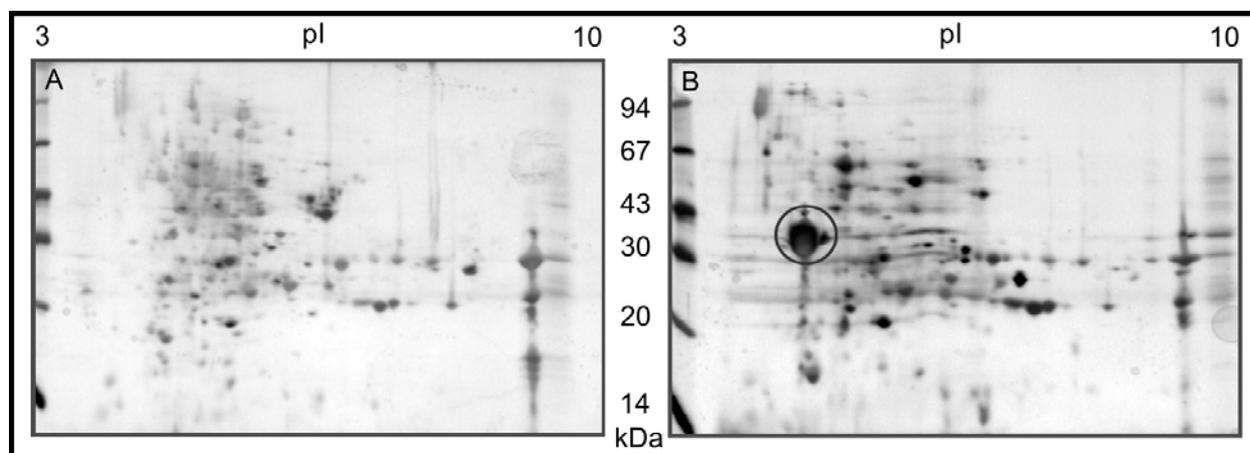
Mass spectrometry analysis of cleavage products of β-casein shows that the substrate specificity of Pst is similar to that of other enzymes from the thermolysin family. Preferentially, peptide bonds preceding hydrophobic residues are hydrolyzed (e.g., I, L or F at P1′ position, nomenclature according to (Schechter & Berger, 1967)). The enzyme is not absolutely specific and — less frequently — also hydrophilic residues (e.g., E, T or Q) can occupy the P1′ position. Residues at other positions seem to be much less important for the enzyme specificity.

To investigate the influence of Pst on the profile of bacterial extracellular proteins, 2D electrophoresis was performed. The bacteria were grown in liquid media with or without supplementation with 5 mM CaCl$_2$. Proteins from overnight culture liquids were subjected to electrophoresis. Comparison of 2D gels showed profound differences in protein patterns (Fig. 2). A total of 206 and 208 protein spots were identified on the gel with the proteins from the growth medium non- and supplemented with calcium ions, respectively. There were 118 unmatched protein spots between the gels. A spot corresponding to the protease (confirmed by N-terminal sequencing) was only observed on the gel with the proteins from the growth medium supplemented with calcium ions.

Gene sequence of the protease confirms it is a novel member of the thermolysin family

PCR was first applied to obtain an internal fragment of the *aur*-like gene by using the genomic DNA of *S. pseudintermedius* strain 9p and the degenerated primers coaF-1 and coaR (Table 1). These were chosen on the basis of reverse translation from the most conserved amino-acid sequences of the metalloproteases from *S. aureus*, *S. chromogenes* and *S. epidermidis* at positions 351 to 356 and 431 to 436, respectively, in the translation product of the *aur* gene (Sabat *et al.*, 2000). PCR was performed and the resulting 260-bp amplicon was sequenced. The predicted amino-acid sequence encoded by the fragment revealed significant homology with amino-acid sequences of staphylococcal members of the thermolysin family of metalloproteases (Teufel & Gotz, 1993; Sabat *et al.*, 2000).

