Lysis of staphylococcal mastitis pathogens by bacteriophage phi11 endolysin

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Abstract

The *Staphylococcus aureus* bacteriophage phi11 endolysin has two peptidoglycan hydrolase domains (endopeptidase and amidase) and an SH3b cell wall-binding domain. In turbidity reduction assays, the purified protein can lyse untreated staphylococcal mastitis pathogens, *Staphylococcus aureus* and coagulase-negative staphylococci (*Staphylococcus chronogenes*, *Staphylococcus epidermidis*, *Staphylococcus hyicus*, *Staphylococcus simulans*, *Staphylococcus warneri and Staphylococcus xylosus)*, making it a strong candidate protein antimicrobial. This lytic activity is maintained at the pH (6.7), and the 'free' calcium concentration (3 mM) of milk. Truncated endolysin-derived proteins containing only the endopeptidase domain also lyse staphylococci in the absence of the SH3b-binding domain.

Introduction

Bovine mastitis, an infection of the mammary gland, exists on every dairy farm, often with one-third of the animals affected. This worldwide problem costs two billion dollars annually to the US dairy industry (Sordillo & Streicher, 2002). Coagulase-negative staphylococcus (CoNS) and *Staphylococcus aureus* are major US mastitis pathogens, accounting for 22% and 18% of cases, respectively, in a study of New York and Pennsylvania dairy herds (Wilson *et al.*, 1997). Antibiotics are the standard treatment but are often less than 50% effective, at times resulting in premature culling (Deluyker *et al.*, 2005).

Antibiotic resistance in mastitis treatment is of concern because mastitis is the single most common reason for antimicrobial use in dairy herds (Erskine *et al.*, 2002). In a broad study of nine European countries, the USA and Zimbabwe, 57% of 811 *Staphylococcus aureus* isolates from bovine mastitis showed resistance to penicillin (De Oliveira *et al.*, 2000), and 44% of the mastitis strains identified in an Ohio study demonstrated resistance to at least one antibiotic (Rajala-Schultz *et al.*, 2004). FDA, USDA and CDC promote the development of antimicrobials that reduce risk of resistance development (CDC Action Plan: http://www.cdc.gov/drugresistance/actionplan/html/product.htm). The use of pathogen-specific antimicrobials is expected to reduce the incidence of resistance development (Walsh, 2003; Nathan, 2004).

To reduce the use of broad-range antibiotics and thus decrease the chance of antibiotic resistance development, our goal is to develop pathogen-specific antimastitis agents that can be expressed in mammary glands of transgenic cattle (Kerr & Wellnitz, 2003; Donovan *et al.*, 2005; Wall *et al.*, 2005). Bacteriophage endolysins specifically degrade the peptidoglycan of their host cell wall, thus lysing the bacteria, and allowing infective phage to escape. Bacteriophage endolysins are of interest as antimicrobials against Gram-positive organisms (Loessner, 2005) due to their high host specificity and reports that Gram-positive bacteria are highly unlikely to develop resistance to the peptidoglycan hydrolyzing action of their bacteriophage endolysins (Loeffler *et al.*, 2001; Schuch *et al.*, 2002). Peptidoglycan hydrolases have been proposed for human antimicrobial applications (Schuch *et al.*, 2002; Fischetti, 2003, 2005) and have demonstrated efficacy in mouse models of human disease (Nelson *et al.*, 2001; Jado *et al.*, 2003; Cheng *et al.*, 2005) as well as transgenic murine (Kerr *et al.*, 2001) and bovine (Wall *et al.*, 2005) mammary glands.

