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A novel  $\alpha$ -defensin of the horse: gene transcription, recombinant expression and  
characterisation of the structure and function

Oliver Bruhn<sup>\*</sup>, Petra Regenhard<sup>\*</sup>, Matthias Michalek<sup>†, ‡</sup>, Sven Paul<sup>\*</sup>, Christoph Gelhaus<sup>‡</sup>, Sascha  
Jung<sup>†</sup>, Georg Thaller<sup>\*</sup>, Rainer Podschun<sup>§</sup>, Matthias Leippe<sup>‡</sup>, Joachim Grötzinger<sup>†</sup>, and Ernst Kalm<sup>\*1</sup>

<sup>\*</sup>Institute of Animal Breeding and Husbandry, University of Kiel, Hermann-Rodewald-Straße 6,  
24118 Kiel, Germany

<sup>†</sup>Institute of Biochemistry, University of Kiel, Rudolf-Höber-Straße 1, 24118 Kiel, Germany

<sup>‡</sup>Department of Zoophysiology, Zoological Institute, University of Kiel, Am Botanischen Garten 1-9,  
24118 Kiel, Germany

<sup>§</sup>Institute for Infection Medicine, University of Kiel, Brunswiker Straße 4, 24105 Kiel, Germany

<sup>1</sup>**Corresponding author:** Tel. 0049-(0)431-880-2586; Fax. 0049-(0)431-880-2588; E-Mail:

[ekalm@tierzucht.uni-kiel.de](mailto:ekalm@tierzucht.uni-kiel.de)

## SYNOPSIS

Defensins are a predominant class of antimicrobial peptides, which act as endogenous antibiotics. Defensins are classified into three distinct sub-families,  $\theta$ -,  $\beta$ -, and  $\alpha$ -defensins. An  $\alpha$ -defensin synthesis is confirmed only in primates and glires to date and is presumably unique for a few tissues including neutrophils and Paneth cells of the small intestine. Antimicrobial activities of these peptides were shown against a wide variety of microbes including bacteria, fungi, viruses, and protozoan parasites. Here, we report the characterisation of the equine  $\alpha$ -defensin DEFA1. Transcription analysis revealed that the transcript of the gene is present in the small intestine only. An alignment with known  $\alpha$ -defensins from primates and glires displayed a homology to Paneth cell specific  $\alpha$ -defensins. DEFA1 was recombinantly expressed in *Escherichia coli* and subsequently analysed structurally by circular dichroism and molecular modelling. To examine the antimicrobial properties a radial diffusion assay was performed with 12 different microorganisms and the LD<sub>90</sub> and MBC values were examined. DEFA1 showed an antimicrobial activity against different gram-positive and gram-negative bacteria and against the yeast *Candida albicans*. Using viable bacteria in combination with a membrane-impermeable fluorescent dye as well as depolarisation of liposomes as a minimalistic system, it became evident that membrane permeabilisation is at least an essential part of the peptide's mode of action.

**Keywords:** Antimicrobial peptide, defensin, horse, innate immunity

**Running Title:** An  $\alpha$ -defensin of the horse

## INTRODUCTION

An important component of the innate immunity next to the complement pathway and phagocytes are antimicrobial peptides [1, 2], which play a pivotal role as key effectors of the immune system and act as endogenous antibiotics [2, 3]. Antimicrobial peptides are synthesised by circulating phagocytic cells, leukocytes and epithelial cells of mucosal tissues. In mammals, defensins are a predominant class of these peptides that exhibit a direct activity against a broad range of bacteria [4], fungi [5] and enveloped viruses [6].

Defensins are cationic and cysteine-rich peptides with molecular masses ranging from 3 to 5 kDa. They contain six highly conserved cysteine residues forming characteristic intramolecular disulfide bonds. The disulfide array is specific for the three defensin sub-families in mammals:  $\alpha$ -,  $\beta$ -, and  $\theta$ -defensins [7]. An  $\alpha$ -defensin gene expression is confirmed only in primates and glires to date. They were found initially in human neutrophil granulocytes, and identified as natural peptide antibiotics [4]. Further studies showed a gene expression also in mice [8], rhesus macaques [9], rats [10], rabbits [11], guinea pigs [12], and hamsters [13]. Additionally,  $\alpha$ -defensin synthesis is presumably unique for a few tissues. The peptides were found in neutrophils, in epithelial cells of the human female urogenital tract [14], in the kidney of rabbits [15], and in the Paneth cells of different species [16]. Paneth cells are secretory epithelial cells, which are most abundant as specialised ileal cells in the distal small intestine at the base of the crypts of Lieberkuhn. Human Paneth cells synthesise two different  $\alpha$ -defensins, HD5 (DEFA5) and HD6 (DEFA6) [17, 18] in addition to the neutrophil human  $\alpha$ -defensin peptides 1 to 4 (HNP-1, -2, -3, -4). All  $\alpha$ -defensins are synthesised *in vivo* as inactive precursor proteins. A proteolytic excision of the N-terminal inhibitory anionic propeptide is required for maturation and activation. The signal peptides and propeptides are necessary for correct subcellular sorting and trafficking of the defensins [19, 20], and the propeptide is thought to be the major contributing factor that inhibits the antimicrobial activity by neutralisation of cationic charges of the mature peptide [20].

The active mature  $\alpha$ -defensin peptides consist of 29-35 amino-acid residues with a molecular mass of 3-4 kDa. The primary structure shows highly conserved residues, which are indispensable for the

structural stability. Among them are six invariant cysteines, necessary for the typical  $\alpha$ -defensin intramolecular disulfide-bond connectivity (Cys<sub>1</sub>-Cys<sub>6</sub>, Cys<sub>2</sub>-Cys<sub>4</sub> and Cys<sub>3</sub>-Cys<sub>5</sub>); two charged amino-acid residues Arg<sub>5</sub>, and Glu<sub>13</sub>, forming a conserved salt bridge [21], and Gly<sub>17</sub>, which constitutes a signature structural motif essential for correct folding [22]. The tertiary structure is a triple-stranded  $\beta$ -sheet with a beta hairpin that contains cationic amino-acid residues [23].

The antimicrobial activity of  $\alpha$ -defensins was shown against the gram-negative bacteria *Escherichia coli*, *Salmonella typhimurium*, and *Enterobacter aerogenes*, the gram-positive bacteria *Staphylococcus aureus*, *Bacillus cereus*, *Listeria monocytogenes*, the fungal pathogens *Candida albicans*, and *Cryptococcus neoformans*, and against the protist *Giardia lamblia* [9, 24-28]. Furthermore, antiviral activity, particularly an anti-HIV-1 activity was observed [29]. Defensins are thought to kill bacteria by an initial electrostatic interaction with the negatively charged phospholipids of the microbial cytoplasmic membrane, followed by membrane permeabilisation [30, 31] and lysis of the microbes [32]. An amphipathic character of the peptide is essential for inserting into the membrane [33].

In this study, we report the transcription of an equine  $\alpha$ -defensin gene in the intestine. The peptide was recombinantly expressed in *E. coli* and its biological activity against various microorganisms was determined. In particular, the mechanism of killing was examined by monitoring membrane-permeabilising activity of the equine defensin against viable bacteria and liposomes.

