Characterization of a bacteriophage lysin (Ply700) from *Streptococcus uberis*

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**Abstract**

The antibacterial properties of bacteriophage lytic enzymes may be of importance in future mastitis control programs. A prophage was isolated from a strain of *Streptococcus uberis* (ATCC 700407) following exposure to mitomycin C. Partial sequencing of the phage DNA revealed a putative lysin based on sequence similarity to other streptococcal phage lysins. The putative lysin (Ply700) was recombinantly expressed in *Escherichia coli*, and chromatographically purified. Addition of the purified Ply700 to bacterial suspensions of *S. uberis*, *Streptococcus pyogenes*, and *Streptococcus dysgalactiae* caused a rapid, calcium-dependent lysis while there was little activity against *Streptococcus agalactiae*, *Staphylococcus aureus*, or *E. coli*. Killing of *S. uberis* in milk by Ply700 (50 \(\mu\)g/ml) was confirmed by plate count assay. Activity was related to the initial concentration of bacteria in that 31% killing (\(P < 0.05\)) was observed with an inoculating dose of approximately 4500 cfu/ml, while 81% killing (\(P < 0.01\)) was observed when the inoculum was reduced to approximately 600 cfu/ml. In contrast, complete sterilization was observed in parallel cultures suspended in assay buffer indicating that factors in milk are able to neutralize the lysin. Functional characterization of the C-terminal domain, as a component of a GFP fusion protein, revealed its calcium-dependent ability to bind to *S. uberis*. The C-terminal domain may have utility in targeting *S. uberis* while it remains to be determined if the lysin by itself has sufficient potency in milk for effective use in the control of *S. uberis* mastitis.

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**1. Introduction**

Bovine mastitis is a significant impairment to the efficient production of high-quality milk, costing the U.S. dairy industry approximately $2 billion/year (Sordillo and Streicher, 2002). Current practices for the control of mastitis include the use of antibiotics in conjunction with other preventative management techniques. These strategies have worked very well
against some pathogens such as *Streptococcus agalactiae* but new strategies are required to further prevent or cure mastitis due to other pathogens. Concern regarding the exposure of consumers to antibiotics in milk has led to the imposition of a strict post-treatment milk discard period. At the same time, there has been growing public concern that the use of antibiotics in animal agriculture may contribute to the emergence of antibiotic resistant human pathogens (Smith et al., 2002). Antimicrobial enzymes may play a role in mastitis control by substituting for antibiotics currently used in human medicine (Bramley and Foster, 1990; Oldham and Daley, 1991). These enzymes are commonly produced by bacteria as autolysins or bacteriocins, and as bacteriophage-encoded lysins during bacteriophage-mediated cell lysis.

Bacteriophage lytic enzymes are generally peptidoglycan hydrolases and are responsible for lysis of the host bacterial cell. In recent studies, treatment with bacteriophage lysins has proven to be effective in killing several different types of Gram-positive bacteria both *in vitro* as well as *in vivo* (Loeffler et al., 2001; Nelson et al., 2001; Schuch et al., 2002; Cheng et al., 2005). Although not evaluated *in vivo*, the *S. agalactiae* bacteriophage B30 phage lysin has been shown to have lytic activity against mastitis-causing pathogens including *S. agalactiae* and *Streptococcus dysgalactiae* (Pritchard et al., 2004), and also *Streptococcus uberis* (Donovan et al., 2006a). However, its activity is reduced in the presence of milk-whey. The potential use of a *S. uberis* bacteriophage lysin for the treatment of mastitis led us to characterize a lytic enzyme from a prophage induced from this pathogen.