To obtain the remaining part of the *aur*-like gene and flanking regions, inverse PCR was applied. The genomic DNA was restricted with *Csp*6I or *Cfo*I, the fragments produced were circularized with T4 DNA ligase, and PCR was performed with two outward-pointing oligonucleotide primers, InvF56 and InvR57, complementary to sequences inside the internal 260-bp. The resulting PCR products of 0.7 and 1.1 kb for the *Csp*6I and *Cfo*I inverse PCRs, respectively, were sequenced. Analysis

Figure 2. 2D electrophoresis maps of extracellular proteins from overnight cultures of *S. pseudintermedius.*
Bacteria were grown in TSB medium non-supplemented (A) and supplemented (B) with 5 mM CaCl$_2$. The spot corresponding to the metalloprotease (verified by N-terminal analysis) is encircled.


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                  *        20         *        40         *        60
B.the : MKMKMKLASFGLAAGLAAQVFLPYNALASTEHVTWNQQFQTPQFISGDLLKVNGTSPEEL :  60
S.aur : MRKFSRYAFTSMATVTLLSSLTPAALASDTNHKPATSDINFEITQKSDAVKALKELPK-- :  58
S.pse : MFKLSKKVLFGTLCLSVLS-LSPNVHAESSSEKPLEKNFKTTVKSKADANKALKELP--- :  56


                  *        80         *       100         *       120
B.the : VYQYVEKNENKFKFHENAKDTLQLKEKKNDNLGFTFMRFQQTYKGIPVFGAVVTSHVKDG : 120
S.aur : ----SENVKNHYQDYS-------VTDVKTDKKGFTHYTLQPSVDGVHAPDKEVKVHADKS : 107
S.pse : -----SDVKDNYKDYD-------VVDALTDDLGYTHYTLQPVVDGVHAKDQEVKVHVDKK : 104


                  *       140         *       160         *       180
B.the : TLTALSGTLIPNLDTKGSLKSGKKLSEKQARDIAEKDLVANVTKEVPEYEQGKDTEFVVY : 180
S.aur : GKVVLIN---GDTDAKKVKPTNKVTLSKDEAADKAFNAVKIDKNKAKNLQDDVIKENKVE : 164
S.pse : GQVVLIN---GEVNAEKVDPTNSVSISKDAALSTAFKAVDMKQSEASNLDEDVVKVNDVE : 161


                  *       200         *       220         *       240
B.the : VNGDEASLAYVVNLNFLTEEPGNWLYIIDAVDGKILNKFNQLDAAKPGDVKSITGTSTVG : 240
S.aur : IDGDSNKYIYNIELITVTPEISHWKVKIDADTGAVVEKTNLVKEA-----------AATG : 213
S.pse : IDGEDNKYVYHVELVTMEEDVSHWDLKVDAETGEVVEKTDLVAHA-----------AATG : 210


                  *       260         *       280         *       300
B.the : VGRGVLGDQKNINTTYSTYYYLQDNTRGNGIFTYDAKYRTTLPGSLWADADNQBFASYDA : 300
S.aur : TGKGVLGDTKDININSIDGGFSLEDLTHQGKLSAYNFNDQTGQATLITNEDENFVKDDQR : 273
S.pse : TGRGVLGDTKRININSIDGGYSLEDVTGSAVMATYAFNPASGSADLITDPDTNETDDYQR : 270


                  *       320         *       340         *       360
B.the : PAVDAHYYAGVTYDYYKNVHNRLSYDGNNAAIRSSVHYS-----QGYNNAFWNGSQMVYG : 355
S.aur : AGVDANYYAKQTYDYYKNTFGRESYDNHGSPIVSLTHVNHYGGQDNRNNAAWIGDKMIYG : 333
S.pse : AGVDANYYAKKVYDYYASKFDRRSYDNRDSDIMSIVHVNNFQGQDNRNNAAWIGDKMIYG : 330