## EXPERIMENTAL

### Transcriptional analysis

Based on sequence informations of the equine BAC-clone CHORI 241-245H5, Acc.no. AY170305 [34], primers were constructed to amplify a cDNA representing the potential  $\alpha$ -defensin. Total RNA was isolated from 13 epithelial tissues (skin, teat, vagina, tongue, gingiva, esophagus, trachea, lung, small intestine, large intestine, umbilical cord, uterus, meninges) and blood (RNeasy Mini-Kit, QIAGEN, Hilden, Germany). The cDNA was generated by performing RT-PCR using a oligo-dT-primer (OligoT-Bio), 5'-ACT CTA TGA GAA TTC GAT GAG CGA TCT GTT T<sub>(n=21)</sub> TTV. PCR

reactions were performed using the forward primer (U130\_L1), 5'-ATC TCC TTT GCA GGG GAT GAA C paired with the reverse primer (L282\_L1), 5'-ACG ACA GCA GAG CCT GTA AAT GA and the cDNA as template. The reaction mixtures were incubated at 94 °C for 3 min, followed by 33 cycles at 60 °C for 50 s, 72 °C for 50 s, and 94 °C for 1 min. Finally, agarose gel electrophoresis was performed to determine the transcription.

### **Molecular cloning and sequencing of *DEFA1***

The generated product of the small intestine was extracted from the gel with the Gel-Extraction Kit (QIAGEN) and purified with the Reaction Cleanup Kit (QIAGEN). The sequences were determined (DLMBC, Berlin, Germany) by using the forward primer and reverse primer previously used for expression analysis. The resulting sequences revealed sequence overlays (data not shown). New primers were generated, the forward primer (DEFA5L\_up1), 5'-TTG ACT CCC AGC CAT GAG (coding sequence is underlined) and the reverse primer (TailPrimer3'), 5'-ACT CTA TGA GAA TTC GAT GAG CGA TCT G (complementary to the OligoT-Bio primer) to determine the complete cDNA sequence. A 3'-RACE [35] was performed with cDNA from the small intestine as template under the following PCR conditions: 94 °C for 3 min, followed by 33 cycles at 58 °C for 50 s, 65 °C for 45 s and 94 °C for 1 min. The amplified product was purified by gel extraction (QIAGEN). For sequencing, 68 ng of the purified product was subcloned in the pDRIVE cloning vector according to the manufacturer's specifications (Cloning-Kit, QIAGEN). A test PCR with the forward primer (DEFA5L\_up1) and the reverse primer (DEFA5L\_lo300), 5'-ACG ACA GCA GAG CCT GTA AAT G was performed with 20 of the grown clones as a template. Positive clones were sequenced (DLMBC, Berlin, Germany). The obtained sequences were aligned (ClustalW, [www.ebi.ac.uk/clustalw/](http://www.ebi.ac.uk/clustalw/)) and analysed.

### **Preparation of the cDNA for recombinant peptide expression**

The cDNA coding for the mature peptide of the  $\alpha$ -defensin, corresponding to nucleotides 193 to 296 of the equine  $\alpha$ -defensin cDNA (Acc. no. EF379126) was amplified using the forward primer (DEA6\_mat\_U2), 5'-GGT ATT GAG GGT CGC TCC TGC ACC TGC AGA CGT, paired with the

reverse primer (DEA6\_mat\_L1), 5'-AGA GGA GAG TTA GAG CC TCA GCG ACG ACA GCA GAG (peptide specific sequence is underlined) and the pDRIVE cloning vector containing the complete cDNA as template. The reaction was performed by incubating the reaction mixture at 94 °C for 3 min, followed by 33 cycles at 61 °C for 30 s, 68 °C for 1 min and 94 °C for 45 s. The sample was purified by gel extraction. The purified PCR products (0.2 pM) were treated 30 min at 22 °C with 0.16 U T4 DNA Polymerase (QIAGEN) and 3 mM dGTP to generate the overhangs for the annealing reaction. The product was ligated into the vector pET-30 Xa/LIC (NOVAGEN, Darmstadt, Germany).

### **Recombinant expression of DEFA1**

The vector was transformed into the *E. coli*-strain BL21 (DE3) by heat-shock (90 s at 42 °C). The cells were grown at 37 °C to an OD<sub>600</sub> of 0.5 in 1.5 l Luria-Bertani (LB)-Medium (ROTH, Karlsruhe, Germany) containing 30 µg/ml kanamycin [36]. The expression of the fusion peptide was induced with 1 mM IPTG (isopropyl-D-1-thiogalactopyranoside) followed by an incubation for 4 h at 37 °C. Bacterial cells were harvested by centrifugation at 6000 x g for 20 min at 4 °C and lysed by resuspending the bacterial cell pellet in 40 ml 6 M guanidine HCl in 100 mM Tris-HCl, pH 8.0 followed by sonication at 70% power for 3 min (BANDELIN Sonopuls, Berlin, Germany). The lysate was clarified by centrifugation at 30,000 x g for 30 min at 4 °C, supernatant was sterilised using a membrane filter with a pore-size of 0.45 µm (SARTORIUS, Göttingen, Germany) prior to peptide purification.

### **Purification of the recombinant peptide**

The His<sub>6</sub>-tagged α-defensin fusion peptide was purified by performing nickel-nitrilotriacetic acid metal-affinity chromatography using prepacked Ni-NTA superflow columns (QIAGEN). Clarified lysates were transferred to the column and drained by gravity flow. Washing was performed with 6 M guanidine HCl in 100 mM Tris-HCl, pH 8.0 including 15 mM imidazole, and the His<sub>6</sub>-tagged peptide was eluted with 6 M guanidine HCl in 100 mM Tris-HCl, pH 8.0 including 750 mM imidazole. The samples were stored at -20 °C.

The buffer of the dissolved fusion peptides was exchanged by using desalting columns PD-10 (GE HEALTHCARE, Uppsala, Sweden, bed volume 8.3 ml). Ni-NTA purified peptides were transmitted to a PD-10 column and eluted with cleavage buffer consisting of 20 mM Tris-HCl, 50 mM NaCl, 1 mM CaCl<sub>2</sub>, pH 6.5. The buffer exchange procedure was repeated twice. The eluted His<sub>6</sub>-tagged peptide was quantified photometrically by measuring the UV absorption at 280 nm based on the extinction coefficient [37].

The fusion peptide was cleaved for 12 h at room temperature with 10 U Factor Xa protease (QIAGEN) per 150 µg peptide in cleavage buffer.

### **Reversed-phase chromatography**

After cleavage, the mature peptide was purified immediately to apparent homogeneity by using reversed-phase high performance liquid chromatography (RP-HPLC). The sample was applied to a C-18 preparative RP-HPLC VP 250/10 Nucleosil 300-7 column (MACHEREY-NAGEL, Düren, Germany), and the peptide was eluted after 16 min using a 22-40% acetonitrile gradient developed over 90 min. Finally, the peptide was lyophilised, and redissolved in 0.01% trifluoroacetic acid (TFA) for storage at -20 °C and further studies.

### **Circular dichroism (CD spectroscopy)**

CD measurements were carried out on a JASCO J-720 spectropolarimeter (JAPAN SPECTROSCOPIC CO., Ltd., Tokyo, Japan) calibrated according to reference [38]. The spectral bandwidth was 2 nm. Three scans were recorded between 190 nm and 250 nm with 1 nm acquisition steps and the spectrum was baseline corrected. Measurements were carried out at room temperature and cuvettes of 1.0 mm optical path length were used. The peptide concentration was 15 µM in 0.01% TFA.

### **Mass spectrometry**

The molecular identity and homogeneity of the preparation was verified by matrix-assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF MS). The samples were mixed



with a saturated matrix solution of sinapinic acid prepared in 80% acetonitrile/0.1% TFA and spotted on a stainless steel sample target. Mass spectra were obtained on a MALDI-TOF/TOF mass spectrometer (4700 Proteomics Analyser, Applied Biosystems, Framingham, MA, USA) in the positive ion linear mode and calibrated externally. The average mass of the proteins were compared to the theoretical average mass of DEFA1 considering potential disulfide formation.