### 2. Materials and methods

#### 2.1. Bacterial strains

Ten different *S. uberis* isolates were used in this present study. Three of the strains were purchased from The American Type Culture Collection (ATCC; 27958, 700407, and BAA-854), while the others were clinical isolates from the University of Vermont collection that were originally obtained from milk of infected cows. The clinical isolates were confirmed to be *S. uberis* via PCR of the 16S and 23S ribosomal RNA (Hassan et al., 2001). Other strains including *Streptococcus pyogenes* D471, *S. agalactiae* (NCTC 11244, NCTC 11254, and NCTC 11248), *S. dysgalactiae* 26RP66, and *Staphylococcus aureus* RN4220 are part of The Rockefeller University collection. All streptococcal and staphylococcal isolates were grown in Todd Hewitt Broth (THB) at 37 °C. All *Escherichia coli* strains used were grown in Luria–Bertani media (LB) at 37 °C.

#### 2.2. Prophage induction

To induce the production of prophage, a single colony of *S. uberis* (ATCC 700407) was used to inoculate 5 ml of THB media, and grown overnight at 37 °C. The overnight culture was diluted 1:100 the following day in fresh THB media, and incubated for 4 h at 37 °C to ensure logarithmic growth. After the initial incubation, mitomycin C, which is a DNA crosslinker, was added to the culture (1.0 μg/ml), followed by a further incubation period of 4 h at 37 °C. Following prophage induction, the phage DNA was isolated from the bacterial culture using the Lambda Phage DNA prep kit (Qiagen). Phage DNA was visualized by ethidium bromide gel electrophoresis.

#### 2.3. Phage DNA sequencing

A plasmid library of phage genomic DNA was constructed in pUC18, grown in *E. coli*. The phage DNA was digested with *HindIII* and ligated into the pUC18 vector using the Quick Ligase kit (New England Biolabs). Selected plasmids were then used for sequencing portions of the phage genome. Additional sequencing was performed on DNA obtained by amplification of phage genomic DNA with the Genomiphi kit (Amersham Biosciences). Briefly, 1 μl (10 ng) of purified bacteriophage DNA was combined with 9 μl of sample buffer containing random hexamer primers, heated to 95 °C for 3 min, and then cooled on ice. The denatured sample was combined with 9 μl of reaction buffer, containing salts and dNTPs, as well at 1 μl of enzyme mix, containing the Phi29 DNA polymerase. The sample was incubated at 30 °C for 18 h, followed by heat inactivation at 65 °C for 10 min. The amplified phage genomic DNA was purified by alcohol precipitation,
with final resuspension in double distilled water. The final product was stored at −20 °C.

2.4. Identification, cloning and expression of the phage lysin

Phage DNA sequences were translated into putative open reading frames (ORFs) using Gene Runner (version 3.01). A database search of proteins (blastn; BLAST, and NCBI) was then used to identify similar sequences (see results). Primers were designed to amplify the putative lysin gene by PCR (forward 5′TAGTCGCTAGCAGGAGGAATTAACCATGACGACGGAGGAATTCAATGACAGACAGTTT; reverse 5′GATCGGTTACCTTACATTCTGTGGCATCAA) from the purified phage genomic DNA using the Platinum Taq DNA polymerase kit (Invitrogen). The amplified lysin gene was then cloned into the pBAD 18 prokaryotic expression vector (Guzman et al., 1995) using the NheI and KpnI restriction sites incorporated into the PCR primers (underlined). The resulting plasmid (pBAD18700lys) was transformed into TOP 10 chemically competent cells (Invitrogen) according to the standard procedure. Plasmids were purified from positive transformants (Qiaprep; Qiagen), verified by restriction digest, and then sequenced.

2.5. Lysin production

The E. coli containing the recombinant plasmid was grown overnight in LB media supplemented with 50 µg/ml ampicillin at 37 °C. This culture was diluted 1:100 the following day and grown to an OD600 of 0.5. Recombinant protein expression was induced for 4 h at 37 °C by the addition of L-arabinose to a final concentration of 0.20%. Cells were harvested by centrifugation (3000 × g, 10 min, 4 °C), resuspended in 10 ml of chilled lysis buffer (10 mM Tris–HCl, pH 8.0, 1 mM EDTA, 0.1 mg/ml DNase I, and 10 mM CaCl2), and placed on ice. The samples were sonicated on ice for six 10 s bursts, with a 30 s cooling period in between each burst to prevent overheating. After sonication, the crude lysate was centrifuged (10,000 × g, 10 min, 4 °C) to remove bacterial debris.