                  *       380         *       400         *       420
B.the : DGDGQTFIPLSGGIDVVAHELTHAVTDYTAGLIYQNESGAINEAISDIEGTLVEFYANKN : 415
S.aur : DGDGRTFTNLSGANDVVAHELTHGVTQETANLEYKDQSGALNESFSDVEGYFVD-----D : 388
S.pse : DGDGTFTALSGADDVVAHEITHGVTQETAGLIYRGQPGALNESMSDTEAYFMD-----P : 385


                  *       440         *       460         *       480
B.the : PDWEIGEDVYTPGISGDSLRSMSDPAKYGDPDHYSKRYTGTQDNGGVHINSGIINKAAYL : 475
S.aur : EDFLMGEDVYTPGKEGDALRSMSNPEQFGQPSHMKDYVYTEKDNGGVHTNSGIPNKAAYN : 448
S.pse : GDTLIGEDVYTPRRPGDALRSMSNPENQPGHMRDYVNTSSDHGGVHTNSGIPNKAAYL : 445


                  *       500         *       520         *       540
B.the : ISQGGTHYGVSVVGIGRDKLGKIPYRALTQYLTPTSNFSQLRAAAVQSATDLYGSTSQEV : 535
S.aur : VIQ---------AIGKSKSEQIYYRALTEYLTSNSNFKDCKDALYQAAKDLYD--EQTA : 496
S.pse : TVN---------KIGQEQAEQIYYRALTSYLTSTSNFADAKASLVRAALDLYG--EDVA : 493


                  *
B.the : ASVKQAFDAVGVK : 548
S.aur : EQVYEAWNEVGVE : 509
S.pse : RQVEAAWNEVGV- : 505
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Figure 3. Alignment of amino-acid sequences of selected metalloproteases from the thermolysin family.
B.the, thermolysin precursor from *Bacillus thermoproteolyticus*; S.aur, aureolysin precursor from *Staphylococcus aureus*; S.pse, metalloprotease precursor from *S. pseudintermedius* strain 9p. The conserved amino-acid residues among the thermolysins and between S.aur and S.pse are black- and grey-shadowed, respectively.

revealed that the complete 3'-terminal region of the *aur*-like gene was included in the *Cfo*I inverse PCR product, whereas the 5' terminus was lacking in both inverse PCR products. In order to obtain the full-length *aur*-like gene, a third inverse PCR with outward-pointing primers, InvF56 and InvR59, and *Hpa*II-digested, ligated chromosomal DNA was conducted, resulting in the amplification of a 1.2-kb fragment. The combined sequence information revealed a coding sequence (CDS) of 1515 nucleotides encoding a preproenzyme of 505 amino acids (Fig. 3) with a molecular mass of 55170 Da and a putative ribosome binding site (AGGAGT) seven nucleotides upstream of the CDS. We propose to name this gene *pst*.

For the sequence upstream of *pst*, we used two outward-pointing oligonucleotide primers, InvF61 and InvR62, located before the *pst* gene, and an endonuclease *Apo*I cleavage site, which is recognized in the near vicinity but downstream of the forward primer InvF61. For the region downstream of *pst*, we used two outward-pointing primers, InvF67 and InvR68, located after the *pst* gene, and a site recognized by endonuclease *Hpa*II in the near vicinity but upstream of the reverse primer InvR68. The resulting PCR products of 0.7 and 1.5 kb for the *Apo*I and *Hpa*II inverse PCRs, respectively, were sequenced. One-hundred and eighty nucleotides upstream of *pst*, a putative open reading frame (ORF) a 55 amino acids with no significant sequence similarity to other proteins was identified. On the complementary strand, 383 nucleotides upstream of *pst* part of an ORF coding for oxidoreductase was recognized. Downstream, but in near vicinity, of *pst* no ORFs were identified. The sequences for *pst* together with the flanking regions have been deposited in the EMBL database under accession numbers: AM921781, AM921782, AM921783, AM921784 and AM921785 for the strains 2INT, 6INT, 6p, 9p and CCUG, respectively.

