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11 **Antimicrobial Activity of Bovine Psoriasin**

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26

27 **Abstract**

28 Human psoriasin (S100A7) has originally been described as a member of the family of S100
29 calcium-binding proteins which is overexpressed in patients suffering from psoriasis. The
30 bovine homolog was first identified as a cow-derived respiratory allergen. As *Escherichia*
31 *coli*-mastitis is a common problem in dairy cattle, and human psoriasin was found to exhibit
32 antimicrobial activity preferentially against *E. coli*, we examined whether the bovine mRNA
33 is expressed in the mammary gland.

34 To demonstrate the antimicrobial activity of bovine psoriasin, we isolated cDNA from the
35 udder, cloned the bovine psoriasin gene in a bacterial expression vector, and the recombinant
36 protein was expressed in BL21 cells. The *in vitro* antibacterial activity was tested by
37 performing microdilution susceptibility tests and radial diffusion assays with eight different
38 bacterial strains, thereof three different *E. coli* strains, and one yeast.

39 The antimicrobial activity of the recombinant bovine psoriasin is comparable with human
40 psoriasin and also limited to *E. coli*.

41 Psoriasin appears to be a part of the local host defense mechanism in the udder, is a putative
42 candidate for a cow-specific factor influencing mastitis susceptibility, and a possible
43 alternative to conventional antibiotics.

44 Keywords: antimicrobial peptides, dairy cows, *E. coli* mastitis, psoriasin

45

46 **Introduction**

47 Psoriasin (S100A7), a member of the S100 family of calcium-binding proteins, is abundantly
48 synthesised in keratinocytes of patients suffering from psoriasis (Madsen et al. 1999).
49 Bacterial infections of the skin are uncommon in patients with psoriasis in contrast to patients
50 with atopic dermatitis (Madsen et al. 1999). Gläser et al. (2005) have recently demonstrated
51 that psoriasin exhibits antibacterial activity and protects human skin from *E. coli* infections.
52 The expression of the psoriasin gene is not limited to psoriatic skin, it is also expressed in

53 other epithelial cells like carcinomatous tissues from skin bladder (Celis et al. 1996), stomach
54 (El-Rifai et al. 2002), skin (Alowami et al. 2003), and breast in humans (Moog-Lutz et al.
55 1995, Leygue et al. 1996, Al-Haddad et al. 1999, Enerbäck et al. 2002, Emberly et al. 2004).

56 From a cDNA library of bovine skin, Rautiainen et al. (1995) isolated a clone, named
57 BDA11, coding for an allergen with a predicted 63.4% amino-acid sequence identity to
58 human psoriasin. Hitomi et al. (1996) characterised a calcium-binding protein (CAAF2)
59 abundant in bovine amniotic fluid with the same sequence identity to human psoriasin (Porre
60 et al. 2005).

61 The exact physiological function of psoriasin remains unclear. Because of its antibacterial
62 properties, psoriasin is considered to be part of the innate immune defense against *E. coli*
63 infections (Gläser et al. 2005). Mastitis caused by *E. coli* is a common problem in dairy cattle,
64 and the antimicrobial susceptibility of *E. coli* isolates from clinical bovine mastitis against
65 antibiotics and chemotherapeutics decreases (Srinivasan et al. 2007). We have isolated
66 psoriasin cDNA from the mammary gland, and the recombinant bovine psoriasin shows
67 antibacterial activity against *E. coli*.

68 Bovine psoriasin as an evolutionary ancient component of host defense is an interesting target
69 for immunomodulation of the innate immune response in the udder and a possible alternative
70 to conventional antibiotics.