The supernatant was dialyzed overnight (Spectro/ Por 4, 12–14 kDa MWCO; Spectrum Laboratories) to exchange the cell lysis buffer with the column chromatography buffer (20 mm Bis–Tris, 25 mM NaCl, pH 6.2). The buffer was exchanged twice. The solution was then filtered using a 0.45 µm syringe filter (Milipore) to remove any remaining debris. The filtered lysate was applied to an Econo-Pac High Q Anion Exchange Cartridge (BioRad) and the putative lysin eluted using a linear gradient of NaCl from 0 to 1 M in 20 mM Bis–Tris, pH 6.2. The protein content of all fractions was determined using the Biorad Protein Assay. Samples of all protein containing fractions were examined on SDS-PAGE gels, and those containing the recombinant protein were pooled. The purified protein was desalted and concentrated using a Centriprep −10 ultrafiltration device (Amicon). The buffer was exchanged three times with the final enzyme buffer (20 mM ammonium acetate, 10 mM CaCl2, pH 6.2). Final protein concentration of the pooled fraction was then determined.

2.6. Turbidity reduction assay

Streptococcal, staphylococcal, and E. coli cultures were grown in THB or LB media at 37 °C. Overnight cultures were diluted 1:100 in fresh media and grown until reaching mid-exponential phase. The cells were harvested by centrifugation (3000 × g, 10 min, 4 °C), washed 3 × with enzyme buffer, and then resuspended in enzyme buffer to a final OD600 of approximately 0.7.

The activity of putative lysin was first tested in buffers containing various concentrations of CaCl2 (20 mM ammonium acetate, 0–100 mM CaCl2, pH 6.2). Ply700 was added to 100 µl of the bacterial suspension in a 96 well plate (SpectraMax Plus) to a final concentration of 20–50 µg/ml. Enzyme activity was expressed as the percent decrease in the turbidity of the bacterial suspension. All experiments were performed in triplicate, and control experiments were performed with the addition of enzyme buffer under the same conditions. After determining that 10 mM CaCl2 enhanced activity, it was used for all further testing. The effect of pH on Ply700 activity was determined in a range of pH buffers, all containing 10 mM CaCl2 (20 mM sodium acetate (pH 5.2), 20 mM ammonium acetate (pH 6.2), and 20 mM Tris (pH 7.5 or 8.5)). Likewise, the ability of the putative lysin to lyse S. uberis bacteria harvested at different stages of growth (early exponential, mid-exponential, and late exponential) was also tested using this
technique with an optimized enzyme assay buffer (20 mM ammonium acetate, 10 mM CaCl₂, pH 6.2). Lastly, this method was also used to determine the ability of the putative lysin to lyse clinical isolates of *S. uberis* as well as other bacterial species including *S. pyogenes, S. agalactiae, S. dysgalactiae, Staphylococcus aureus,* and *E. coli.*

2.7. Plate count assay to evaluate killing activity in milk

An overnight culture of *S. uberis* 700407 was diluted 1:100 in fresh THB and grown until reaching mid-exponential phase (4 h). The cells were harvested by centrifugation (3000 × g, 10 min, 4 °C), resuspended with assay buffer, and 100 μl of serial dilutions plated for overnight growth to retrospectively verify the viable cfu content in the starting culture. Based on prior experience, dilutions of the culture were added to bovine milk containing a final concentration of 50 μg/ml of putative lysin and estimated initial bacterial counts of approximately 5000, 500, and 50 cfu/ml. The cultures were incubated for 15 min at 37 °C after which 100 μl aliquots were plated for enumeration after 16 h incubation.

