



Published in final edited form as:

*FEMS Microbiol Lett.* 2013 May ; 342(1): 30–36. doi:10.1111/1574-6968.12104.

## Chimeric Ply187 endolysin kills *Staphylococcus aureus* more effectively than the parental enzyme

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### Abstract

Peptidoglycan hydrolases are an effective new source of antimicrobials. A chimeric fusion protein of the Ply187 endopeptidase domain and LysK SH3b cell wall binding domain is a potent agent against *Staphylococcus aureus* in four functional assays.

### Keywords

Bacteriophage; endolysin; CHAP domain; SH3b cell wall binding domain; *Staphylococcus aureus*

### Introduction

*Staphylococcus aureus* is a pathogen that causes a broad spectrum of human and animal diseases and has adapted to antibiotic selective pressures resulting in a high prevalence of multi-drug resistant strains (de Lencastre *et al.*, 2007). The spread of these antibiotic-resistant strains is a threat to public health and a critical concern to health care providers worldwide. Thus, the search for novel antimicrobials against pathogens that are refractory to resistance development is essential.

Phage endolysins are cell wall hydrolases that are produced near the end of the phage lytic cycle to help the nascent phage escape the infected host. Endolysins are ideally suited as antimicrobials for several reasons as described previously (Loessner, 2005, Donovan *et al.*, 2009). It has been postulated that phage endolysins have co-evolved with their host such that they target cell wall bonds that are believed difficult for the host cell to alter, and thus bacterial resistance to phage endolysins is unlikely (Fischetti, 2005). Due to the absence of an outer membrane as in Gram-negative bacteria, endolysins are able to access the Gram-positive peptidoglycan ‘from without’ and lyse these bacteria when exposed externally.

The *S. aureus* bacteriophage 187 endolysin (Ply187) gene was initially reported by Loessner *et al.* (Loessner *et al.*, 1999). Ply187 consists of 628 amino acids with a calculated molecular mass of 71.6 kDa. Typically, endolysins from a Gram-positive background have a modular structure with an N-terminal catalytic domain for peptidoglycan hydrolysis and a C-terminal cell wall binding domain (CBD) (Loessner, 2005). However, the Pfam domain database indicates that the amino terminus of Ply187 harbors a cysteine, histidine-dependent amidohydrolase/peptidases (CHAP) domain (Bateman & Rawlings, 2003, Rigden *et al.*, 2003) and the C-terminus contains a glucosaminidase domain with no known CBD

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(Loessner *et al.*, 1999) (Fig. 1A). CBDs are essential for some endolysins' lytic activity and often determine specificity (Baba & Schneewind, 1996, Lu *et al.*, 2006, Loessner *et al.*, 2002, Grundling & Schneewind, 2006, Sass & Bierbaum, 2007). Ply187 is, thus, somewhat unique among lysins of Gram-positive phages in that it lacks a CBD. The Ply187 CHAP domain shows weak homology (40% identity) with the CHAP domain of other well-characterized lysins, such as LysK, an endolysin from phage K (O'Flaherty *et al.*, 2005). Previous experimental data indicates that cloned full-length Ply187 is nearly inactive while C-terminal truncated Ply187 (1-157aa; Ply187AN) is much more active than the full-length protein, suggesting an inhibitory domain at the C-terminus (Loessner *et al.*, 1999). In an effort to improve the Ply187 lytic activity the Ply187AN sequences were fused to the LysK SH3b cell wall binding domain (KSH3b) to generate a chimeric Ply187AN-KSH3b fusion protein (Fig. 1A), similar to work reported from this lab with the streptococcal LambdaSa2 (LSA2) endolysin N-terminal lytic domain (Becker *et al.*, 2009). In a series of functional assays we have demonstrated that this fusion protein is much more active than the parental Ply187AN truncated enzyme.

## Materials and Methods

**Strains**—All Gram positive strains used to determine lysin target range are described in Table 1. All plasmid constructs were created and cryopreserved in *E. coli* DH5 $\alpha$  (Invitrogen, Carlsbad, CA) and expressed from freshly transformed *E. coli* BL21 (DE3) (Invitrogen, Carlsbad, CA) as described below.