71

72 **Materials and Methods**

73 **cDNA cloning**

74 RNA was isolated from bovine udder tissue with the RNeasy Midi Kit (Qiagen, Hilden,
75 Germany) according to the manufacturer's instructions. To isolate mRNA, a biotinylated
76 oligo(dT)primer was bound to avidin-coated polystyrene beads. A 5'RACE (rapid
77 amplification of cDNA ends) was performed as described by Schramm et al. (2000). Briefly,
78 first strand cDNA synthesis was performed with mRNA left on the beads using the bound

79 oligo(dT)oligonucleotide as a primer. The reaction conditions were as follows: 50 mM Tris-
80 HCl pH 8.3, 6 mM MgCl₂, 2 mM MnCl₂, 1 mM dNTPs, 0.01 M dithiothreitol, 0.2 mg/ml
81 BSA, 10 pmol cap finder primer (5'-GAGAGAACGCGTGACGAGAGACTGACAGGG-3'),
82 200 U Superscript II (Life Technologies, Karlsruhe, Germany) in a total volume of 20 µl for
83 1 h at 45 °C. After completion of the reaction and washing the cDNA bound to the beads, this
84 preparation was used for 5' RACE and 3' RACE PCR reaction. The sequence of the forward
85 primer for the 5'RACE was 5'-GAGAGAACGCGTGACGAGAGACTGACAG-3', the
86 sequence of the reverse primer of bovine psoriasis was 5'-
87 TGGTAGTCTGTGGCTATGTCC-3'. For the 3' RACE a forward primer with the sequence
88 5'-AAGCCAAGATGAGCGAAA-3' and a reverse primer with the sequence 5'-
89 ACTCTATGAGAATTCGATGAGCGATCTG-3' were used. The reaction conditions were as
90 follows: 100 mM Tris-HCl pH 8.3, 500 mM KCl, 15 mM MgCl₂, 200 µM dNTPs, 2.5 U *Taq*-
91 polymerase (Eppendorf, Hamburg, Germany), 200 nM of each primer in a total volume of
92 15 µl. Amplification was carried out at 94 °C for 3 min, followed by 35 cycles of 94 °C for
93 1 min, 55 °C for 50 s, 72 °C for 1 min, and a 72 °C 10 min final extension. PCR products
94 were analysed by agarose gel electrophoresis and verified by sequencing.

95 **Construction of a psoriasis expression vector**

96 The psoriasis encoding region was amplified by PCR using bovine psoriasis cDNA as
97 template. *Nde*I and *Xho*I sites were introduced in the 5'- and 3'-primers, respectively, to
98 enable cloning of the amplified DNA in the *Nde*I and *Xho*I sites of the procaryotic expression
99 vector pET22b (Novagen, Darmstadt, Germany). The sequence of the forward primers was:
100 5'-GGAATTCCATATGAGCAGCTCTCAGCTTG-3', of the reverse primer:
101 5'-CCGCTCGAGTCACTGATTTCCCCAGAAC-3'. PCR was performed in a total volume
102 of 50 µl of PCR buffer (10 mM KCl, 20 mM Tris HCl pH 8.8, 10 mM (NH₄)₂SO₄, 2 mM
103 Mg₂SO₄, 0.1 % Triton X-100, 0.2 mM of each dNTP) containing 0.25 ng of template DNA,
104 0.5 U of Vent DNA-polymerase (New England Biolabs, Frankfurt a.M., Germany), and 1 µM

105 forward and reverse primer pairs. Amplification of psoriasin cDNA was performed with 35
106 cycles of denaturation (94 °C, 45 s), primer annealing (54 °C, 45 s), and elongation (72 °C,
107 45 s). PCR products were purified, subsequently digested with *NdeI* and *XhoI*, and ligated
108 into the pET22b opened using these enzymes. The psoriasin construct was verified by
109 restriction analysis and DNA sequencing.