### **Antimicrobial activity**

Following bacterial strains were used in a radial diffusion assay as described previously [39]: Gram-negative *Escherichia coli* ATCC 11303, ATCC 11775, ATCC 25922, ATCC 35218, and D31, *Pseudomonas aeruginosa* ATCC 10145, *Burkholderia cepacia* ATCC 25416, and gram-positive *Staphylococcus aureus* ATCC 6538, and ATCC 12600, *Staph. epidermidis* ATCC 14990, *Bacillus megaterium* ATCC 14581, and the yeast *Candida albicans* ATCC 24433. The organisms were grown to mid-log phase in trypticase soy broth (TSB), suspended into agar (1% agarose in 10 mM sodium phosphate pH 7.4 supplemented with a 1:100 dilution of 1 x TSB, and poured into petri dishes (10 ml). After hardening, 5 µl of peptide test solution was pipetted into wells formed with a biopsy punch (diameter: 3 mm) and allowed to incubate at 37 °C overnight. Lysozyme was used as a control peptide, 0.01% TFA served as negative control. Plates were then overlaid with 10 ml of 1% agar supplemented with double strength TSB (caseine peptone 3.4%, tryptic soy peptone 0.6%, glucose 0.5%, NaCl 1%, and K<sub>2</sub>HPO<sub>4</sub> 0.5%). After incubation for 3 h, zones of growth inhibition surrounding the wells were measured.

Additionally, LD<sub>90</sub> and MBC (minimal bactericidal concentration) values of the peptide (≥ 90% or ≥ 99.9% killing of the target organisms) were determined by using the test strain pattern described for the radial diffusion assay with the exception of *E. coli* ATCC 11030. In addition, gram-negative *Klebsiella pneumoniae* ATCC 13883, *Enterobacter cloacae* ATCC 13047, *P. aeruginosa* ATCC 11440, gram-positive *Enterococcus faecalis* ATCC 29212, *Ec. faecalis* PEG 205 (wild type), and *Streptococcus pyogenes* ATCC 12344 were introduced. Briefly, after a 2-3 h growth period in brain heart infusion broth at 36 ± 1 °C, the bacteria were washed three times in 10 mM sodium phosphate buffer (pH 7.4) and adjusted to 10<sup>4</sup>-10<sup>5</sup> colony forming units (cfu) per ml in the same buffer. Ten µl of

peptide solution (range of final concentration tested: 0.05-6.1  $\mu\text{M}$ ) were added to 100  $\mu\text{l}$  of the bacterial suspension and incubated at  $36 \pm 1$   $^{\circ}\text{C}$  for 2 h before colony forming units were determined. The peptide solvent (0.01% TFA) served as negative control.

### **Permeabilisation of bacterial membranes**

Bacteria with compromised membranes were detected by monitoring the fluorescence of the DNA-binding dye SYTOX Green (INVITROGEN, Molecular Probes). *Bac. megaterium* ATCC 14581 in mid-logarithmic phase was washed twice with and resuspended in 10 mM HEPES, pH 7.4 containing 25 mM NaCl. A flat bottom 96-well microtiter plate (SARSTEDT, Germany) was coated with 0.1% bovine serum albumin (A2153, SIGMA-ALDRICH, Deisenhofen, Germany) for 15 min prior to its use in the assay. Peptides were twofold serially diluted in 10 mM HEPES, 25 mM NaCl, pH 7.4. Bacteria ( $1 \times 10^5$  CFU/50  $\mu\text{l}$ ) were incubated with the diluted peptides (25  $\mu\text{l}$ ) and 2  $\mu\text{M}$  of the fluorescent dye SYTOX Green (25  $\mu\text{l}$ ; in 10 mM HEPES, 25 mM NaCl, pH 7.4) at 20  $^{\circ}\text{C}$  for 1 h. Permeabilisation of the bacterial cytoplasmic membrane allows the dye to cross this membrane and to intercalate with the DNA. When excited at 495 nm, the binding of the dye to DNA resulted in an increase of emitted fluorescence at 538 nm, which was measured in a microtiter plate reader (Fluoroskan II; Labsystems). Membrane-permeabilising activity of the peptides was expressed as percentage of permeabilised bacteria. For monitoring the activity at pH 5.2, 20 mM MES, 25 mM NaCl was used. As a control antimicrobial peptide, cecropin P1 (in HPLC-grade; SIGMA-ALDRICH) was used in parallel. For maximum permeabilisation of the bacteria (100% value), cells were incubated with 70% ethanol for 10 min. The values were expressed as the mean of two independent experiments, each performed in duplicates.

### **Assay for pore-forming activity**

Pore-forming activity of samples was determined by measuring fluorimetrically the dissipation of a valinomycin-induced membrane potential in liposomes prepared from crude soy bean phospholipids [40]. Fluorescence was measured by a fluorescence spectrophotometer (model LS 50B; PERKIN ELMER) using excitation and emission wavelengths of 620 nm and 670 nm, respectively. Pore-

forming activity was measured as the initial change in fluorescence intensity over time after adding the sample. As a control pore-forming peptide, alamethicin (in HPLC-grade; SIGMA-ALDRICH) was used in parallel.

### **Molecular modelling**

The three-dimensional model for the equine  $\alpha$ -defensin was generated using the X-ray structure of the human  $\alpha$ -defensin HNP-3 (PDB acc. code: 1dfn) as a template. According to the alignment, amino-acid residues were exchanged in the template structure. Insertions and deletions were modelled by using a database search approach included in the software package WHAT IF [41]. The database was searched for a peptide sequence of the appropriate length, which was fitted into the template. All loops were selected from the database so as to give a minimum root mean square distance between the ends of the loops and helices. Loops with unfavourable backbone dihedral angles or van der Waals clashes were excluded. Finally, these model structures were energy minimised, using the steepest descent algorithm implemented in the GROMOS force field [42]. The structural representation was performed with the RIBBON programme [43].

## **RESULTS**

### **Transcriptional analysis**

An amplified product could solely be verified in the small intestine of the horse (FIG. 1). This analysis failed to detect gene transcription in skin, teat, vagina, tongue, gingiva, esophagus, trachea, lung, large intestine, umbilical cord, uterus, meninges and blood. The amplified product of the horse small intestine was approximately 340 base pairs long. The specific transcription of the equine  $\alpha$ -defensin-like gene suggested the existence of an  $\alpha$ -defensin in the intestine of the horse.

### **Determination of the primary structure of the DEFA1 precursor**

Sequence overlays were determined by sequencing the amplified product from the gut with primers used for transcriptional analysis. It could not be excluded that more than one product has been

amplified. Therefore, subcloning was performed with amplified products generated by 3'-RACE to determine the complete coding sequence of the amplified product. According to the transcriptional analysis, cDNA of the small intestine was used for the PCR reaction. Positive clones were sequenced with forward and reverse primers used for 3'-RACE. Two distinct cDNA sequences, *DEFA5L* (already known from the BAC clone CHORI 241-245H5), and a new sequence, *DEFA1* (Acc. no. EF379126) were identified.