Polymorphism and molecular evolution of *pst*

To investigate the conservation of the *pst* gene in different strains, we amplified by PCR the whole gene from ten *S. pseudintermedius* strains using primer pair F1-RFLP–R4-RFLP (Table 1). In all strains, the PCR products were successfully amplified to the expected 1554 bp fragment with the exception of one strain, CCUG, for which the PCR product showed a slightly greater size (1596 bp). For the RFLP analysis, restriction enzymes were chosen based on the nucleotide sequence of the *pst* gene of strain 9p. The amplified DNA was digested with *Alu*I, *Csp*6I, *Hpy*F3I, *Eco*130I, and *Taq*I restriction enzymes. The strains (2INT, 6INTF, 6p, CCUG) which

showed a unique restriction pattern for at least one endonuclease were selected for further sequencing analysis.

The analysis revealed that the length of the *pst* gene of *S. pseudintermedius* strains 2INT, 6INTF, and 6p was identical to that of strain 9p (1518 nucleotides), whereas the gene of strain CCUG was 1560 bp in length due to an insertion corresponding to 14 additional amino acids (VMATYAFNPASGSA) between positions 255 and 268, which were a perfect duplication of the preceding sequence between positions 241 and 254. The nucleotide sequences obtained during this study were aligned (not shown). There were ten (0.6%) polymorphic nucleotide sites and seven (1.3%) variable inferred amino-acid positions among the five sequences. The pairwise differences in the nucleotide and inferred amino-acid sequences between pairs of the five *pst* alleles ranged from one to nine nucleotides (0.1 to 0.6%) and one to six amino acids (0.2 to 1.2%).

Most polymorphisms led to non-synonymous substitutions, indicating a substantially little role of purifying selection in the evolution of the gene. The strength of purifying selection was expressed as the ds/dn ratio, and showed a value of 1.83.

The *pst* transcript is monocistronic

To test if *pst* is expressed as a monocistronic unit, RT-PCR experiment was performed. cDNA and primer pair AurLRT and AurLRTR (Table 1) with sequences corresponding to an internal fragment of the putative ORF upstream of *pst* and reverse complementary to the innate part of *pst*, respectively, were used for the reaction. The failure to obtain an amplicon shows that *pst* and the ORF upstream of the metalloprotease gene are not transcriptionally linked (not shown).

Quantitative analysis of the *pst* transcript revealed that its levels in bacteria grown in medium non-supplemented and supplemented with 5 mM $CaCl_2$ were similar (relative expression 1.03). This, together with the other presented results, suggests that the metalloprotease production undergoes regulation at a post-transcriptional level.

DISCUSSION

We took advantage of the results of a basic microbiological test to conduct studies which resulted in the characterization of a novel member of the thermolysin family. Production of proteases by numerous bacterial pathogens is regarded as an important factor of their virulence (Armstrong, 2006). Therefore, investigation of the profile of bacterial extracellular proteases is often a routine assay dur-

ing identification of clinical isolates. The exceptionally high production of the protease by the bacteria isolated from skin lesions in dogs was a leading reason for investigating the protease profiles of these strains. Surprisingly, no proteolytic activity was detected when the bacteria were grown in regular liquid medium. Supplementation of the medium with various fractions of milk showed clearly that calcium ions are responsible for the expression of the proteolytic activity.

Biochemical studies demonstrated that the enzyme is a zinc-dependent metalloprotease, because its activity was completely abolished by 1,10-phenanthroline and EDTA, common inhibitors of proteases of this catalytic class (Auld, 2004). N-terminal chemical sequencing, further supported by gene cloning, revealed that the protease is a novel member of the thermolysin family, most closely related to aureolysin, the metalloprotease of *S. aureus* (Sabat *et al.*, 2000). During purification, two unexpected features of Pst were noted. Firstly, the commonly applied procedure of protein precipitation with ammonium sulfate resulted in irreversible inactivation of the enzyme. Therefore, clarified culture liquid was concentrated by ultrafiltration prior to purification. Secondly, the protease was strikingly stable in buffers containing calcium ions, even at long-time incubation at 37°C. The latter property was utilized for the proteolytic removal of protein impurities during the isolation procedure, similar to that for elastase (Dubin *et al.*, 1976).