110 **Expression and purification of recombinant bovine psoriasin**

111 Bovine psoriasin was expressed in *E. coli* (BL21). Isopropyl-1-thio- β -D-galactopyranoside
112 (IPTG)-induced log phase bacterial cultures containing the recombinant protein were
113 collected by centrifugation, resuspended in 20 mM sodium acetate buffer, pH 4.5 and
114 sonicated several times. After lysis, the insoluble parts were removed by centrifugation. The
115 supernatant containing the soluble psoriasin was purified by cation exchange chromatography
116 (Source 30S, Amersham Biosciences, Freiburg, Germany). Bovine psoriasin was eluted with
117 a linear salt gradient (20 mM sodium acetate buffer from 0 M to 1 M NaCl), 2 ml fractions
118 were collected and subsequently tested for psoriasin presence by SDS-PAGE analysis.
119 Psoriasin containing fractions were pooled and concentrated by ultrafiltration, re-buffered into
120 sodium acetate buffer, pH 5.4 or phosphate buffered saline (PBS: 130 mM NaCl, 2.68 mM
121 KCl, 6.46 mM Na₂HPO₄ x 2 H₂O, 1.47 mM KH₂PO₄), pH 7.4 and then subjected to size-
122 exclusion chromatography. Size-exclusion chromatography was performed with an
123 equilibrated Superdex 75 (16/60) column (Amersham Biosciences) at a constant flow rate of 1
124 ml/min at 4 °C. The column was calibrated using a mixture of four proteins of known
125 molecular masses, i.e. albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa),
126 and ribonuclease A (13.7 kDa). The column was equilibrated with 20 mM sodium acetate
127 buffer pH 5.4 or phosphate buffered saline, respectively, and loaded with 1.0 ml of protein
128 solution. Fractions of 1.5 ml were collected.

129 **Protein analysis**

130 Purified protein was analysed by SDS-PAGE on a 15 % separation gel and by subsequent
131 Coomassie staining. The correct mass and purity of the recombinant protein were verified by
132 MALDI-TOF mass spectrometric analysis.

133 **Circular dichroism (CD) spectropolarimetry**

134 CD measurements were carried out with a Jasco J-720 spectropolarimeter (Japan
135 Spectroscopic Co., Ltd., Japan), calibrated according to Chen and Yang (1977). The spectral
136 bandwidth was 2 nm. The measurements were carried out at 24 °C.

137 **Protein concentration**

138 Protein concentrations were calculated from UV-absorption spectra in the range of
139 240-320 nm using the method of Waxman et al. (1993).

140 **Assays for antimicrobial activity**

141 *Microdilution susceptibility assay*

142 *Bacteria and yeast.* The bacteria and yeast used were three different *E. coli* strains (ATCC
143 11775, 25922, 35218), *Pseudomonas aeruginosa* (ATCC 15442), *Staphylococcus (Staph.)*
144 *aureus* (ATCC 6538), the mastitis relevant streptococci *Streptococcus (Strept.) uberis* (ATCC
145 19436), *Strept. agalactiae* (ATCC 27956), and *Strept. dysgalactiae* (ATCC 43078), and
146 *Candida albicans* (ATCC 24433).

147 The susceptibility to bovine recombinant psoriasin was tested based on the minimal
148 bactericidal concentration (MBC) and the lethal dose for ≥ 90 % of bacteria (LD₉₀) (Sahly et
149 al. 2003). Cells were grown for 2 to 3 h in brain heart infusion broth at 36 +/- 1°C, washed
150 three times in 10 mM sodium phosphate buffer (pH 7.4), and adjusted to 10⁴ to 10⁵ colony
151 forming units (CFU)/ml in 10 mM phosphate buffer (pH 7.4) supplemented with 1 % tryptic
152 soy broth. A 100 µl volume of the microbial suspension was mixed with 10 µl of psoriasin
153 solution (range of final concentration from 0.0125 to 100 mg/L) and incubated at 36 +/- 1 °C.
154 After 3 h, viable counts were determined by plating serial dilutions. Briefly, 100 µL volumes
155 of test suspensions serially diluted 1:10 and 1:100 in 10 mM phosphate buffer were plated

156 onto tryptic soy agar. After overnight incubation at 36 +/- 1 °C, CFU were counted. Microbial
157 suspensions supplemented with 10 µl of phosphate buffer served as negative controls. Results
158 are given either as MBC ≥99.9% killing or as LD₉₀s. Arbitrarily, a strain was defined as
159 sensitive to bovine psoriasin if MBC levels were < 10 mg/L, as intermediately sensitive if
160 MBC levels were 10 to 100 mg/L, or as resistant at MBC levels of >100 mg/L.