Peptidoglycan hydrolases can be divided into three general types: glycosidases, amidases and endopeptidases (Lopez & Garcia, 2004), and are often encoded by highly conserved domains (Loessner, 2005). The conserved domains are ~200 amino acids in size (Bateman & Rawlings, 2003; Huard *et al.*, 2003; Rigden *et al.*, 2003), and are readily identified using common domain databases (Uniprot: http://www.pir.uniprot.org/index.shtml; Pfam: http://www.sanger.ac.uk/cgi-bin/Pfam/getacc?PF04650; SMART: http://smart.embl-heidelberg.de/, or NCBI conserved domain database). The domains are referred to as modules because they can often function independently of the remainder of the native endolysin (Navarre *et al.*, 1999; Morita *et al.*, 2001; Yokoi *et al.*, 2005; Donovan *et al.*, 2006), and maintain activity when fused to create novel, recombinant, fusion hydrolases (Diaz *et al.*, 1990; Garcia *et al.*, 1990; Donovan *et al.*, 2006).

The *Staphylococcus aureus* (NCTC 8325) phi11 prophage endolysin gene was first isolated from the *Staphylococcus aureus* genome as the LytA amidase (Wang *et al.*, 1991) and was later shown to be a phage lytic enzyme. It contains two peptidoglycan hydrolase activities: a cysteine, histidine-dependent amidohydrolases/peptidase (CHAP) endopeptidase (Bateman & Rawlings, 2003; Rigden *et al.*, 2003) that cleaves at D-alanyl-glycyl moieties, and an amidase domain that cleaves at *N*-acetylmuramyl-L-alanyl bonds (Navarre *et al.*, 1999). The phi11 endolysin also contains a C-terminal SH3b cell wall-binding domain (Baba & Schneewind, 1998). Many related endolysins with high amino acid sequence identity to the phi11 endolysin sequence (Genbank Accession # P24556) have been identified from staphylococcal phage genomes [phi 80 alpha: 99% (AAB39699); bacteriophage 69: 91% (YP_239596); bacteriophage 29: 91% (YP_240560); bacteriophage 92: 90% (YP_240773); bacteriophage 52A: 90% (YP_240634); bacteriophage 55: 90% (YP_240484); bacteriophage mu 50: 89% (BAB57075); bacteriophage 88: 89% (YP_240699); and bacteriophage 37: 76% (YP_240103)] (Kwan *et al.*, 2005).

A weakly homologous (40% identical) *Staphylococcus warneri* M phage phi WMY endolysin was recently described and shown to share the same domain organization [N-terminal CHAP endopeptidase – amidase – C-terminal SH3b] as the phi11 endolysin (Yokoi *et al.*, 2005). Via site-directed and deletion mutagenesis analysis, it was shown that both the CHAP endopeptidase domain and the amidase domains of the phi WMY endolysin contribute to degradation of autoclaved staphylococci in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) zymograms. Despite knowledge of the peptidoglycan hydrolase enzymatic activities of this and other *Staphylococcus aureus* bacteriophage endolysins, only one published phage endolysin, LysK, also sharing a similar modular organization as previously described, has been shown to kill untreated staphylococci. LysK also kills many CoNS and clinically relevant, multi-drug-resistant strains of *Staphylococcus aureus* (O'Flaherty *et al.*, 2005).

We have examined the 481 amino acid phi11 endolysin for its ability to lyse untreated *Staphylococcus aureus* and CoNS, and for its pH and calcium concentration optima. We have also truncated the phi11 endolysin to remove the SH3b domain and thus test for its role in 'lysis from without'.

Materials and methods

Bacterial strains and culture conditions

Cloning and vector constructs were maintained in *Escherichia coli* DH5α cells (Invitrogen, Carlsbad, CA) or BL21 (DE3) (EMD Biosciences, San Diego, CA). *Staphylococcus aureus* (ATCC 29740), and the mastitis isolates *Streptococcus agalactiae*, *Staphylococcus chromogenes*, *Staphylococcus epidermidis*, *Staphylococcus hyicus*, *Staphylococcus simulans*, *Staphylococcus warneri and Staphylococcus xylosus* (USDA) were grown at 37 °C in Brain Heart Infusion (DIFCO, Franklin Lakes, NJ).