The cDNA sequence of the *DEFA1* gene showed a open reading frame of 297 nucleotides including start and stop codon (FIG. 2 A). In comparison with sequences available in public databases, the region of the putative propeptide and mature peptide was predicted. The putative coding region of both, the signal peptide and the propeptide is 192 nucleotides in length, the coding region of the mature peptide consists of 105 nucleotides. Alignment (ClustalW) of the *DEFA1* amino acid sequence with the equine *DEFA5L* sequence (CAJ01789) and sequences of the human *DEFA5* (EAW80489), *DEFA6* (AAC50382) and *HNP-3* (NP\_005208), chimpanzee *DEFA5* (NP\_0010112657), rhesus macaque  $\alpha$ -defensin 5 (AAW51369), rat *DEFA5* (P82106),  $\alpha$ -defensin 6 (AAT91348), and *NP-3* (Q62713) showed a relative conserved region of the signal peptide and a more divergent region of the mature peptide (FIG. 2 B). The highest identity was found to equine *DEFA5L* with 84% amino acid sequence identity and to the human and chimpanzee *DEFA5* as well as to the rat neutrophil peptide (*NP-3*) with 48% sequence identity to each. The rhesus macaque  $\alpha$ -defensin 5 showed an identity of 44% to *DEFA1*, the rat Paneth cell specific  $\alpha$ -defensins *DEFA5* and defensin 6 an identity of 39%, human *DEFA6* of 37%, whereas the lowest identity was found to human *HNP-3* (35%). The six cysteine residues (Cys<sub>2</sub>, Cys<sub>4</sub>, Cys<sub>10</sub>, Cys<sub>20</sub>, Cys<sub>31</sub> and Cys<sub>32</sub>) are completely conserved in the peptides. Equally, the arginine and glutamine residues (Arg<sub>5</sub> and Glu<sub>13</sub>), necessary for the intramolecular salt bridge, are conserved as well as the glycine residue at position 17, essential for correct folding. The 34 amino-acid residues long putative mature peptide starts with a serine residue followed by the first cysteine. Including the signal peptide and propeptide the complete primary structure of the *DEFA1* precursor consists of 98 amino-acid residues.

### Recombinant expression and purification of DEFA1

Expression of recombinant DEFA1 was performed by cloning the cDNA sequence of the mature  $\alpha$ -defensin peptide (FIG. 2 A) into the pET30-Xa/LIC vector and transfecting *E. coli* BL21 (DE3) with the vector. The fusion peptide was exclusively synthesised in inclusion bodies and therefore was only detected in the pellet of cells after cell lysis and centrifugation at 30,000  $\times g$  (FIG. 3 A). Analysis of bacterial extracts by SDS-PAGE at different time points revealed a peptide synthesis in inclusion bodies already during the first 30 min after induction of gene expression. A further increase of peptide concentration after induction was observed after an expression time of up to four hours (FIG. 3 B). The recombinant product, confirmed by SDS-PAGE, had a molecular mass of approximately 10 kDa closely to the theoretical mass of 9.046 kDa. After nickel-nitrilotriacetic acid metal-affinity chromatography, the cleavage of the product with Factor Xa resulted in a peptide of approximately 5 kDa, determined by Tricine-PAGE (FIG. 3 C). This result is in good agreement with the expected mass of 4.071 kDa.

The recombinant DEFA1 was purified to homogeneity by performing RP-HPLC. Three conspicuous peaks appeared at elution times of 13.4, 14.0, and 15.7 min (FIG. 4 A). The fractions were analysed by MALDI-TOF mass spectrometry. The fraction F1 showed an unknown peptide mixture with peptide masses of approximately 4090, 5003 and 5035 Da. Fractions F2 and F3 contained peptides with the mass of 4071.8 Da (FIG. 4 B). Notably, for the mature DEFA1 peptide an average mass of 4071.8 Da was calculated, provided that the six cysteine residues are involved in disulfid bonds. Whereas in fraction F2 the His<sub>6</sub>-tag (4.987 kDa) was eluted in addition to the mature peptide in fraction F3 homogeneous material representing the DEFA1 peptide was found. The accurate monoisotopic mass of the peptide was also determined (4068.9 Da) and confirmed the molecular identity of DEFA1. F3 eluates were used for further studies. The eluates were lyophilised and the purified peptide was resuspended in 0.01% TFA for further investigations. Finally, MALDI-TOF mass spectrometry was performed to proof the purity of the DEFA1 peptide (data not shown).

### Structural characterisation

The recombinantly expressed DEFA1 was structurally characterised by CD spectroscopy. The CD spectrum (in the range of 190 nm and 250 nm) is shown in FIG. 5 and displays a minimum at 203 nm. The shape of the spectrum is indicative for a  $\beta$ -sheet structure with a high content of P<sub>2</sub> elements [44]. This is in agreement with the known  $\alpha$ -defensin structures.

### Antimicrobial activity of DEFA1

To assess the antimicrobial activity of DEFA1, a radial diffusion assay was performed at first. As shown in FIG. 6, treatment of microorganisms with the recombinant equine  $\alpha$ -defensin resulted in a visible inhibition of the growth (zones of clearing) against all of the target organisms with the exception of *B. cepacia* (data not shown). The relative potential of the peptide varied depending on the microorganisms tested. Whereas DEFA1 was only lightly active against *P. aeruginosa* ATCC 10145, potent activity was found against *Bac. megaterium*, all *E. coli*-strains, *Staph. epidermidis* and *Staph. aureus*-strains. Against seven test strains (all *E. coli*-strains, *Staph. epidermidis* and *Candida albicans*), peptide concentrations as low as 4.7  $\mu$ M led to growth inhibition zones. The *Staph. aureus* strains were inhibited at concentrations of  $\geq 9.3 \mu$ M, *Candida albicans* was susceptible at  $\geq 18.7 \mu$ M. Additionally, the minimal concentration of the equine DEFA1 that killed 90% (LD<sub>90</sub>) and 99.9% (MBC) of 17 target microorganisms is shown in TABLE 1. All organisms were tested with various defensin concentrations (0.05 to 6.1  $\mu$ M). This range was sufficient to determine the lethal peptide dose (LDs) of the target organisms. DEFA1 displays a broad spectrum of antimicrobial activity against fermenting and non-fermenting gram-negative strains, against gram-positive cocci (genera *Staphylococcus*, *Streptococcus*, and *Enterococcus*), spore-forming *Bac. megaterium*, and the yeast *C. albicans*. *Bac. megaterium* was more susceptible to DEFA1 than any of the other strains tested. In general, *E. coli* strains were more susceptible to the peptide than other fermenting gram-negatives such as *K. pneumoniae* and *Eb. cloacae*, which are weakly susceptible to the peptide. Non-fermenting gram-negative bacteria such as *P. aeruginosa* and *B. cepacia* were only weakly susceptible or showed no sensitivity to DEFA1. The gram-positive cocci such as *Staph. aureus*, *Staph. epidermidis*, *Ec. faecalis*, and *Strep. pyogenes* exhibited a high sensitivity to DEFA1 at reliable peptide concentrations.

The yeast *Candida albicans* showed a MBC value of 0.8  $\mu\text{M}$ , *B. cepacia* was not killed up to peptide concentrations of 6.1  $\mu\text{M}$ . These results correspond with the results from the radial diffusion assay.

### Characterisation of the peptide's mode of action

To investigate the mode of action of DEFA1, we used *Bac. megaterium* and the fluorescent dye SYTOX Green. As soon as the dye can enter the bacterial cell it intercalates with the DNA and its fluorescence will be enhanced. This assay confirmed that DEFA1 is potentially active against these Gram-positives (FIG. 7 A). Notably, it demonstrated that the peptide rapidly permeabilises bacterial membranes. As DEFA1 contains a histidine residue in its primary structure, we evaluated the effect of a more acidic pH at which the histidine residue is protonated on the membrane-permeabilising activity. The efficacy of the horse defensin to induce membrane lesions in viable bacteria was found to be very similar at pH 5.2 and pH 7.4 and comparable to that of the well-known antimicrobial peptide cecropin P1 (FIG. 7 B).

We also measured the pore-forming activity of DEFA1 to further characterise its mode of action by using a minimalistic membrane system. More precisely, we monitored the dissipation of a membrane potential induced in liposomes composed of azolectin, a crude phospholipid mixture from soy bean. It became evident that the equine defensin displayed pore-forming activity (FIG. 8), although only moderately when compared to alamethicin, the prototype of a pore-forming peptide acting according to the barrel-stave model [45].