161 *Radial diffusion assay*

162 Bacterial strains used in a radial diffusion assay previously described by Lehrer et al. (1991)
163 included: *E. coli* ATCC 35218, *E. coli* ATCC 11775, *E. coli* ATCC 25922 *Staph. aureus*
164 ATCC 6538, *Ps. aeruginosa* ATCC 15442, *Strep. uberis* ATCC 19436, *Strep. agalactiae*
165 ATCC 27956, and *Candida albicans* ATCC 24433. Bacteria and yeast were grown to mid-log
166 phase in trypticase soy broth (TSB), suspended into agar (1% agarose in 10 mM sodium
167 phosphate pH 7.4, supplemented with 1% TSB), and poured into petri dishes (10 ml). After
168 hardening, 5 µl of each protein solution pipetted into wells formed with a biopsy punch
169 (diameter: 3 mm), and plates were incubated overnight at 37 °C. Lysozyme was used as a
170 control peptide, the buffers in which psoriasin was solubilized served as negative controls
171 (PBS pH 7.4 respectively 20 mM sodium acetate buffer). Plates were overlaid with 10 ml
172 sterile 1% agar supplemented with double concentrated TSB. Following incubation for 3 h,
173 the diameter of the zone surrounding each well was measured. Inhibition zones were recorded
174 as radial diffusion units (mm inhibition zone – mm well diameter x 10).

175

176 **Results**

177 **Cloning of bovine psoriasin**

178 Using bovine udder cDNA, the complete open reading frame of bovine psoriasin was
179 obtained by RACE cDNA amplification, and the complete nucleotide sequence of bovine
180 psoriasin gene was determined and compared with the mRNA sequence of *Bos taurus* S100

181 calcium binding protein A7 (S100A7), accession number NM_174596 (GenBank). The
182 sequences are 100 % identically.

183 **Comparison of bovine and human psoriasin sequences**

184 The deduced amino-acid sequence of bovine psoriasin was compared with that of human
185 psoriasin (UniProtKB/Swiss-Prot Accession number P31151). An alignment of the sequences
186 is shown in Fig. 1. The bovine psoriasin protein has a 68.3% sequence identity with human
187 psoriasin.

188 **Recombinant expression and purification of bovine psoriasin**

189 A cDNA fragment encoding the complete sequence of bovine psoriasin was amplified by
190 PCR and cloned into the bacterial expression vector pET22b (Novagen). The construct was
191 expressed in *E. coli* BL21. After disruption of the cells by sonication, the soluble cytoplasmic
192 protein was purified by cation exchange and size-exclusion chromatography. The purified
193 product had an apparent molecular mass of 11 kDa on SDS-PAGE, which was expected from
194 its calculated molecular mass. The recombinant bovine psoriasin appears homogeneous after
195 cation-exchange chromatography (Fig. 2).

196 **Size-exclusion experiments and structural characterisation**

197 The recombinant protein was further purified by size-exclusion chromatography. Size-
198 exclusion experiments in PBS, pH 7.4 and in 20 mM sodium acetate buffer, pH 5.4 were
199 performed to investigate whether psoriasin as a protein of the S100 protein family forms
200 homodimers or even oligomers, and whether this oligomerisation is pH-dependent. At pH 7.4
201 bovine psoriasin elutes as a dimer in presence of EDTA. In addition, size-exclusion
202 chromatography was performed at pH 7.4 without EDTA and at pH 5.4. In all cases the
203 protein eluted at the same elution volume, indicating that there is no influence of pH and
204 presence of divalent cations on dimerisation. Fractions containing the protein were analysed
205 by SDS-PAGE. We further identified and characterised the recombinantly expressed bovine
206 psoriasin by MALDI-TOF mass spectrometry. The measured mass corresponds to the

207 expected mass, the purity of the recombinant bovine psoriasin was approved. In order to
208 characterise the recombinant protein structurally we recorded circular dichroism (CD) spectra.
209 The CD-spectrum of the purified recombinant protein in the far UV is indicative of a protein
210 in a folded state. Secondary structure analysis of the far UV CD-spectrum reflects the α -
211 helical character of the protein (Provencher and Glockner 1981).