2.8. Fusion of the Ply700 cell wall-binding domain to green fluorescent protein

To test the ability of the putative cell wall-binding domain (CBD) to adhere to the *S. uberis* bacterial cell wall, a new modular protein was expressed from a fusion gene encoding green fluorescent protein (GFP; eGFP, and Clontech) fused to the CBD of the putative lysin. The GFP coding region without the stop codon was amplified by PCR (forward 5’TAGTCGCTAGCAGGAGGAATTAACCATGGTGACAAAGG; reverse 5’TACGTGTACCTTTGATACG GCTCGTCCATGCGC) using *NheI* and *KpnI* restriction sites incorporated into the primers (underlined). Recombinant protein expression was induced as previously described and lysates were prepared in the presence or absence of 10 mM CaCl₂.

2.9. Fusion protein binding assay

An overnight culture of *S. uberis* 700407 was diluted 1:100 in fresh THB and grown until reaching mid-exponential phase. The cells were harvested by centrifugation (3000 × g, 10 min, 4 °C), washed once in enzyme buffer, and then resuspended in enzyme buffer to 1/10 their original volume. A 500 μl aliquot of the bacterial suspension was harvested by centrifugation (16,000 × g, 30 s), then resuspended in 500 μl of the pBAD18 crude lysate, the pBAD18GFP-700CBD crude lysate, or the enzyme buffer alone, and incubated at room temperature for 30 min. For fluorescence microscopy, the cells were pelleted by centrifugation and washed twice with enzyme buffer. They were then resuspended in 50 μl of enzyme buffer, and viewed using an Olympus BX41 microscope. In a separate assay, fluorescence was quantitated using a fluorescence plate reader (Biotek Synergy HT). Briefly, the cells were prepared as above, and then resuspended in a final volume of 200 μl which was transferred to a 96 well plate to determine fluorescence (excitation 485 nm, emission 530 nm).

2.10. Statistics

Data comparing the activity of the putative lysin under different culture conditions were analyzed by one-way analysis of variance, then means separated by Tukey test. The activity of the lysin in milk was analyzed by one-tailed paired *T*-test comparing final viable cfu count resulting after exposure to lysin or buffer control.
3. Results

3.1. DNA sequencing and lysin identification

Initial attempts to isolate bacteriophage from nine of the 10 *S. uberis* isolates (BAA-854 not tested) by standard plaque assay techniques were unsuccessful. Furthermore, attempts to recover bacteriophage from dairy farm environmental samples such as bedding, pasture, or manure collection sites were not successful. However, a prophage was induced from *S. uberis* 700407 by the addition of mitomycin C to a mid-log culture of bacteria, and the phage DNA isolated using a commercial kit. The phage DNA was subsequently digested and cloned to generate a library of phage DNA for sequencing. One particular plasmid, P15, contained an approximately 10 kb insert that was sequenced by primer walking. New primers were developed from both ends of the P15 insert to perform additional sequencing directly from the phage DNA or from the phage genome amplified using a commercial kit. A total of approximately 13 kb were sequenced and deposited into GenBank (accession #DQ198146).

The DNA sequence was translated into putative ORFs, and those larger than 10 kDa were characterized based on sequence homology revealed with a database search (tblastn, BLAST, and NCBI). Using this method, 11 contiguous putative ORFs were identified and seven of these assigned putative functions based on similarity to other characterized phage proteins (Table 1). We were unable to assign functions to the remaining ORFs, either because there was no similarity to previously characterized proteins, or the sequences were similar to uncharacterized proteins. The most 3’ ORF (#11) encodes a protein with a predicted molecular size approximately 26.5 kDa that has substantial similarities (tblastn expectation values of 2e−26, 6e−26, and 4e−07, respectively) with confirmed or putative phage derived lysins from a number of sources including the *S. agalactiae* phage NCTC11261 (Cheng et al., 2005), the *S. agalactiae* phage B30 (Pritchard et al., 2004), and the *S. pneumoniae* phage Dp-1 (Sheehan et al., 1997). This protein encoded by ORF #11 is referred to as Ply700 to reflect a phage lysin from ATCC strain #700407.