### Construction of expression vectors

The Ply187 protein sequence is available (Y07740) through GenBank. The truncated Ply187 N-terminal domain (Ply187AN; 1-157aa) was commercially synthesized based on published sequence (Loessner *et al.* 1999). To enhance the heterologous expression of Ply187 endolysin in *E. coli*, the sequences encoding the Ply187AN were converted to an *E. coli* codon bias, commercially synthesized and subcloned into pUC57 with engineered 5' NdeI (CATATG; ATG = start of translation) and 3' XhoI (CTCGAG; codes for aa's LE) restriction enzyme sites (Genscript; Piscataway, NJ). These sites did not introduce translational stop codons and were used to subclone the protein coding sequences into pET21a for expression and purification in *E. coli* B21 (DE3) (EMD Biosciences, San Diego, CA). Subcloning of the Ply187AN construct into the pET21a expression vector was via conventional means, effectively adding six C-terminal His codons to the protein coding sequences, using a strategy similar to previous constructs from the Donovan lab (Becker *et al.* 2009). Similarly, the chimeric Ply187AN (Ply187AN-KSH3b) was fused to the LysK SH3b by subcloning the Ply187AN NdeI-XhoI DNA fragment harboring all coding sequences into a similarly digested pre-constructed pET21a-KSH3b vector described previously (Becker *et al.* 2009). Recombinant LysK was used in this work as a positive control (Becker *et al.* 2009).

### Recombinant protein expression and purification

For protein expression, *E. coli* B21 (DE3) (EMD Biosciences) expression cultures were grown at 37°C in Luria-Bertani (LB) broth containing ampicillin (100 $\mu$ g/ml) to an OD<sub>600nm</sub> of 0.4 – 0.6, chilled on ice for 30 min., induced by addition of isopropyl-b-D-thiogalactopyranoside (IPTG) to a final concentration of 1mM, and grown at 10°C for 20 hrs. *E. coli* harvested from 500 ml cultures were lysed via sonication and His-tagged proteins isolated using nickel-chromatography Ni-NTA (Qiagen, Valencia, CA) as described previously (Becker *et al.* 2009). Briefly, wash and elution profiles were empirically determined to be, 10 ml of 10 mM imidazole, 20 ml of 20 mM imidazole and elution with 0.5 ml of 250 mM imidazole in the same phosphate-buffered saline (PBS) (50 mM

NaH<sub>2</sub>PO<sub>3</sub>, 300 mM NaCl, pH8.0) with 30% glycerol to prevent precipitation of the purified protein. All samples were then desalted with Zeba desalting column (Pierce, Rockford, IL) equilibrated in 2X PBS buffer and filter sterilized. Sterilized protein preparation was stored at 4°C in 2X PBS buffer until the time of assay.

### SDS-PAGE and zymogram analysis of Ply187 constructs

4µg per lane of each construct and Kaleidoscope protein standards (Bio-Rad) were analyzed using 15% SDS-PAGE. Zymograms were prepared identically as the SDS PAGE gels, but with 300 ml culture equivalents of mid log phase *S. aureus* cells (OD<sub>600nm</sub> of 0.4–0.6). The SDS-PAGE and zymogram were running simultaneously. SDS gels were Coomassie stained and zymograms were washed in excess water for 1 hr and incubated at room temperature in PBS, resulting in areas of clearing in the turbid gel where a lytic protein had localized.

### Plate lysis assay

Plate lysis assays are described previously (Becker et al, 2009). In brief, purified proteins for each construct were sterilized and diluted in sterile PBS buffer with 30% glycerol. 10µl containing 10, 1 or 0.1 µg of test protein were spotted onto a freshly spread lawn of mid-log phase (OD<sub>600</sub> ~ 0.4–0.6) of either *S. aureus* Newman or *S. aureus* 305 cells that had air-dried for 10 min on tryptic soy agar plates. The spotted plates were air-dried for 10 min in laminar flow hood, and incubated overnight in a 37 C environment. Scoring of the cleared spots occurred with 20 h of plating the cells.