212 **.Antimicrobial activity**

213 Antimicrobial activity of the recombinant bovine psoriasin was first tested in a microdilution
214 susceptibility assay at pH 7.4. The type strain *E. coli* ATCC 11775, and *E. coli* ATCC 35218,
215 a control strain tested in the Institute of Infection Medicine, University of Kiel for
216 susceptibility to human psoriasin, were both intermediately sensitive to bovine psoriasin. The
217 other tested strains including *E. coli* ATCC 25922, a quality control strain widely used for
218 antimicrobial susceptibility testing, were resistant to bovine psoriasin. The only yeast tested
219 *C. albicans* (ATCC 24433) was intermediate sensitive with a lower LD₉₀ value of 25 mg/L.
220 Values of MBC and LD₉₀s are reported in Table 1.

221 Antibacterial activities of bovine psoriasin were further evaluated by radial diffusion assays.
222 Activities of the protein against *E. coli* ATCC 35218, ATCC 11775, and ATCC 25922, *Staph.*
223 *aureus* ATCC 6538, *Ps. aeruginosa* ATCC 15442, *Strep. uberis* DSMZ 20569, *Strep.*
224 *agalactiae* DSMZ 6784, and *Candida albicans* ATCC 24433 were examined in PBS, pH 7.4
225 (Fig. 3 A) and acetate buffer, pH 5.4 (Fig. 3 B). The results of the assay demonstrated that
226 clear zones appear only at exposure concentrations of psoriasin, whereas at lower
227 concentrations the growth is inhibited, but not totally reduced. Psoriasin was only inhibitory
228 against *E. coli* growth, the concentration needed for this effect varies from one strain to
229 another. Additionally, antibacterial activity appears to be pH-dependent. Increased activity
230 was observed in the radial diffusion assay conducted at pH 7.4 than at 5.4. The growth of the
231 other tested strains relevant for mastitis, *Strep. uberis* (ATCC 19436) and *Strep. agalactiae*
232 (ATCC 27956), was not affected (data not shown).

233 **Discussion**

234 Nothing is known to date about the physiological role of psoriasin in cattle and particularly in
235 the udder. Other antimicrobial peptides such as beta defensins are postulated to play a role in
236 local host defense during udder infections, including mastitis. Because of the recent
237 observation that human psoriasin exhibits antibacterial activity against *E. coli* (Gläser et al.
238 2005), and the isolation of bovine psoriasin from natural sources is quite intensive, we
239 decided to express bovine psoriasin recombinantly and to test the protein for antimicrobial
240 activity.

241 In humans, psoriasin expression appears to be restricted to epithelial cells (Madsen et al.
242 1999, Østergaard et al. 1999, Alowami et al. 2003, Emberly et al. 2003, Emberly et al. 2004,
243 Bryborn et al. 2005). Much less is known about the presence of bovine psoriasin in different
244 tissues. This is the first report that psoriasin mRNA is expressed in the udder and might be
245 part of the innate immunity.

246 The mode of action even of human psoriasin still remains unclear. Antimicrobial peptides or
247 proteins may have a direct effect on the microbe, such as by damaging or destabilising the
248 bacterial membrane or acting on other targets. Regardless of their actual target of action, all
249 antibacterial peptides must first interact with the bacterial cytoplasmic membrane.

250 Comparing the amino acid sequences, highly homologous regions in both proteins contain an
251 identical calcium-binding motif, and the zinc-binding site consisting of three histidines and an
252 aspartate is also conserved. The S100 protein family comprises closely related proteins
253 containing a conserved EF-hand motif, many of the S100 proteins additionally bind Zn^{2+} ions.

254 Gläser et al. (2005) proposed psoriasin kills *E. coli* by sequestering zinc ions essential for
255 bacterial growth, but the exact mechanism still remains to be elucidated.

256 The aim of the recombinant expression was to test whether bovine psoriasin exhibits
257 antimicrobial activity and whether this activity is limited to *E. coli*. Compared with the native
258 human psoriasin, the bovine recombinant protein appeared to be less potent against *E. coli* by

259 a factor of 10. In both assays tested, the inhibitory effect of psoriasin was restricted to *E. coli*
260 strains. As naturally occurring psoriasin including bovine psoriasin is N-terminally acetylated
261 (J. M. SCHRÖDER, personal communication), the loss of activity may be explained by the
262 recombinant bacterial expression system which is incapable of accomplishing
263 posttranslational modifications. The second possibility for the need of a higher concentration
264 to inhibit the growth of *E. coli* may be due to the fact that the protein from cattle has the same
265 biological function, but is less potent.