Zymographic analysis showed that Pst is responsible for the whole proteolytic activity of the culture liquid of *S. pseudintermedius*. The observation is in contradiction with the profile of extracellular proteases expressed by *S. aureus*, where at least four proteases from three catalytic classes have been identified (Arvidson, 1973, Shaw *et al.*, 2004). For the latter bacteria, it was shown that the secreted proteases alter extracellular proteins, host-derived as well as produced by the microorganism (Dubin, 2002). Moreover, reports suggest that through the shedding of bacterial cell surface proteins, such as fibronectin-binding protein and protein A, proteases also play a role in the transition of *S. aureus* cells from an adhesive to an invasive phenotype (McGavin *et al.*, 1997; Karlsson *et al.*, 2001). Indeed, only proteolytic strains of *S. aureus* showed a remarkable decrease in fibronectin-binding when grown in casein-rich medium (Miedzobrodzki *et al.*, 1989). Therefore, it was reasonable to investigate whether the production of Pst by the *S. pseudintermedius* strains affects the profile of their extracellular proteins. Indeed, comparison of 2D gels showed that around 60% protein spots were unmatched. This demonstrates profound changes in the protein content of culture liquid depending on the presence or absence of the protease.

Unfortunately, a detailed identification of the differentiating proteins was impossible due to the lack of genomic data on *S. pseudintermedius*. The involvement of Pst in modulation of the profile of extracellular proteins is in agreement with the broad specificity of the protease. Similarly to other enzymes from the thermolysin family, Pst prefers cleavage of peptide bonds before bulk hydrophobic amino-acid residues.

Several reports have indicated that protease production is dependent on environmental conditions such as carbohydrate and nitrogen sources, temperature, and ions. Particularly, calcium ions were shown to be necessary for enhanced production of bacterial metalloproteases (Ghorbel-Frikha *et al.*, 2005, Sarkisova *et al.*, 2005). However, in the case of the protease from *S. pseudintermedius* the dependence on calcium ions in the growth medium was even stricter, the proteolytic activity was detected only when the bacteria were cultivated in the presence of calcium. A similar situation was observed for a metalloprotease from the fish pathogen *Flavobacterium psychrophilum* (Secades *et al.*, 2001). This may be explained either by the induction of the protease expression by calcium or by the fact that the ions are indispensable for the stabilization of the enzyme active conformation after synthesis. Although the first hypothesis is more encouraging in terms of bacterial ecology, it must be rejected because, regardless of the medium composition, the level of the metalloprotease gene (*pst*) transcript was comparable. Thus, in the case of Pst the second explanation has to be favored. Endopeptidases from the M4 family possess 2–4 calcium binding sites. Two Ca^{2+} ions are bound within a double cooperative site (Ca(1), Ca(2)), additionally two independent binding sites (Ca(3) and Ca(4)) can bind the next two Ca^{2+} ions (nomenclature according to Colman *et al.*, 1972). Thermodynamic data shows that Ca(1) and Ca(2) bind Ca^{2+} weaker than Ca(3) and Ca(4) (Buchanan *et al.*, 1986). The role of the cooperative calcium site in thermolysins is well known, calcium bound within these sites stabilizes the overall fold of enzymes by linking the N-terminal and C-terminal subdomains. In low concentrations of calcium, those sites release calcium ions, leading to denaturation and autodegradation of the enzyme (Corbett & Roche, 1983). In the crystal structure of the highly homologous aureolysin from *S. aureus*, three Ca ions occupy the Ca(1), Ca(2) and Ca(4) sites (Banbula *et al.*, 1998).