3.2. Production of Ply700 and characterization of enzyme activity

The Ply700 gene was amplified by PCR, and cloned into the L-arabinose inducible pBAD18 prokaryotic expression plasmid. Recombinant phage lysin (Ply700) was purified by anion exchange column chromatography, and when examined by SDS-PAGE revealed a band of approximately 27 kDa, which closely reflects the predicted mass of 26.5 kDa (Fig. 1).

Based on the high degree of homology between Ply700 and the calcium-dependent bacteriophage B30 endolysin (Pritchard et al., 2004), the effect of calcium ion concentration on enzyme activity was determined.

### Table 1

Open reading frames of a DNA segment isolated from a prophage derived from *S. uberis* 700407 translated into proteins and then assigned putative function following database search (tblastn; NCBI)

<table>
<thead>
<tr>
<th>ORF</th>
<th>Base pairs</th>
<th>Base pair position</th>
<th>Hypothetical size (kDa)</th>
<th>Putative protein function</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>725</td>
<td>119–844</td>
<td>25.2</td>
<td>Tape measure</td>
</tr>
<tr>
<td>2</td>
<td>299</td>
<td>936–1235</td>
<td>10.7</td>
<td>Unknown</td>
</tr>
<tr>
<td>3</td>
<td>1127</td>
<td>1297–2424</td>
<td>41.2</td>
<td>Tape measure</td>
</tr>
<tr>
<td>4</td>
<td>398</td>
<td>2838–3236</td>
<td>14.6</td>
<td>Unknown</td>
</tr>
<tr>
<td>5</td>
<td>416</td>
<td>3902–4318</td>
<td>15.2</td>
<td>Tail component</td>
</tr>
<tr>
<td>6</td>
<td>275</td>
<td>4461–4736</td>
<td>10.3</td>
<td>Tail component</td>
</tr>
<tr>
<td>7</td>
<td>2345</td>
<td>4824–7169</td>
<td>86.4</td>
<td>Tail host specificity</td>
</tr>
<tr>
<td>8</td>
<td>2042</td>
<td>7538–9580</td>
<td>73.8</td>
<td>Structural</td>
</tr>
<tr>
<td>9</td>
<td>371</td>
<td>9593–9964</td>
<td>13.9</td>
<td>Unknown</td>
</tr>
<tr>
<td>10</td>
<td>305</td>
<td>10,116–10,421</td>
<td>11.8</td>
<td>Unknown</td>
</tr>
<tr>
<td>11</td>
<td>711</td>
<td>10,609–11,320</td>
<td>26.5</td>
<td>Lysin</td>
</tr>
</tbody>
</table>

*Base pair position is relative to the 13,000 bp sequenced and not the entire genome.

*Putative function based on results of tblastn search of NCBI data base.*
It was found that the activity of Ply700 was dependent on the concentration of calcium in the system (Fig. 2A). Considerable activity was attained with a calcium ion concentration of 10 mM. However, at higher (100 mM) or lower (1 mM or 0 mM) concentrations there was a significant ($P < 0.05$) decrease in activity. Given this, 10 mM CaCl$_2$ was added to all subsequent enzyme buffers. Using the turbidity reduction assay, a pH profile of enzyme activity was also constructed. Greatest enzymatic activity was obtained at a relatively neutral pH (Fig. 2B). Activity of the lysin at pH 5.2 or 8.5 was significantly ($P < 0.05$) reduced in comparison to pH 6.2 or 7.5.

Susceptibility of S. uberis to Ply700 was also dependent on the growth stage of the culture. By harvesting samples of S. uberis 700407 at different time points throughout growth, it was determined that the bacterial cells were most susceptible to the lyasin enzyme during the early and mid-exponential stages of growth. The susceptibility of the cells to lysis by the enzyme significantly ($P < 0.05$) decreased as the culture reached the late-exponential stage of growth (data not shown).