### Turbidity reduction assay

Turbidity reduction assays are described previously (Becker et al, 2009). In brief, staphylococcal strain *S. aureus* Newman grown to logarithmic phase (OD<sub>600nm</sub> = 0.4–0.6) at 37°C in Brain Heart infusion broth (DIFCO, Franklin Lakes, NJ) was performed in a 96-well dish and analyzed in a plate reader. Rates of lysis that occur with 0.2 ml of *S. aureus* cells at an OD<sub>600nm</sub> = 1.0 in a 96 well plate were determined every 20 seconds by a plate reader [ $\Delta$ OD<sub>600nm</sub>] for five minutes. Peaks in OD are determined on a sliding scale and reported results are within the linear range of the assay. Results are presented as Specific activities ( $\Delta$ OD<sub>600nm</sub>/µg/min).

### Minimal inhibitory concentration assay

A classical microdilution broth method for determination of the MIC was used (Jones et al 1985) with modifications as described previously (Becker et al 2009). All MIC values represent four assays with 3 replicates in each assay.

**Milk Assays**—Fresh milk samples obtained from the USDA BARC, Beltsville, MD dairy herd was pasteurized for 30 minutes at 63°C. The milk was brought to 37°C and was spiked with exponentially growing (OD<sub>600nm</sub> = 0.4 – 0.6) cells of the mastitis causing *S. aureus* strain Newbould 305 at a concentration of  $\sim 1 \times 10^3$  cfu/ml. Immediately after inoculation, purified enzyme was added at a concentration of 8 µM, and the milk was incubated at 37°C without shaking for 3 hours. Samples were taken immediately before and immediately after addition of enzyme, as well as after 30, 60, 120, and 180 minutes, and the CFUs determined by direct plating on duplicate TSA plates. A sample spiked with lysin buffer alone, no enzyme served as a control. The absence of CFUs in pasteurized milk was verified by direct plating on TSA plates.

## Results

### SDS PAGE and Zymogram analysis of Ply187-derived lysins

Nickel chromatography purified proteins were analyzed using 15% SDS-PAGE and Kaleidoscope protein standards (Bio-Rad, Hercules, CA) (Fig. 1B), with or without 300 ml culture equivalents of mid log phase *S. aureus* cells ( $OD_{600nm} = 0.4-0.6$ ) embedded in the gel as described previously (Becker *et al.*, 2009), to verify the absence of co-purifying lytic contaminants. Coomassie-stained SDS-PAGE of each purified protein CHis-Ply187AN and C-His-Ply187AN-KSH3b indicated that the two constructs were able to be expressed in *E. coli* and purified at greater than 95% purity (Becker *et al.*, 2009). Zones of lysis on the zymogram gel run in parallel with the SDS-PAGE indicate that the predicted protein in each preparation is the only protein with staphylolytic activity (Fig. 1B).

### Chimeric Ply187AN-KSH3b lysin is active in killing live *S. aureus* in plate lysis, turbidity reduction and MIC assays

To evaluate the fusion constructs' potential as an antimicrobial agent, we verified and quantitated the lytic activity of Ply187AN-KSH3b, the parental truncation (Ply187AN), and a strong antimicrobial endolysin, LysK (Becker *et al.*, 2009, O'Flaherty *et al.*, 2005) in three different antimicrobial assays. The plate lysis assay results in Fig. 2A demonstrate that both 10  $\mu$ g and 1  $\mu$ g LysK (0.2 and 0.02  $\mu$ mol, respectively; molecular weight (MW): 55.8 kD) produce a zone of clearing, indicating that 1  $\mu$ g LysK in 10  $\mu$ l of buffer is effective at eliminating the *S. aureus* lawn, consistent with previous reports (Becker *et al.*, 2009, O'Flaherty *et al.*, 2005). In contrast, only 10  $\mu$ g of Ply187AN (0.5  $\mu$ mol; MW: 18.9 kD) produced a zone of clearing in the plate lysis assay, indicating that Ply187AN is less effective than LysK in this assay. Surprisingly, Ply187AN-KSH3b produces a zone of clearing at 10, 1  $\mu$ g, and 0.1  $\mu$ g (0.3, 0.03, and 0.003  $\mu$ mol, respectively; MW: 30.6 kD), indicating that Ply187AN-KSH3b is more active than Ply187AN and LysK. All bacterial strain susceptibility tests are described in Table 1. The staphylococcal strains included bovine mastitis isolates, MRSA strains, and coagulase negative staphylococci. Both the fusion protein and LysK were able to lyse all staphylococcal strains tested, with Ply187AN-KSH3b exhibiting higher activity than LysK against many strains when compared on a molar basis. In contrast, both enzymes were inactive against non-staphylococcal strains with the exception of Ply187AN-KSH3b showing weak activity against *Streptococcus dysgalactiae*.