Based on homology modelling data, the majority of interactions in Pst seem to be similar to those around the equivalent calcium sites in aureolysin from *S. aureus* (Fig. 4). However, in the cooperative site, there is a substitution of amino acids interacting with Ca(2). In aureolysin from *S. aureus* E389 interacts with Ca^{2+} through the carboxylate oxygen atom
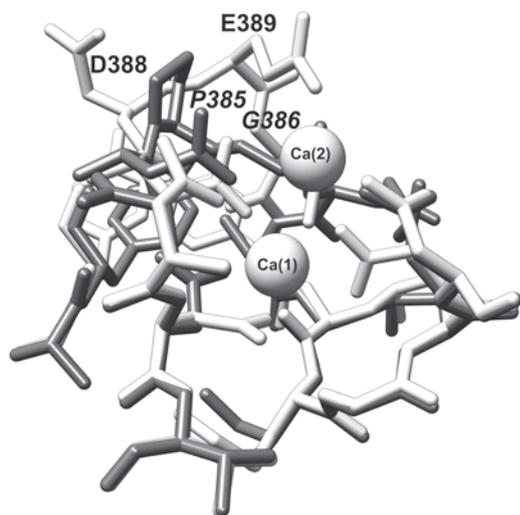
Figure 4. Superposition of residues from the area of calcium ion binding.
Aureolysin is in light- and Pst in dark-gray, respectively. The spheres represent calcium ions. Labels of residues in the cooperative site of aureolysin and Pst are bolded and italicized, respectively. Pairwise alignment of the sequences of mature Pst and aureolysin gives 69% of identical positions (78% of similar positions).

(Oε1) (Banbula *et al.*, 1998), whereas in Pst the corresponding position is occupied by G386 (numbering as in Fig. 3). Consequences of the substitution of D388 E389 (in aureolysin) by P385 G386 (in Pst) are twofold. Firstly, the interaction corresponding to Oε1 E389 with Ca^{2+} is abolished. Secondly, the *cis*-peptide bond probably formed by the proline residue disturbs conformation of the backbone in proximity of the Ca(2) binding site. Therefore, binding of Ca^{2+} to the Ca(2) binding site may be impaired and stabilization by Ca^{2+} is either weaker or rather occurs at higher concentrations of calcium ions. This fact may explain the strong dependence of the protease stability on the presence of Ca^{2+} ions in the environment.

It is very difficult to differentiate between species that have overlapping phenotypic characteristics. Moreover, commercial kits are not available for the identification of *S. pseudintermedius*. As more and more staphylococcal species are described and associated with human infection (Van Hoovels *et al.*, 2006; Trulzsch *et al.*, 2007), their identification becomes more important, particularly when resistance to certain antimicrobial drugs becomes evident (Descloux *et al.*, 2008, Schwarz *et al.*, 2008). In our study only sequencing of the internal fragment of the *rpoB* gene allowed for the final identification of the *S. pseudintermedius* species. Therefore, the accurate identification of *Staphylococcus* species can be obtained only using appropriate nucleic acid targets. Very recently, a new molecular diagnostic test based on sequencing of the internal fragment of the *gap* gene encoding glyceraldehyde-3-phosphate dehydrogenase was reported (Ghebremedhin *et al.*, 2008). The polymorphism of the partial *gap* sequences was found to be higher than those of other targets (16S rRNA, *rpoB*, *hsp60*, and *sodA*) currently used for identification of staphylococcal species. Therefore, partial sequencing of the *gap* gene seems to be sufficient for correct identification to the species level of the *Staphylococcus* genus.

In summary, we showed gene cloning and biochemical characterisation of a novel member of the thermolysin family expressed by *S. pseudintermedius*, Pst. Production of Pst is strictly dependent on the presence of calcium ions in the environment of bacterial growth. This broad-specific protease significantly alters the profile of extracellular proteins, and is the only agent responsible for the exceptionally high proteolytic activity of the strains. Therefore, it can not be excluded that in favourable circumstances Pst may be responsible for the development of staphylococcal infection.

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