### 3.3. Specificity of Ply700 activity

A turbidity reduction assay was performed to determine lytic activity against seven isolates from clinical S. uberis mastitis, three ATCC S. uberis strains, and representatives of group A (S. pyogenes), group B (S. agalactiae), and group C (S. dysgalactiae) streptococci. In each case the bacterial cells harvested at mid-log phase and suspended in a 20 mM ammonium acetate buffer, with 10 mM CaCl$_2$, pH 6.2 and exposed to Ply700 at a concentration of 50 $\mu$g/ml. After 30 min a maximal reduction in turbidity of approximately 80% was observed for five of the 10 S. uberis stains, the S. pyogenes strain, and the S. dysgalactiae strain (Fig. 3). The remaining turbidity in these cultures was presumably due to debris from cell
walls and bacterial contents. The apparently reduced activity against five of the S. uberis strains is not easily explained although by 60 min the turbidity was further reduced in these strains, and at this time no viable colonies were recovered after plating 100 μl aliquots on blood agar plates. Only modest activity of the enzyme was observed against three group B streptococcal (S. agalactiae) strains (~10% decrease in turbidity in 30 min), while Ply700 had no activity against representative Staphylococcus aureus and E. coli strains (data not presented).

Evaluation of the activity of Ply700 in cow’s milk relied on direct measurement of bacterial viability using a plate count assay because turbidity measurements could not be taken in milk samples. Milk was spiked with Ply700 (50 μg/ml) and then inoculated with dilutions of log phase cultures of S. uberis (ATCC 700407) at levels thought to represent conditions in early stage mastitis (approximately 5000, 500, and 50 cfu/ml). Following a 15 min incubation period the actual cfu/ml were determined by plate counting. This revealed that the Ply700 lysin is quite effective in killing S. uberis in milk (Fig. 4), although it was not able to completely sterilize the cultures as it did in parallel assays in buffer (data not presented). The activity in milk was related to the initial concentration of bacteria in that 31% killing (P < 0.05) was observed with an inoculating dose of approximately 4500 cfu/ml, while 81% killing (P < 0.01) was observed when the inoculum was reduced to approximately 600 cfu/ml. However, even at a lower inoculum of approximately 90 cfu/ml complete sterilization was only observed in two out of six culture replicates.

3.4. Characterization of the C-terminal domain of Ply700

The ability of the C-terminal domain of the lysin (amino acids 156–236) to function as a cell wall-binding domain was evaluated by construction of a new modular protein containing GFP fused with the C-terminal domain of Ply700 (GFP-CBD). Cells exposed to the fusion protein became readily visible by fluorescence microscopy (Fig. 5, insert). The binding of the fusion protein to the bacterial cell surface was also found to be dependent on the presence of CaCl₂ in the system. When calcium was
removed from the system the apparent binding was reduced \((P < 0.05)\) as confirmed by quantifying the fluorescence (Fig. 5).

4. Discussion

Current therapy for mastitis is based on the intramammary administration of antibiotics typically used in human medicine. Concerns over animal agricultural use of these antibiotics which may contribute to development of drug-resistance human pathogens have led us to explore alternative strategies. The effectiveness of phage lysins in clearing certain infections has been well documented in mouse models (Loeffler et al., 2001; Nelson et al., 2001; Schuch et al., 2002; Loeffler et al., 2003; Cheng et al., 2005). These enzymes have been targeted as potential therapeutics because of their ability to kill bacteria combined with a low probability of bacteria to develop resistance. For example, it was found that repeated exposure of \(S. pneumoniae\) to low doses of the lytic enzyme Pal, in liquid culture or on agar plates, did not lead to the recovery of resistant strains (Loeffler et al., 2001). It has also been demonstrated that repeated exposure of \(B. anthracis\) to the lytic enzyme PlyG, did not lead to the recovery of resistant strains (Schuch et al., 2002). In fact, while chemically enhanced mutation of \(B. anthracis\) was capable of increasing the frequency of resistance to streptomycin 10,000-fold, no phage lysin resistant mutants were detected. It has been speculated that the evolution of phage lysins in conjunction with bacteria has allowed them to target, either through binding or hydrolysis, structures which are essential to the survival of the bacteria, making it difficult for resistance to develop (Loeffler et al., 2001).