To further quantify the degree of lytic enhancement obtained from the fusion of the Ply187 CHAP domain to the KSH3b domain we compared the staphylolytic activities in both turbidity reduction (Fig. 2B) and minimal inhibitory concentration (MIC) assays (Fig. 2C). A standardized turbidity assay modified from (Donovan *et al.*, 2006) with staphylococcal strains grown to logarithmic phase ( $OD_{600nm}=0.4-0.6$ ) at 37° C in Brain Heart infusion broth (DIFCO, Franklin Lakes, NJ) was performed in a 96-well dish and analyzed in a plate reader as described previously (Becker *et al.*, 2009). The specific activity for LysK, Ply187AN and Ply187AN-KSH3b is  $1.2 \pm 0.4$ ,  $0.08 \pm 0.03$ , and  $1.2 \pm 0.2 OD_{600nm} \mu\text{mol}^{-1}\text{min}^{-1}$ , respectively. Consistent with the plate lysis assay, Ply187AN was only about 10% as active as LysK. However, the addition of the KSH3b domain to the Ply187 CHAP domain yields a ten-fold increase in specific activity (Fig. 2B). The resulting activity of Ply187AN-KSH3b is similar to that of the recombinant LysK protein. A classical microdilution broth method for determination of the MIC was used (Jones et al 1985) with modifications as described previously (Becker *et al.*, 2009) to determine the MIC for each construct. In the MIC assay, LysK inhibits growth of *S. aureus* Newman at concentrations of  $35.6 \pm 10.5 \mu\text{g ml}^{-1}$ , corresponding to  $0.6 \pm 0.2 \mu\text{mol ml}^{-1}$ , which is comparable to previous results (Becker et al 2009). The MIC for Ply187AN is  $53.5 \pm 12.0 \mu\text{g ml}^{-1}$  ( $2.8 \pm$

0.6  $\mu\text{mol ml}^{-1}$ ). Similar to the prior two antimicrobial assays, Ply187AN-KSH3b is more active than Ply187AN, with an MIC of only  $7.6 \pm 2.9 \mu\text{g ml}^{-1}$  ( $0.24 \pm 0.09 \mu\text{mol ml}^{-1}$ ), indicating that this CHAP domain yields a ~ten-fold increase in specific activity compared to Ply187AN. The fusion MIC is five-fold lower than that of LysK's (and only 2-fold lower when compared on a molar basis). We know that LysK is a very potent antibacterial, showing higher activities in turbidity reduction assays than lysostaphin (Becker *et al.*, 2009). These data indicate that Ply187AN-KSH3b is a potent staphylolytic agent.

### Chimeric Ply187AN-KSH3b lysin is active in pasteurized cows milk

The lytic activity of Ply187-AN-KSH3b, Ply187-AN, and LysK were determined via serial diluting plating of timed samples of milk inoculated with *S. aureus*, and either a lytic enzyme or enzyme buffer alone (Fig. 3). Immediately after addition of enzyme, Ply187-AN-KSH3b samples showed immediate eradication of all CFUs at time zero and remained undetectable throughout the 3-hour period. Ply187-AN and LysK showed no significant difference from the buffer alone control at any time point.