## DISCUSSION

The existence of an  $\alpha$ -defensin peptide in the small intestine of the horse was indicated by transcriptional analysis, and the obtained amino-acid sequence aligned to other Paneth cell specific  $\alpha$ -defensin sequences. Interestingly, an  $\alpha$ -defensin expression in other species next to primates and rodents was not reported to date. The horse is now added to the group of animals expressing  $\alpha$ -defensins, and this may lead to a new perception in the evolution of defensins. A possible ancestral gene for DEFA5 may already have been present before euarchontoglires (including glires and

primates) and Laurasiatheria (including Perissodactyla amongst others) evolved to two different subgroups. It is unlikely that the identified equine DEFA1 has developed independently as indicated by the high identity of the amino acid sequences between the equine DEFA1 and other known  $\alpha$ -defensins (FIG. 2 B). This is supported by the finding of genomic  $\alpha$ -defensin like sequences in basal mammals [46].

DEFA1 revealed the typical conservation of a signal- and negatively charged prosegment, and the  $\alpha$ -defensin characteristic conserved amino-acid residues, including the six cysteines, Arg<sub>5</sub>, Glu<sub>13</sub> and Gly<sub>17</sub>. Most  $\alpha$ -defensins contain no or only one charged or polar uncharged amino-acid residue following the last two cysteines. C-terminal tails, rich in charged or polar uncharged amino-acid residues are predicted to be exposed to the surface to influence the overall charge and amphipathicity of the peptide. The equine defensins DEFA1 and DEFA5L have two C-terminal Arg residues (FIG. 2 B). An extended C-terminus is also shown on rhesus macaque  $\alpha$ -defensin 5. In previous studies, a general relationship between higher defensin cationicity and a greater microbicidal potency was noted [5]. It was supposed that the net charge covers a different antimicrobial spectrum and efficiency of these peptides and is strongly correlated with antimicrobial activity of the defensins [47]. Equine DEFA1 is less cationic (+ 2) than human DEFA5 (+ 5), and Paneth cell  $\alpha$ -defensins from rhesus macaque small intestine (+ 4.5 to + 7.5), but equine DEFA1 showed at least similar antimicrobial activities. Accordingly, the relative cationicity did not appear to be a major factor in the killing of bacteria. This result is consistent with those of previous studies demonstrating that the overall microbicidal activity of  $\alpha$ -defensins are not merely a function of their net charge [48], but may influence a strain specificity.

A three-dimensional model of the DEFA1 structure is shown in FIG. 9. The model displays a small  $\beta$ -sheet consisting of three  $\beta$ -strands flanked by large unstructured regions. The long loop connecting the first and the second  $\beta$ -strand contains several cationic amino-acid residues. Together with the two arginines located at the C-terminus, this loop forms an epitope with a high positive charge density.

Alignment of DEFA1 and DEFA5L with other amino-acid sequences revealed a homology to human DEFA5. The antimicrobial properties of human DEFA5 have been investigated comprehensively [24]. In summary, the authors established that human DEFA5 efficiently kills gram-negative and gram-



positive bacteria at comparable peptide concentrations and showed a consistently high activity against a broad spectrum of microorganisms including fungi. Human DEFA5 was potent in almost the same manner as the neutrophil peptides HNP 1-4 against gram-positive *Staph. aureus*, but displayed a conspicuous higher activity against *E. coli*. By comparing the activities of equine DEFA1 against *E. coli*, *Staph. aureus* and *Staph. epidermidis* we also determined a higher activity against *E. coli*. Other strains of gram-negative bacteria such as *Eb. cloacae*, *K. pneumoniae*, *P. aeruginosa* and *B. cepacia* are weakly sensitive. Similarly, gram-positive cocci such *Staph. aureus*, *Staph. epidermidis*, *Strep. pyogenes*, and *Ec. faecalis* are highly sensitive to DEFA1 at reliable peptide concentrations. Collectively, despite the broad antimicrobial spectrum of DEFA1, the most sensitive targets of this peptide are gram-positive bacteria. It appears that a preference for particular bacteria applies for both, human DEFA5 and equine DEFA1.

The mode of action of Paneth cell  $\alpha$ -defensins was not studied until date. For antimicrobial peptides in general a two-step mechanism is proposed to explain their killing effect on microorganisms. Most cationic peptides bind to the target cell surface due to their content of negatively charged phospholipids or other moieties. Many of them subsequently disturb the membrane integrity [2]. Likewise, killing of bacteria by the equine defensin was accompanied with permeabilisation of the bacterial cytoplasmic membranes similar to the action of other antimicrobial peptides such as cecropin P1. Moreover, in a minimalistic system using the depolarisation of liposomes as a measure of pore-forming activity it became evident that DEFA1 is moderately active in creating pores in artificial membranes, a property described previously for human alpha-defensins [49].

In recent studies, a specific deficiency of human DEFA5 was observed in patients with ileal Crohn's disease, a chronic disease of the intestine characterised by inflammation of the gut. The horse shows similar disease patterns, for example anterior enteritis (duodenitis) or colitis X, catarrhal-haemorrhagic inflammations of the gut. It will be interesting to examine a possible relationship of these infectious diseases with a possible deficiency of equine Paneth cell  $\alpha$ -defensins.

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## FIGURE LEGENDS

### FIG. 1. Transcriptional analysis of *DEFA1* in various equine tissues.

*DEFA1* is solely transcribed in the small intestine (lane 9, bottom). The product size is approximately 340 bp in length.  $\beta$ -actin was used as a positive control (top), water instead of cDNA template as a negative control (lane 15). *bp*, base pair

### FIG. 2. *DEFA1* nucleotide sequence and the amino-acid sequence and alignment of the *DEFA1* primary structure with those of other $\alpha$ -defensins.

**A.** *DEFA1* cDNA sequence is depicted in small type letters, amino acid sequence in capitals. The sequence (nucleotides and amino acids) of the mature peptide is depicted in bold characters the start codon is underlined.

**B.** Comparison of the *DEFA1* amino acid sequence with those of equine *DEFA5L*, human  $\alpha$ -defensins *DEFA5*, *DEFA6*, and *HNP-3*, chimpanzee *DEFA5*, rat *DEFA5*, *NP-3* and defensin 6, and rhesus macaque  $\alpha$ -defensin 5. The boxes indicate the highly conserved residues among the primary translation products of equine defensin and of the other  $\alpha$ -defensin genes. The connectivity of the cysteines typically present in  $\alpha$ -defensins is indicated by lines. The length of the peptides is denoted at the C terminus. *ECA*, *Equus caballus*; *RNO*, *Rattus norvegicus*; *HSA*, *Homo sapiens*; *PTR*, *Pan troglodytes*; *MMU*, *Macaca mulatta*

### FIG. 3. Recombinant expression of *DEFA1*.

**A.** SDS-PAGE of the cell pellet prior to (lane 2) and after induction (lane 3), and of the supernatant of unstimulated (lane 4) and stimulated cells (lane 5). Lane 1 shows the marker. The His<sub>6</sub>-tagged fusion peptide was only detected in the pellet of stimulated cells and shows a mass of approximately 10 kDa.

**B.** Time dependent analysis of peptide expression of the supernatant (top) and the pellet (below) after 0.5 h (lane 2), 1 h (lane 3), 1.5 (lane 4), 2 h (lane 5), 2.5 h (lane 6), 3 h (lane 7), 3.5 h (lane 8), and 4 h (lane 9). The marker is shown in lane 1.