PCR cloning

The Amp^R plasmid pTZ18R contains the phi11 endolysin on a 3 kb EcoRI fragment of the phi11 bacteriophage genome (a gift from R. Jayaswal) (Jayaswal *et al.*, 1990). The endolysin gene was subcloned into pET21a (EMD Biosciences, San Diego, CA) via PCR of the pTZ18R plasmid template. High-fidelity PCR (Deep Vent Polymerase; New England Biolabs, Ipswich, MA) with primers that introduce unique restriction enzyme (RE) sites NdeI (CATATG, includes an ATG translation initiation site) and XhoI (at the translational stop sequence, amino acid 481) were used to amplify the full-length phi11 endolysin-coding sequences (phi11-481). Primers: F LytA NdeI, 5′-GTG GCG CAT ATG CAA GCA AAA TTA AC-3′ and R LytA XhoI 481 5′-T GAC TAT GTC CTC GAG ACT GAT TTC-3′ (the introduced RE sites are underlined). These sites were designed to allow subcloning into pET21a such that a six Histidine tag (in pET21a) was added at the C-terminus of the phi11 protein. Gradient thermocycling determined optimal primer annealing temperature and PCR conditions. Gel-purified (Qiaex, Qiagen, Valencia, CA) PCR products were digested, desalted (Micro Bio-Spin 30 columns) (BioRad, Hercules, CA) or gel-purified and ligated to a similarly digested, dephosphorylated (Shrimp Alkaline Phosphatase, Roche, Basel, Switzerland) and purified recipient vector (pET21a) using standard techniques. In order to subclone truncated phi11 endolysin protein-coding sequences, two reverse primers that introduce XhoI sites at amino acid 194 and 389 were utilized [R LytA XhoI 194: 5′-ACT ACC ACG CTC GAG TAG GTC -3′; R LytA XhoI 389: 5′-AGT ACC ATA TTT CTC

GAG TTT CCA TGC-3′] with the NdeI forward primer F LytA NdeI (above). These primers create a truncated protein coding sequence that, when cloned into pET21a, fused the truncated phi11 endolysin to the six x His tag codons of the pET21a vector. Ligations were transformed into *E. coli* DH5α (Invitrogen, Carlsbad, CA), DNA isolated (Qiagen, Valencia, CA), RE site mapped and retransformed into BL21(DE3) (EMD Biosciences, San Diego, CA) for expression and purification of protein products. All constructs tested positive for peptidoglycan hydrolase activity (in lieu of DNA sequence analysis).

Extract preparation and protein purification

Escherichia coli BL21(DE3) cells harboring plasmid constructs were grown in 500 mL Superbroth (Becton Dickenson, Franklin Lakes, NJ) supplemented with 100 μg mL^{-1} ampicillin at 37 °C with shaking. Mid log phase ($OD_{600\,nm}$ of 0.4–0.6) cultures were induced with 1 mM IPTG (isopropyl-beta-D-thiogalactopyranoside), followed by 4 h shaking at 37 °C. Cells were pelleted, washed with lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM Imidazole, pH 8) and pellets frozen at −80 °C. Extracts were prepared according to a modified procedure of Pritchard *et al*., 2004 (Neville *et al*., 1994). For nickel column-purified protein, 500 mL culture cell pellets were resuspended in 10 mL Lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM Imidazole, pH 8), sonicated (15 × 5 s pulses with 15 s rest between), centrifuged at 6800 r.p.m. in a Sorvall HS4 rotor (8500 ***g***), supernatant decanted and added to 5 mL Ni-NTA (nickel matrix) slurry according to the manufacturer's instructions (Qiagen, Valencia, CA) with gentle rocking for 1 h at 4 °C. The matrix was washed and protein batch eluted according to the manufacturer's instructions. Protein eluates were desalted in Micro Bio-Spin 30 columns (BioRad) prior to protein determination with BCA protein assay (Pierce, Rockford, Ill.). The various protein fractions and Kaleidoscope protein standards (Invitrogen, Carlsbad, CA) were analyzed with 15% SDS-PAGE run in Tris-Glycine buffer at 131 V for 1.5 h in the BioRad Mini-PROTEAN three gel apparatus, according to the manufacturer's instructions. Gels were stained in BioSafe Coomassie stain (BioRad, Hercules, CA) for 1 h and then destained in 50% methanol/10% Acetic Acid solution in distilled water overnight.