## Discussion

Although the bacterial SH3b domain is readily identified in multiple domain databases, it is still poorly understood at the level of the site of binding. SH3b domains often determine an endolysin's specificity (Baba & Schneewind, 1996, Lu *et al.*, 2006, Grundling & Schneewind, 2006, Sass & Bierbaum, 2007, Becker *et al.*, 2009). Low *et al.* have proposed a model to explain the role of the SH3b domain in lysin lytic activity (Low *et al.*, 2005). This model suggested that the SH3b domain folds back, binds to and inhibits the lytic domain until it recognizes and binds to peptidoglycan when it releases the lytic domain allowing digestion of the peptidoglycan. Although believed to play a role in substrate recognition and binding specificity, its role must be empirically determined. In deletion experiments, Horgan *et al.*, (Horgan *et al.*, 2009) suggested that deletion of the LysK SH3b domain enhanced LysK CHAP domain enzymatic activity while Becker *et al.* (Becker *et al.*, 2009) showed that fusion of the LysK SH3b domain to the LysK CHAP domain was necessary for CHAP domain activity. Other labs have demonstrated that lysostaphin SH3b domain bind to the *S. aureus* pentaglycine bridge (Baba & Schneewind, 1996, Lu *et al.*, 2006, Grundling & Schneewind, 2006). Despite the lack of a consensus binding site, SH3b domains and other CBDs have been used for decades to develop novel antimicrobials (Diaz *et al.*, 1991, Diaz *et al.*, 1990, Croux *et al.*, 1993, Lopez *et al.*, 1997, Sheehan *et al.*, 1996). Recently, the Fischetti group used a non-SH3b cell wall binding domain to generate a chimeric staphylolytic lysin using the Twort phage endolysin CHAP domain (Daniel *et al.*, 2010). Our lab used staphylococcal SH3b domains in fusions with the streptococcal phage LambdaSA2 endolysin endopeptidase domain to shift the activity from Streptococcus-specificity to an enzyme that recognized both streptococcal and staphylococcal cell walls (Becker *et al.*, 2009). Despite the numerous CBD fusion constructs reported, the Ply187-KSH3 fusion in this study represents the first time that the activity of a lytic domain from an endolysin that naturally lacks a cell wall binding domain was enhanced by adding a known cell wall binding domain.

In conclusion, we have generated a potent chimeric Ply187AN-KSH3b protein by fusing the CHAP endopeptidase domain of endolysin Ply187 from phage 187 and SH3b CBD of LysK from phage K. This chimeric Ply187AN-KSH3b is a more effective antimicrobial than the full length Ply187, the Ply187 truncation (Ply187AN) and also outperforms the known high activity lysin, LysK, in three out of four functional assays.

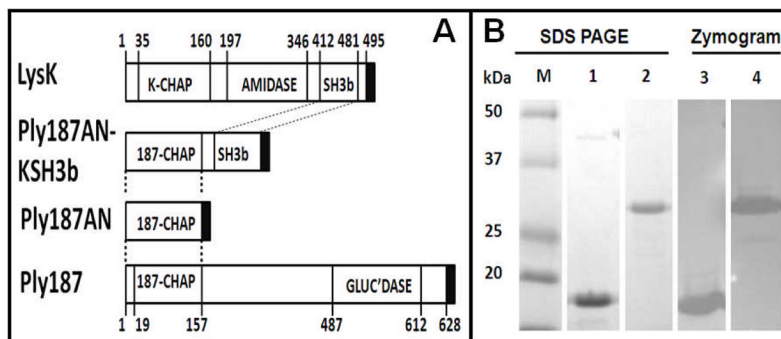
## Acknowledgments

This work was supported in part by Viridax, Inc. Baco Raton, FL, as well as NIH grant1RO1AI075077-01A1; NRI grant 2007-35204-18395 and US State Dept funds supporting a US-Pakistani and US Russian collaboration. All awards are to DMD. Mentioning of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the United States Department of Agriculture.