C. Digestion of the recombinant peptide with Factor Xa. Lane 1 shows the marker, lane 2 the uncleaved peptide with a mass of approximately 10 kDa and lane 3 represents the cleaved product. The mature peptide reveals a molecular mass of approximately 5 kDa. *kDa*, kilodalton; *SN*, supernatant

**FIG. 4. RP-HPLC purification of DEFA1 and MALDI-TOF mass spectrometry of the eluted fractions.**

A. The chromatogramme on the left shows three conspicuous peaks appearing at elution times of 13.4, 14.0 and 15.7 min. Eluted fractions were termed F1 to F3 and analysed by mass spectrometry. An RP-HPLC analysis of the undigested peptide is shown on the right, the eluted fraction was termed F0.

B. MALDI-TOF mass spectrometry of the fractions F0 to F3. Fraction F0 shows a mass peak of 9046.1 Dalton of the uncleaved product. Fraction F1 shows an unknown peptide mixture with peptide masses of approximately 4090, 5003 and 5035 Dalton. Fractions F2 and F3 contain peptides of the calculated average mass of DEFA1 (4071.8 Dalton), whereas in fraction F2 the His<sub>6</sub>-tag (4987.5 Dalton) was eluted in addition to the mature peptide. In fraction F3 a homogenous peptide without any contaminants was detected.

**FIG. 5. CD spectrum of DEFA1.**

The spectrum was recorded between 190 nm and 250 nm in 0.01% TFA displaying a minimum at 203 nm.

**FIG. 6. Antimicrobial activities of DEFA1 in the radial diffusion assay.**

The diagram shows the peptide concentration (abscissa) plotted against the diameter of microbial growth inhibition zone (ordinate) after incubation for 12 h.

A. Growth inhibition of various *E. coli* strains.

**B.** Growth inhibition of *Staph. aureus*, *Staph. epidermidis*, *P. aeruginosa*, *Bac. megaterium*, and *C. albicans*. The arrangement of measuring points are a result of adjusted peptide concentrations of 4.7, 9.3, 18.7, and 37.3  $\mu\text{M}$ . ATCC, American type culture collection

**FIG. 7. Membrane permeabilisation of *Bac. megaterium* induced by DEFA1.**

Membrane damage of the bacteria was measured fluorometrically using the dye SYTOX Green. The binding of the dye to the DNA in membrane-compromised target cells resulted in an increase of fluorescence. Antibacterial activity of the peptides is expressed as a percentage of permeabilised bacteria. (A) Time kinetics of membrane permeabilisation induced by DEFA1 measured for different doses at various incubation periods. (B) Comparison of the membrane-permeabilising effects of DEFA1 (rhombs) and cecropin P1 (squares) after 30 min of incubation of *B. megaterium* with each peptide at various concentrations, at pH 5.2 (closed symbols) and at pH 7.4 (open symbols).

**FIG. 8. Time course of pore formation induced by DEFA1.**

The dissipation of a valinomycin-induced diffusion potential in vesicles of soy bean phospholipids after addition of DEFA1 (1.4 nmol; arrow) (1), control peptide alamethicin (0.1 nmol; trace 2), and the peptide solvent (trace 3) were recorded. Pore-forming activity is reflected by the increase of fluorescence as a function of time.

**FIG. 9. Three-dimensional model of DEFA1.**

$\beta$ -sheet structures are displayed as arrows, the N-terminus is marked with N, the C-terminus with C. Disulfide bonds are depicted in yellow.

**TABLE 1. LD<sub>90</sub> and MBC values of DEFA1 against different microorganisms.**

LD<sub>90</sub> and MBC (minimal bactericidal concentration) values are defined as lethal peptide dose in  $\mu\text{M}$  that killed 90% (LD<sub>90</sub>) or 99.9% (MBC) of the target organisms. The highest peptide concentration tested was 6.1  $\mu\text{M}$ . >, no killing found at the indicated concentration

Test organism	Gram stain reaction	LD <sub>90</sub> [ $\mu\text{M}$ ] of DEFA1	MBC [ $\mu\text{M}$ ] of DEFA1
<i>E. coli</i> ATCC 11775	-	0.2	0.4
<i>E. coli</i> ATCC 25922	-	0.2	0.4
<i>E. coli</i> ATCC 35218	-	0.05	0.4
<i>E. coli</i> D31	-	0.4	0.8
<i>K. pneumoniae</i> ATCC 13883	-	3	>6.1
<i>Eb. cloacae</i> ATCC 13047	-	6.1	>6.1
<i>P. aeruginosa</i> ATCC 10145	-	3	>6.1
<i>P. aeruginosa</i> ATCC 11440	-	1.5	3
<i>B. cepacia</i> ATCC 25416	-	>6.1	>6.1
<i>Staph. aureus</i> ATCC 6538	+	0.4	0.8
<i>Staph. aureus</i> ATCC 12600	+	0.4	1.5
<i>Staph. epidermidis</i> ATCC 14990	+	0.8	1.5
<i>Ec. faecalis</i> ATCC 29212	+	0.2	6.1
<i>Ec. faecalis</i> PEG 205 (wild type)	+	0.4	3
<i>Strep. pyogenes</i> ATCC 12344	+	0.8	0.8
<i>Bac. megaterium</i> ATCC 14581	+	0.05	0.2
<i>C. albicans</i> ATCC 24433		0.8	0.8

FIG. 1

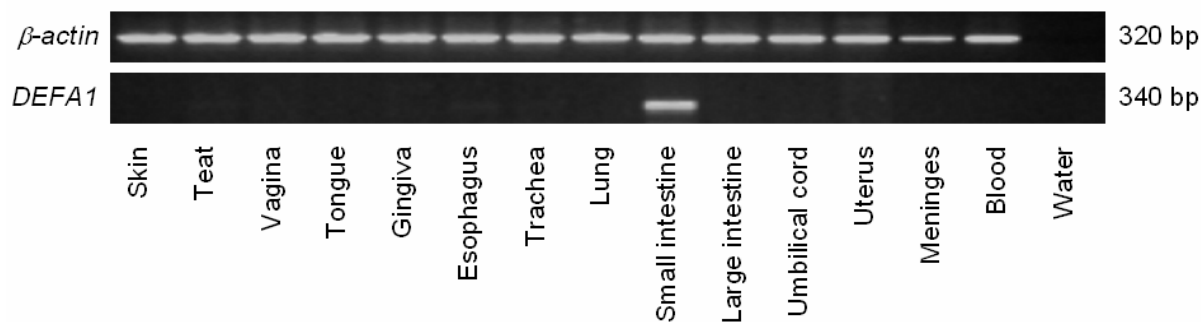


FIG. 2

A

```

atgaggaccctcacccctcctcactgcacttctcctcttggccctccagggtccagactcag
M R T L T L L T A L L L L A L Q V Q T Q
agtcttgaagagacagccgaccagggttcctgcccaggaccagcctggggccgaggcccag
S L E E T A D Q V P A Q D Q P G A E A Q
gacataaaccatctcctttgcaggggatgaacgctctgctcgagaggcttcaaaatccctt
D I T I S F A G D E R S A R E A S K S L
ataggaacagcctcctgcacctgcagacgtgcctggatctgcagatgggggtgagcggcac
I G T A S C T C R R A W I C R W G E R H
tctgggaagtgtatagaccaaaaaggctccacatacaggetctgctgtcgtcgtga
S G K C I D Q K G S T Y R L C C R R .
    
```