Turbidity assay

The turbidity assay measures a decline in optical density due to lysis of the target bacteria with the phage endolysin-derived protein. Mid log phase ($OD_{600\,nm}$ of 0.4–0.6) target cells were grown in Brain Heart Infusion (Becton Dickenson, Franklin Lakes, NJ) and concentrated in lysin buffer A (LBA, 50 mM ammonium acetate, 10 mM $CaCl_2$, 1 mM DTT pH 6.2) to an $OD_{600\,nm}$ ∼2.0. Turbidity assays were performed with 25 μg of purified phi11 endolysin-derived proteins in a cuvette and were initiated, by addition of target cells to a final $OD_{600\,nm}$ of ∼1.2 at room temperature. Changes in OD were recorded for 1 h $OD_{600\,nm}$. Changes in the cells alone sample were subtracted from samples with lysin, before calculating the activity (change in $OD_{600\,nm}$ mg protein^{-1} min^{-1}). Optimum pH and Ca^{2+} concentration were determined with modified LBA. Changes in pH were performed with Glacial acetic acid or ammonium hydroxide. $CaCl_2$ was added at the appropriate concentrations to LBA minus $CaCl_2$. Results are often reported as 'representative' plots due to the instability of the phi11-derived proteins, and the resulting high degree of variability from assays performed on different days. However, the trends identified were consistent among the multiple preparations.

Results

C-terminal truncations of the phi11 endolysin

The full-length (481 codon) phi11 endolysin protein-coding region was subcloned between the NdeI and XhoI sites of pET21a. All constructs described in this work were derived from this parental clone (Fig. 1). With the long-term goal to express the phi11 endolysin in transgenic cattle mammary glands, a series of C-terminal deletions were created in order to 1) reduce the amount of protein engineering necessary to express a bacterial protein in a eukaryotic system, and 2) to determine whether the SH3b domain was essential for pathogen lysis. The C-terminal truncation phi11-194 contains the entire CHAP domain, bisects the amidase domain and lacks the SH3b domain. The phi11-389 construct contains both the CHAP domain and the amidase domain, but lacks the SH3b domain. Partially purified nickel column preparations of full length and truncated gene products are

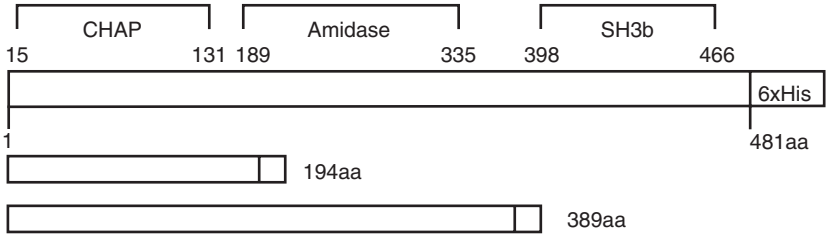

Fig. 1. Schematic of phi11 endolysin protein structure and deletion constructs. phi11 194 and phi11 389 deletion constructs are illustrated, along with the approximate location of the CHAP, amidase and SH3b cell wall-binding domains.