266 The antimicrobial peptides interact with negatively charged phospholipids head groups on the
267 outer surface of bacterial membranes via electrostatic and hydrophobic interactions.
268 Consequently- it is argued- it will be difficult for bacteria to develop resistance (Zasloff
269 2002). However, lower concentrations of anionic phospholipids may be a modification that
270 will enhance resistance and could be responsible for the decreased susceptibility of *E. coli*
271 ATCC 25922.

272 The recombinant bovine psoriasin was characterised by CD spectroscopy, mass spectrometry,
273 and size exclusion chromatography. Under physiological conditions, most S100 proteins
274 including psoriasin form dimers. The examination of recombinant protein by size exclusion
275 chromatography revealed the dimeric association of bovine psoriasin. The experiments
276 showed that the dimerisation was independently of pH and metal binding in aqueous solution.

277 Dairy cattle with clinical mastitis caused by *E. coli* exhibit a wide range of disease severity,
278 from mild, with only local inflammatory changes of the mammary gland, to severe, with
279 significant systemic derangement. A high degree of genotypic variability is characteristic of
280 *E. coli* strains. Many *E. coli* strains differ in their serotype and virulence. However Wenz et
281 al. (2006) were unable to find any correlation between *E. coli* virulence genes and systemic
282 disease severity. They concluded that specific cow factors might play a more important role in
283 determining disease severity, and we speculate that psoriasin might be such an additional cow
284 specific factor.

285 **Conclusion**

286 Psoriasin appears to be a part of the local host defense mechanism in the udder, is a putative
287 candidate for a cow-specific factor influencing mastitis susceptibility, and a possible
288 alternative to conventional antibiotics.

289

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296

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Figure captions:

Figure 1: Sequence alignment of human and bovine psoriasin protein. The bovine protein (Q28050) shows 68.3% sequence identity compared to human psoriasin (P31151). Amino-acid identities between human and bovine sequences are marked with an asterisk. Amino-acid residues involved in Zn-binding are highlighted in green. Conserved cysteines are coloured yellow.

Figure 2: SDS-PAGE analysis of purified recombinant bovine psoriasin (M: Marker, lane 1: starting material after disruption of bacterial cells, lane 2-5: eluted fractions after cation exchange chromatography), the arrow indicates the position of bovine psoriasin.

Figure 3: Radial diffusion assay: 5 A: in PBS, pH 7.4 ; 5 B: in 20 mM sodium acetate buffer, pH 5.4. An increase in zone size represents the zone size measured at each psoriasin concentration minus the size of the central well (3 mm) multiplied with factor 10 (RDU). Data represent the means of three independent experiments.

Table 1: Susceptibilities to recombinant bovine psoriasin

Species	Strain	LD₉₀	MBC
		[mg/L]	[mg/L]
<i>E. coli</i>	ATCC 11775	6.25	50
<i>E. coli</i>	ATCC 35218	12.5	50
<i>E. coli</i>	ATCC 25922	100	>100
<i>Ps. aeruginosa</i>	ATCC 15442	>100	>100
<i>Staph. aureus</i>	ATCC 6538	>100	>100
<i>Strep. uberis</i>	ATCC 19436	>100	>100
<i>Strep. agalactiae</i>	ATCC 27956	>100	>100
<i>Strep. dysgalactiae</i>	ATCC 43078	>100	>100
<i>C. albicans</i>	ATCC 24433	25	>100

```
human  MSNTQAERSI IGMIDMFHKYTRRDDKIDKPSLLTMMKENFPNFLSACDKKGTN
bovine  MSSSQLEQAITDLINLFHKYSGSDTIEKEDLLRLMKDNFPNFLGACEKRGRD
** * * * * * **** * * * * * ** * * * * * * * * * * * * * * *
human  YLADVFEKKDKNEDKKIDFSEFLSLLGDIATDYHKQSHGAAPCSGGSQ
bovine  YLSNIFEKQDKNKDRKIDFSEFLSLLADIATDYHNHSHGAQLCSGGNQ
**   *** *** * ***** ***** ***** ***** * * * * *
```