B

ECA_DEF1	MRTLTLL	L	TALLLL	AL	QVQTQSLEETAD	-----	QVPAQ-D	QPGA	EAQD	ITISFAG	DE	RS
ECA_DEF15L	MRTLTLL	L	LAALLRL	AL	QVQTQSLEETAD	-----	QVPAQ-D	QPGA	EAQD	MTISFAG	DE	RS
RNO_NP-3	MRTLTLL	L	TTLLLL	AL	LHTQAES	PQGSTK	-----	EAPD	-----	EEQDI	SVFFGG	DKGTA
HSA_DEF15	MRTIAIL	L	AAIILLV	AL	QAQAESL	QERAD	-----	EATTQ	-KQSG	EDNQD	LAISFAG	NGLSA
PTR_DEF15	MRTIAIL	L	AAIILLV	AL	QAQAESL	QERAD	-----	EATTQ	-KQSG	EDNQD	LAISFAG	NGLSA
MMU_alpha-defensin5	MRTLTIL	L	AAIILLV	AL	QAQAESL	QERAD	-----	EATTQ	-EQPA	EDNQD	FAVSFE	ENGLST
HSA_DEF16	MRTLTIL	L	TAVLLV	AL	QAQAESL	QERAD	-----	EATTQ	-EQPA	EDNQD	FAVSFE	ENGLST
RNO_DEF15	MKKLVLL	L	SALVLL	AL	QVEAEP	TPKTDE	-----	GTKTD	-EQPG	KEDQV	VS	IEGQ
RNO_defensin6	MKKLVLL	L	SALVLL	AL	QVQAEP	TPKTDE	-----	GTKTD	-EQPG	KEDQV	VS	IEGQ
HSA_HNP-3	MRTLAIL	L	AAIILLV	AL	QAQAEP	LQARAD	-----	EVAAA	PEQIA	ADIPE	VVVS	LAWDE

ECA_DEF1	REASKSLIGTAS	C	T	C	R	R	A	W	I	C	R	W	G	E	R	H	I	D	Q	K	G	S	T	Y	R	L	C	C	R	R	98							
ECA_DEF15L	REASKRPI	-	-	V	C	T	C	R	R	P	W	S	C	R	L	G	E	R	H	S	G	T	C	R	D	G	Q	G	L	I	Y	R	L	C	C	R	R	95
RNO_NP-3	LQDAAVKAG-VT	C	S	C	R	-	T	S	S	C	R	F	G	E	R	L	S	G	V	A	C	R	-	L	N	G	R	I	Y	R	L	C	C	R	87			
HSA_DEF15	LRTSGSQAR-AT	C	Y	C	R	-	T	G	R	C	A	T	R	E	S	L	S	G	V	C	E	-	I	S	G	R	L	Y	R	L	C	C	R	94				
PTR_DEF15	LRTSGSQAR-AT	C	Y	C	R	-	I	G	H	C	T	I	L	E	S	L	S	G	V	C	E	-	I	S	G	R	L	Y	R	L	C	C	R	94				
MMU_alpha-defensin5	LKASGSQAR-MIC	L	C	R	-	I	G	R	C	S	W	R	E	A	H	F	G	S	C	T	-	K	M	G	Q	F	A	K	I	C	C	R	R	A	S	97		
HSA_DEF16	LRALGGSTRAFT	C	H	C	R	-	R	-	S	C	Y	S	T	E	Y	S	Y	G	T	C	T	-	V	M	G	I	N	H	R	F	C	C	L	101				
RNO_DEF15	FQD--AVLRDLK	C	F	C	R	-	R	K	S	C	N	W	G	E	G	I	M	G	I	C	K	K	R	Y	G	S	P	I	-	L	C	C	R	93				
RNO_defensin6	FQD--AVLRDLK	C	F	C	R	-	A	K	S	C	N	W	G	E	G	I	M	G	I	C	N	K	R	Y	G	S	L	I	-	L	C	C	R	93				
HSA_HNP-3	PKHPG-SRKNMDC	Y	C	R	-	I	P	A	C	I	A	G	E	R	R	Y	G	T	C	I	-	Y	Q	G	R	L	W	A	F	C	C	94						