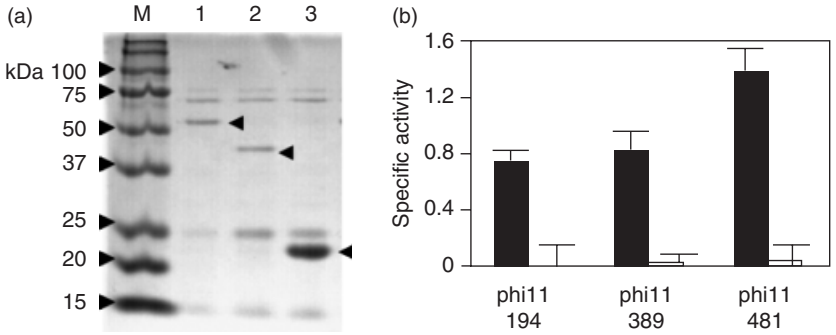

Fig. 2. SDS-PAGE and Turbidity assay results of full length and phi11 endolysin truncations. (a) 3 μg of nickel column-purified phi11 endolysin-derived proteins. Lane M, Markers; 1, phi11-481; 2, phi11-389; 3, phi11-194. Carrots indicate phi11-derived proteins. (b) 25 μg of purified endolysin-derived proteins are active against *Staphyloccocus aureus* (black bars). There is no activity against *Streptococcus agalactiae* (open bars). Specific Activity ($\Delta OD_{600\,nm}\,mg^{-1}\,min^{-1}$). Extracts from *Escherichia coli* harboring just pET21a vector do not have lytic activity in the turbidity assays against *Streptococcus agalactiae* or *S. aureus* (data not shown).

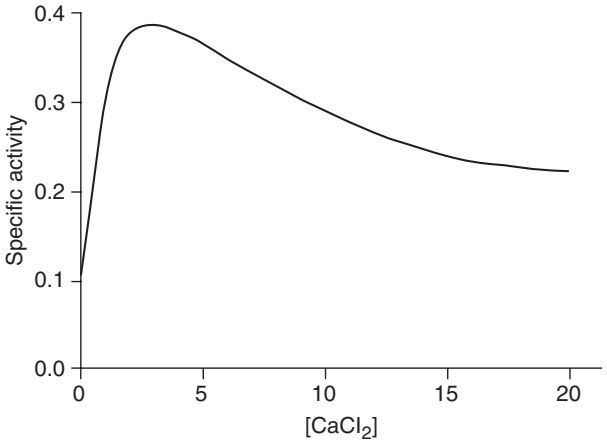

Fig. 3. Effect of $CaCl_2$ concentration on lytic activity of phi11-481 endolysin. Specific Activity = $OD_{600\,nm}\,mg^{-1}\,min^{-1}$. (Graph is a representative plot.) Twenty-five micrograms endolysin per sample.

visualized in SDS-PAGE (Fig. 2a). Both truncations and the full-length protein lyse *Staphylococcus aureus* in turbidity assays (Fig. 2b). None of the constructs were lytic towards *Streptococcus agalactiae* (Fig. 2b). The CHAP domain's ability to lyse *Staphylococcus aureus* in the absence of the amidase domain is consistent with the peptidoglycan hydrolase activity of a previous phi11 endolysin deletion, which removed the amidase domain from the center of the protein, fusing the phi11 CHAP domain to the SH3b cell wall-binding domain (Navarre *et al.*, 1999). The lytic activity of the phi11-194 construct indicates that the CHAP domain alone is sufficient for exolysis ('lysis from without') of the target bacteria. The intact phi11-481 protein has significantly greater activity than either the phi11-194 or phi11-389, suggesting that the SH3b domain may not be essential, but does improve the exolytic activity.

The phi11-389 construct has a slightly higher exolytic activity compared with phi11-194, suggesting that the amidase domain does contribute to exolysis of *Staphylococcus aureus*, as opposed to the Acm 'mid-protein' domain of the streptococcal bacteriophage B30 endolysin, which is essentially silent during exolysis of streptococci (Donovan *et al.*, 2006).

phi11 endolysin is active at the physiological pH and Ca^{2+} concentration of milk

The physiological pH and free calcium concentration of bovine milk is 6.7 and 3 mM, respectively (http://www.foodsci.uoguelph.ca/dairyedu/chem.html#overview) (Pritchard *et al.*, 2004). The phi11-481 endolysin has peak activity in the turbidity assay at 2–3 mM $CaCl_2$ (Fig. 3), and is active at a broad range of physiologically relevant pHs (Fig. 4).