In the current report we have performed initial characterization of a bacteriophage lysin that we identified in a strain of \(S. uberis\). The lysin was identified through sequencing prophage DNA obtained following mitomycin C induction of phage production from \(S. uberis\) (ATCC 700407). A portion of the bacteriophage genome was sequenced and 11 ORFs were assigned putative functions based on their similarity to other phage proteins in the NCBI.
translated protein database. A putative lysin, termed Ply700, was revealed in ORF11 on the basis of substantial similarity with confirmed or putative phage derived lysins from a number of sources including the S. agalactiae phage NCTC11261 (Cheng et al., 2005), the S. agalactiae phage B30 (Pritchard et al., 2004), and the S. pneumoniae phage Dp-1 (Sheehan et al., 1997).

Cloning of the ply700 gene into a prokaryotic expression system, and purification of the recombinantly expressed lysin using ion exchange column chromatography allowed for the characterization of enzyme activity. Using a turbidity reduction assay and direct plate counting of viable bacteria, we found that the Ply700 enzyme was capable of lysing S. uberis cells in vitro, and this was accompanied by bacterial cell death. Further experiments showed that the activity of this enzyme was dependent on the presence of calcium in the medium, greatest at neutral pH, and was greater against actively dividing cells.

Ply700 possessed potent activity in THB against all clinical isolates and ATCC strains of S. uberis, as well as S. pyogenes and S. dysgalactiae. Additionally, Ply700 had slight activity against S. agalactiae. Important to its potential use in mastitis control, this enzyme was active in cow’s milk where it caused a decrease in viability of S. uberis 700407 in 15 min. However, the killing activity in milk was much reduced as compared to killing activity in assay buffer. Milk is a complex fluid containing both an aqueous phase and a colloidal phase consisting of casein micelles and membrane-bound fat droplets. Some antibacterial enzymes such as lysostaphin function quite effectively in mouse (Bramley and Foster, 1990) and bovine (Oldham and Daley, 1991) milk, however, Donovan et al. (2006b) found that the activity of the S. agalactiae bacteriophage B30 endolysin was reduced in milk-whey while activity in whole milk was not reported. It would appear that the lysins’ activity is being neutralized by absorption to milk components. Alternatively both the Ply700 and the B30 endolysin (Pritchard et al., 2004) are dependent on the calcium in the assay buffer and perhaps milk calcium is not accessible to the lysins in sufficient quantities. The normal concentration of ionized calcium in bovine milk is approximately 3 mM (Neville, 2005) while other forms such as calcium citrate, calcium phosphate and casein-bound calcium bring total milk calcium to approximately 30 mM. Although this seems sufficient for enzyme activity, we did not determine if calcium supplementation of milk enhanced the activity of Ply700. Alternatively, given the sub-optimal activity noted with 100 mM calcium there is the possibility that an excess of divalent cations in milk is limiting lysin activity. It has recently been found that the activity of a peptide derived from bactericidal/permeability-increasing protein is severely impaired in milk and this can be remedied by the addition of the divalent chelator EDTA (Chockalingam et al., 2007).