FIG. 3

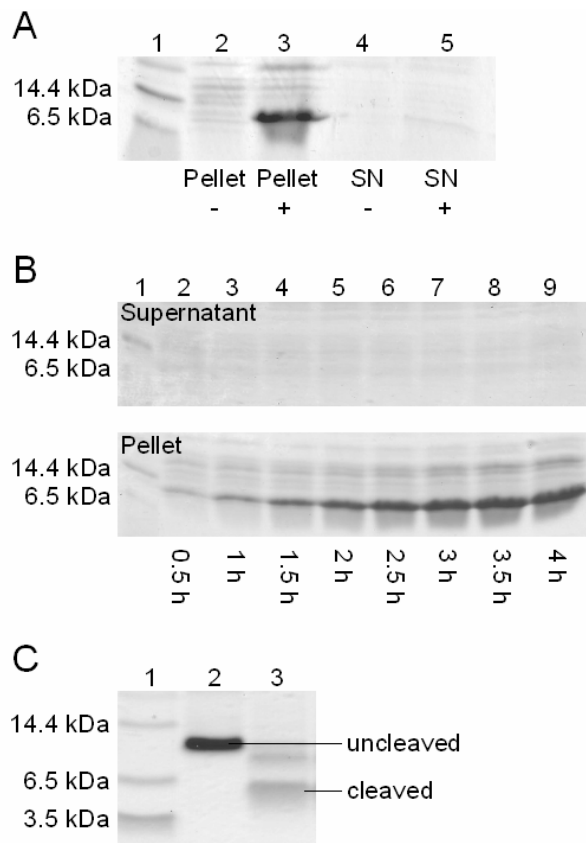


FIG. 4

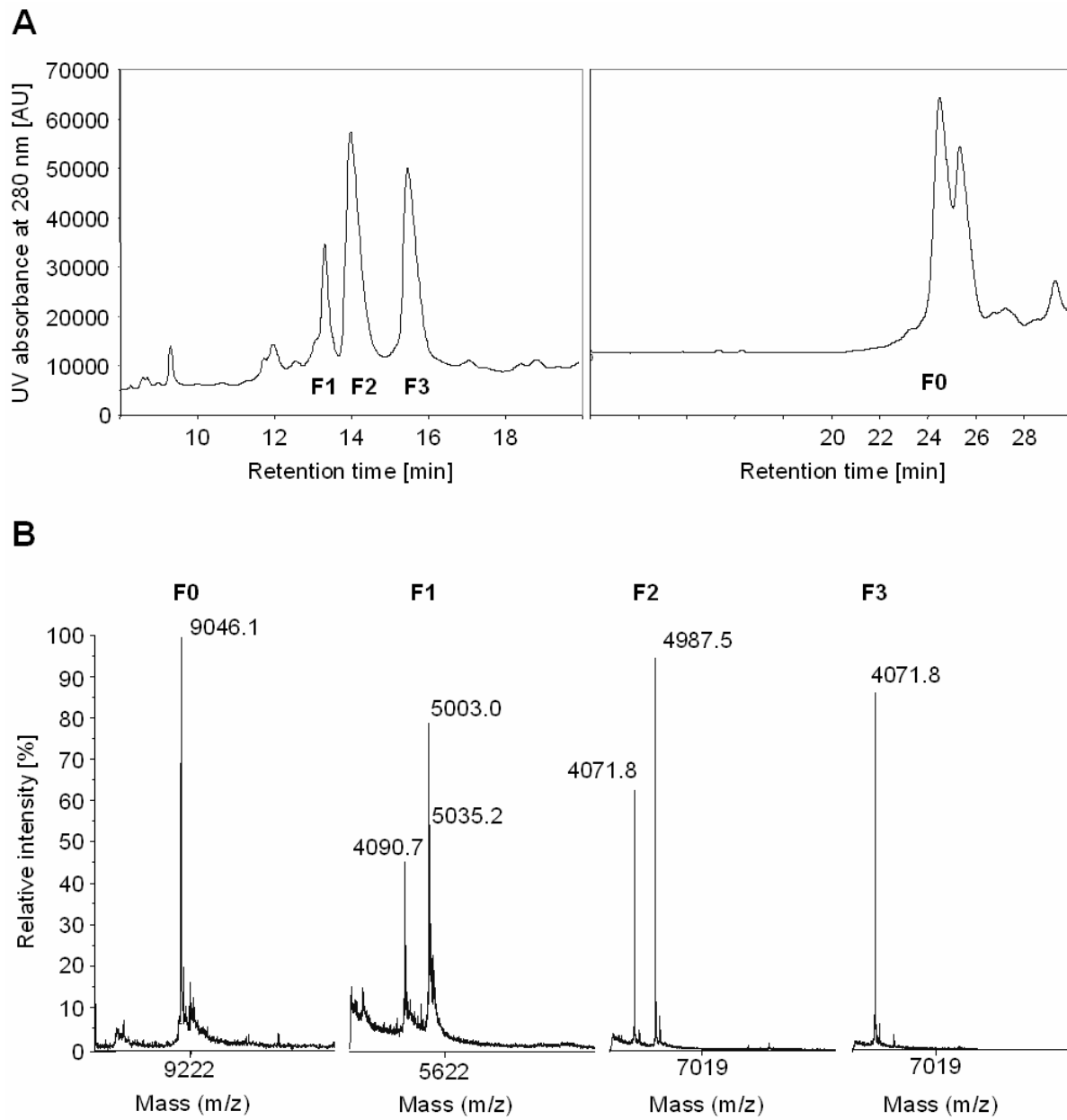


FIG. 5

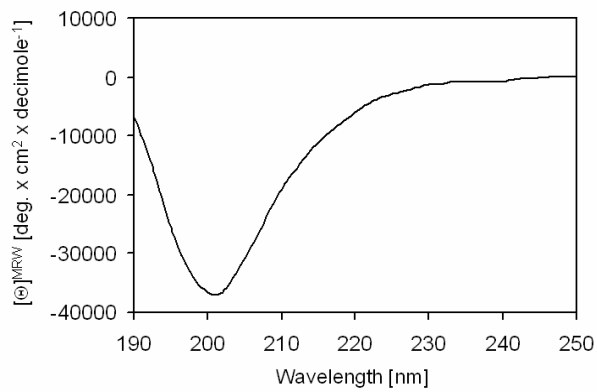


FIG. 6

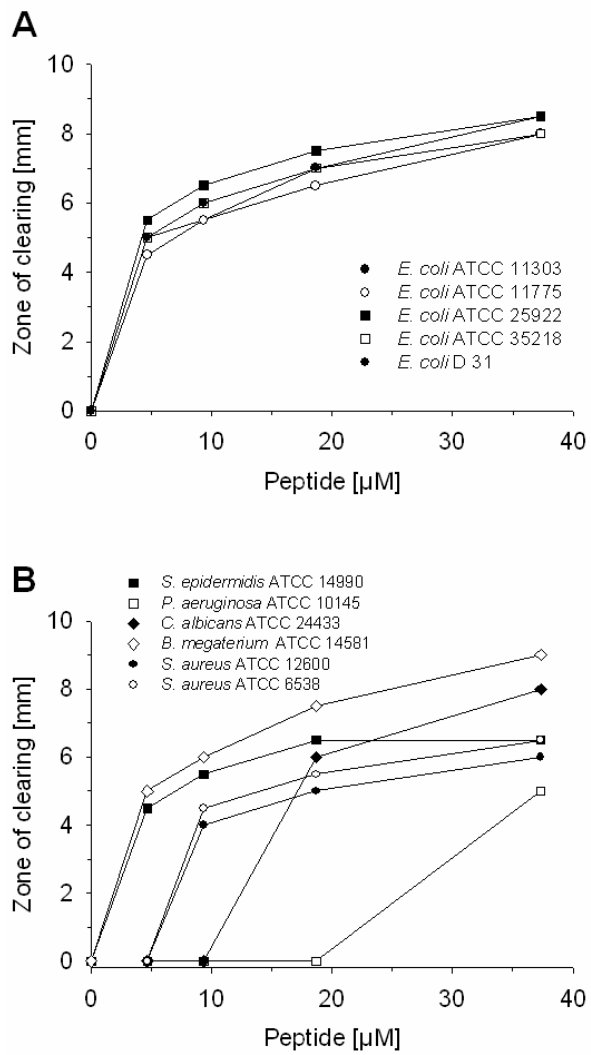


FIG. 7

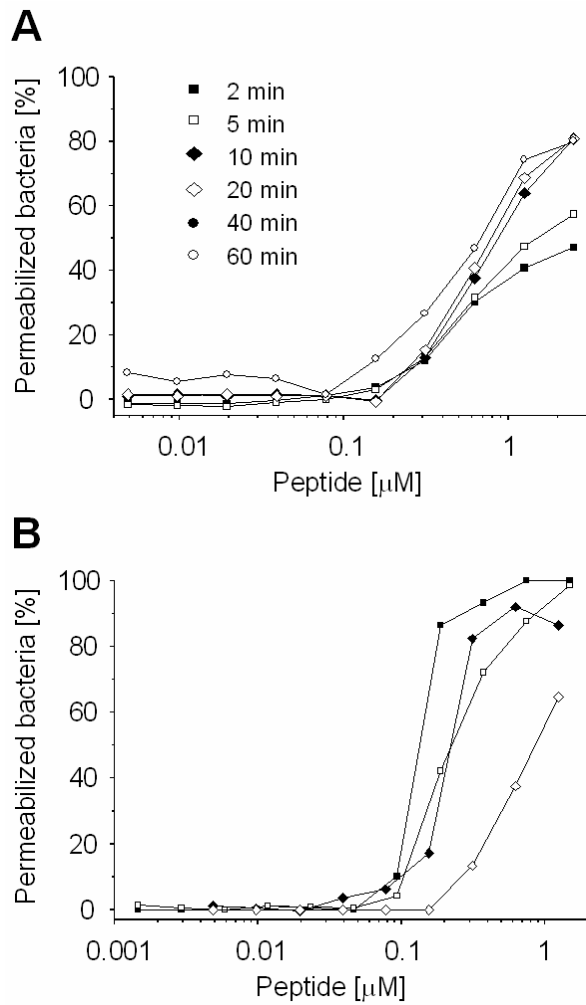


FIG. 8

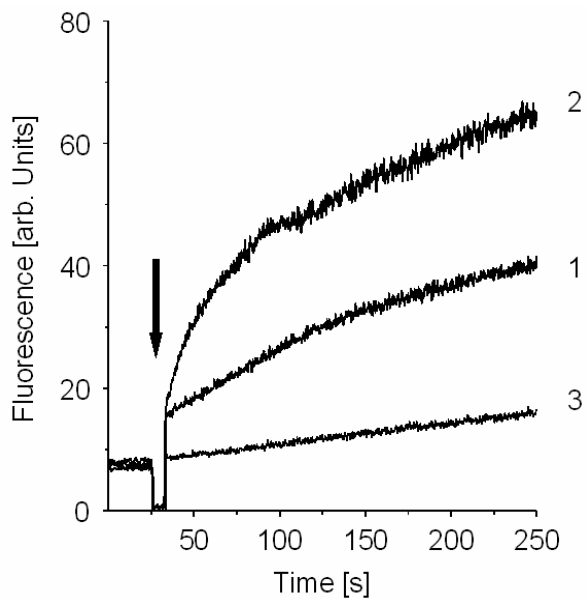




FIG. 9