Phi11 endolysin is lytic towards CoNS

The CoNS have been reported to contribute to 11% of mastitis in one study from Pennsylvania and New York (Wilson *et al.*, 1997), and 12% in another study where *Staphylococcus simulans* accounted for 53% of the 149 CoNS isolates (Waage *et al.*, 1999). The phi11-481 endolysin is lytic for six representative strains (including *Staphylococcus simulans*) that were isolated from mastitis infections (USDA) and identity verified via the University of MD College Park microbiology service (Fig. 5).

Discussion

Our goal is to avoid the use of broad-range antibiotics through the identification of pathogen-specific protein antimicrobials that can be expressed in transgenic cow mammary glands. *Staphylococcus aureus* and CoNS are major mastitis pathogens in the USA. The *Staphylococcus*

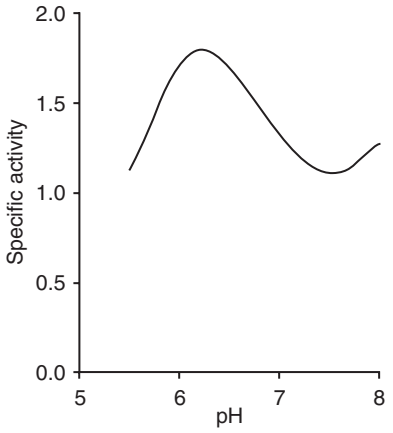

Fig. 4. Effect of pH on lytic activity of phi11-481 endolysin targeting *Staphyloccocus aureus*. [Graph is a representative plot.] Specific Activity ($OD_{600\,nm}\,mg^{-1}\,min^{-1}$). Twenty-five micrograms endolysin per sample.

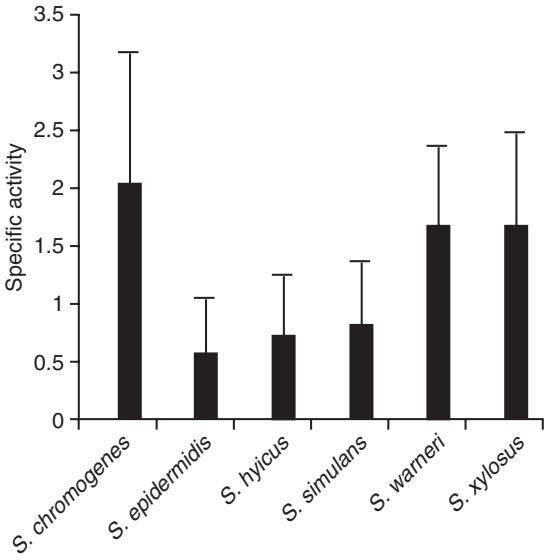

Fig. 5. Turbidity assay results of phi11-481 endolysin lysis of CoNS. Specific Activity ($OD_{600\,nm}\,mg^{-1}\,min^{-1}$). Twenty-five micrograms endolysin per sample.

aureus phi11 endolysin has both an amidase and an endopeptidase domain that are specific for and have been shown to be enzymatically active on cell wall preparations of *Staphylococcus aureus* (Navarre *et al.*, 1999). We have extended this work to demonstrate that the endolysin has exolytic activity toward untreated *Staphylococcus aureus* and mastitis-causing CoNS, and is active at the physiological pH and calcium concentrations found in milk.