Figure 1

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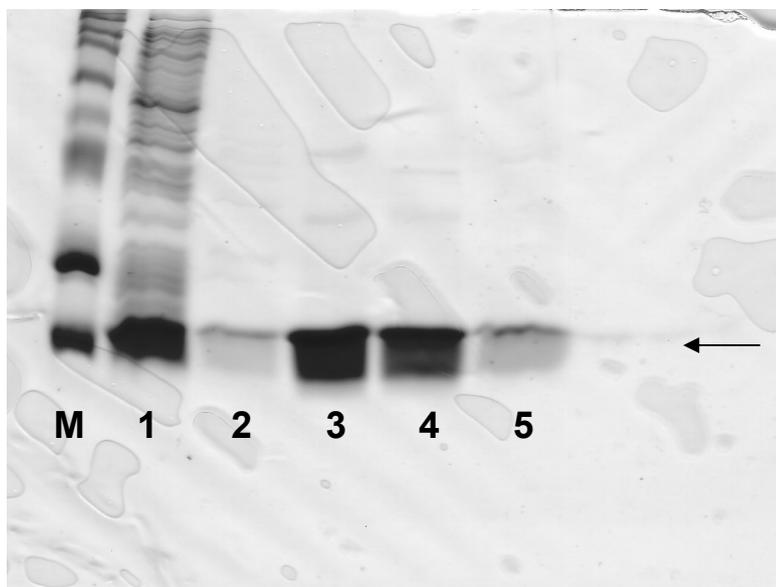


Figure 2

Accepted Manuscript

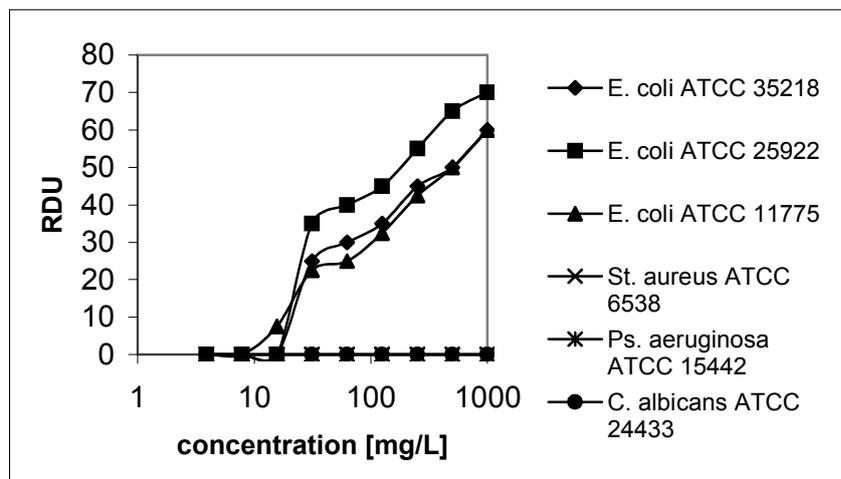


Figure 3 A

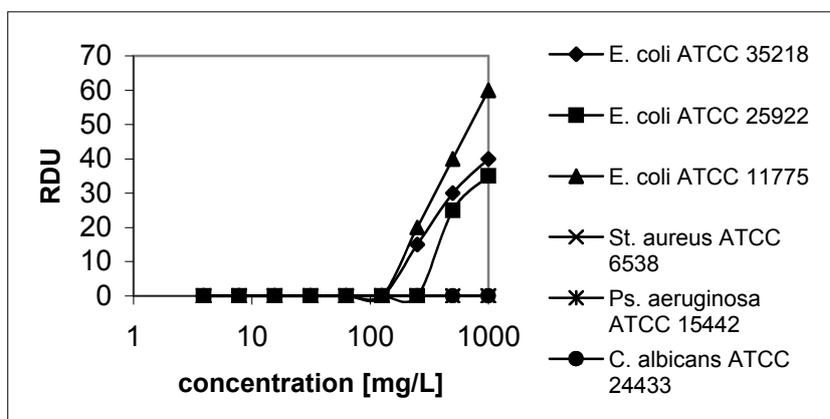


Figure 3 B