Bioinformatic analysis of ORF 11 (Ply700) through a search of the conserved domain data base at the NCBI (Marchler-Bauer et al., 2007) indicated that the amino terminal region (amino acids 16–150) of ORF #11 was homologous to the Amidase_5 family of bacteriophage peptidoglycan hydrolases, while the carboxy terminal portion of ORF #11 (amino acids 184–231) was homologous to the bacterial SH3 domain, a putative cell wall-binding domain. However, further experimentation is required to definitively assign an amidase activity to the N-terminal region of Ply700. The C-terminal portion of the ORF 11 protein (amino acids 156–236) was found to be 72% and 71% identical to the C-terminal portion of the S. agalactiae bacteriophage NCTC11261 lysin PlyGBS (Cheng et al., 2005), and the C-terminal portion of the S. agalactiae bacteriophage B30 endolysin (Pritchard et al., 2004), respectively. Although the exact function of these C-terminal domains is unclear, it has been speculated that they are cell wall-binding domains that direct binding of the lysin to target epitopes on the surface of susceptible bacteria (Pritchard et al., 2004; Cheng et al., 2005). However, in more recent studies it has been found that the lytic activity of the B30 (Donovan et al., 2006b) and PlyGBS (Cheng and Fischetti, 2007) lysins is maintained or even increased by approximately 25-fold, respectively, in engineered lysins in which the SH3 domain has been removed. The marked increase in activity of PlyGBS mutants observed by Cheng and Fischetti (2007) suggest that a similar mutagenesis strategy may be an approach to increase the activity of Ply700 in milk. We have yet pursued Ply700 mutants, but our results with the GFP-CBD fusion protein are consistent with a cell wall-binding function for the C-terminal putative SH3b domain in Ply700. A similar approach was previously used during the characterization of two Listeria monocytogenes lytic enzymes...
(Loessner et al., 2002) where cell surface binding of GFP-CBD fusion proteins to L. monocytogenes was observed. Furthermore, our results also suggest that Ply700’s activity dependence on calcium is at least in part due to its involvement in binding of the lysin enzyme to the bacterial cell wall. The binding of the fusion protein to S. uberis was readily detectable by fluorescence microscopy when CaCl2 was present in the media, but could not be observed in its absence.

To our knowledge, there has been only one other report on the isolation of bacteriophage from S. uberis (Hill and Brady, 1989). The authors reported that 38 S. uberis bacteriophage were obtained from 98 strains of S. uberis following induction with mitomycin C. Phage were identified using a traditional plaque assay. However, the authors did note that when using this method, it was difficult to identify a bacterial propagation strain for the phage. Thus they found that when 120 field strains of S. uberis were challenged with 25 of the isolated phages, only 30% of the strains were susceptible to a least one phage (Hill and Brady, 1989). For our current study, although we attempted to isolate a phage using the same techniques we were unable to identify any visible plaques. Perhaps our attempt to isolate a phage using the plaque assay failed because we did not have an appropriate propagating strain. For this particular study we screened only 10 different S. uberis isolates, which may have limited our ability to identify an inducible prophage as well as an appropriate propagation strain.

The Ply700 enzyme, with its ability to lyse S. uberis, S. agalactiae, and S. dysgalactiae, may potentially serve as a supplement to current antibiotics for the control of streptococcal mastitis. Our results indicate that at a concentration of 50 μg/ml the enzyme has substantial killing activity in milk. To obtain this concentration in milk via intramammary infusion into lactating cows would be problematic. However, the enzyme may prove to be an effective component of an exogenously applied teat dip or a dry cow therapy, which is the intramammary administration of antibiotics immediately after the last milking of lactation. An alternative approach to intramammary infusion is the generation of transgenic animals that can secrete foreign antibacterial enzymes into their milk (Kerr et al., 2001; Wall et al., 2005). This approach has been very successful with the transgenic production of lysozyme to enhance resistance to mastitis caused by S. aureus.

In this study, a S. uberis bacteriophage lysin was identified based on its similarity to other previously characterized lysins. Production of the enzyme in a prokaryotic expression system allowed for demonstration of its lytic capabilities against S. uberis and the finding that the activity was substantially hindered in the presence of milk. Although further work is necessary to determine the effectiveness of this enzyme in an in vivo model, the results of this study suggest only limited potential for the Ply700 enzyme by itself in native form to aid in the prevention or treatment of streptococcal mastitis.

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References