Previous work of Navarre *et al.* (1999) created a deletion construct that removed the amidase mid-region of the protein and fused the N-terminal CHAP endopeptidase to the C-terminal SH3b-binding domain. This work demonstrated that the CHAP domain did not require the amidase domain for enzymatic activity but it was not shown whether or not the CHAP domain requires the SH3b domain for activity and specificity, nor did it demonstrate exolytic activity on living cells. With the phi11-194 construct, we demonstrate that the CHAP domain is sufficient to lyse untreated *Staphylococcus aureus* cells without a need for either the amidase domain or the SH3b domain. The *Staphylococcus warnei* M phage phiWMY endolysin (lysWMY) has a similar CHAP-amidase-SH3b protein organization and is only 43% identical to the phi11 endolysin. The lysWMY CHAP domain has lytic activity towards several SDS-treated staphylococci in zymograms, including many CoNS and *Staphylococcus aureus* (Yokoi *et al.*, 2005), but was not tested on live *Staphylococcus aureus* or CoNS. The *Staphylococcus aureus* K phage endolysin, lysK, also has a similar modular organization, and although only 41% identical to the phi11 endolysin, is the only other staphylococcal phage endolysin that has been reported to lyse untreated *Staphylococcus aureus* and CoNS cells (in a plate lysis assay) (O'Flaherty *et al.*, 2005). For use as an antimastitis agent, it is essential that the endolysin have exolytic activity.

It is also important to our project that the antimicrobial be specific to its pathogen group. The broad-range antibiotics pirlimyacin and penicillin are widely used in the treatment of mastitis (Cattell *et al.*, 2001). Use of broad-range antimicrobials, although successful, is discouraged due to concerns that antibiotic resistance among mastitis pathogens is on the rise (Werckenthin *et al.*, 2001; Rajala-Schultz *et al.*, 2004), and the fear that such strains might find their way from the farm to the clinic (Ferber, 2002, 2003). To reduce concerns about antimicrobial resistance among mastitis pathogens, it is our goal to identify protein antimicrobials that do not have activity against a broad range of pathogens but rather are pathogen-specific (or lyse only closely related species). We have shown that neither the full length nor truncated phi11 endolysin constructs will lyse *Streptococcus agalactiae* in the turbidity reduction assay.

Similarly, it is important that a mastitis antimicrobial be active in a milk environment. We have characterized the full-length phi11-481 endolysin and demonstrate antimicrobial activity at both the pH (6.7) and free-calcium concentration (3 mM) consistent with milk (Pritchard *et al.*, 2004). This activity is comparable to that of lysostaphin (data not shown) and this is encouraging because lysostaphin has a proven track record of preventing *Staphylococcus aureus* mammary infections in transgenic mammary glands in mice (Kerr *et al.*, 2001) and cattle (Wall *et al.*, 2005).

Although the results of lysostaphin expression in transgenic cattle and mice are encouraging, we believe that the phi11 endolysin is potentially a better antimicrobial for several reasons. First, it is known that lysostaphin has a single endopeptidase function cleaving glycyl-glycyl bonds

of the peptidoglycan peptide cross bridge (Browder *et al.*, 1965) and that strains have developed resistance to this activity by inserting serines into the cross bridge (Thumm & Götz, 1997). Second, the phi11 endolysin potentially has two putative antimicrobial activities: an endopeptidase and an amidase, and it is known that for a pathogen to develop resistance to two enzymatic activities would require two simultaneous compensatory mutations in the same cell. This is believed to be a rare event. Third, it is reported that through the coevolution of bacteriophage and their hosts the bacteriophage endolysins target essential bonds in the peptidoglycan, such that despite significant effort to find them, no resistant strains have been identified to those phage endolysins that have been screened (Loessner, 2005). Finally, the phi11 endolysin is potentially a better antimastitis agent, because it lyses multiple mastitis pathogens (both *Staphylococcus aureus* and CNS) while lysostaphin lyses *Staphylococcus aureus* alone.

We are continuing to quantify the activity of the truncated and full-length phi11 endolysins in milk and when expressed in mammalian cells. The highly conserved, yet apparently nonessential role of the SH3b-binding domain of phage endolysins during cellular exolysis raises unanswered questions about these complex modular proteins.

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