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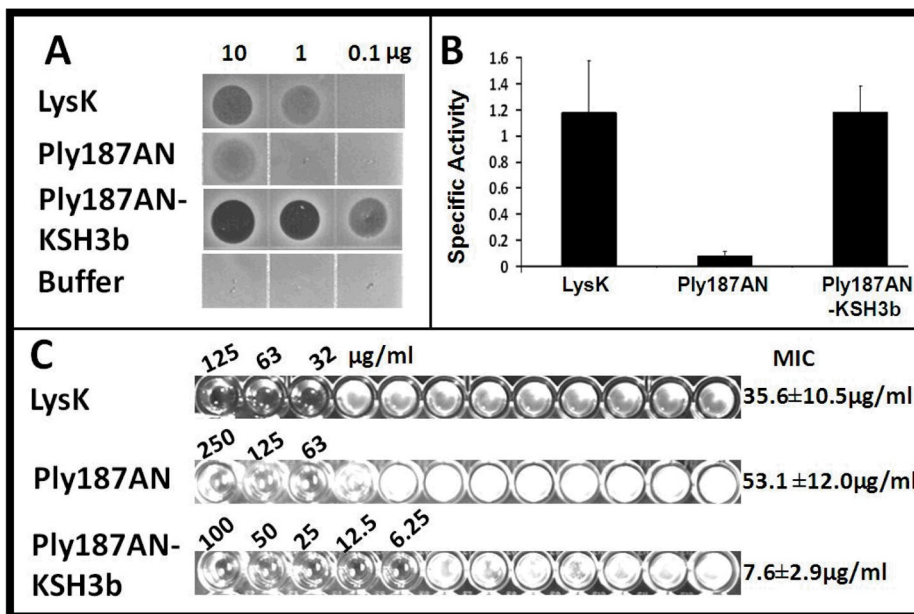
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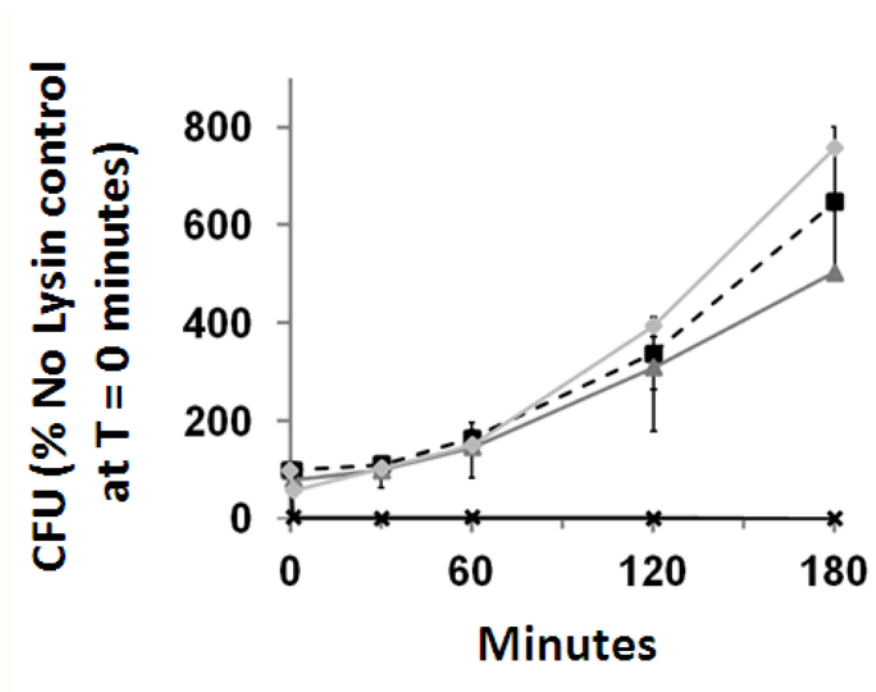
**Figure 1. Constructs utilized, SDS-PAGE and Zymogram analysis of endolysin constructs**  
**A.** Schematic of constructs. Black box = pET21a derived  $6 \times$  His tag; GLUC'DASE = glucosaminidase domain. **B.** SDS-PAGE and zymogram analysis of Ply187 constructs. The proteins migrate as expected for their predicted molecular weights: Ply187AN (Lanes 1 and 3): 18.9kDa, Ply187AN-KSH3b (Lanes 2 and 4): 30.6 kDa.  $4\mu\text{g}$  of each Ni-NTA purified protein was loaded per lane. M = excess prestained Kaleidoscope protein standards (Bio-Rad).





**Figure 2. Fusion of LysK SH3b domain enhances Ply187AN antimicrobial activity in plate lysis, turbidity reduction and MIC assays**

**A.** Representative plate lysis assay with *S. aureus* strain Newman. Zones of clearing represent lysis of the lawn. 10 µg and 1 µg LysK is equivalent to 0.2 and 0.02 µmol, respectively; MW: 55.8 kD. 10 µg of Ply187AN is equivalent to 0.5 µmol; MW: 18.9 kD. 10, 1µg and 0.1 µg of Ply187AN-KSH3b is equivalent to 0.3, 0.03, and 0.003 µmol, respectively; MW: 30.6 kD. **B.** Turbidity reduction assays with *S. aureus* strain Newman. Results are presented as Specific activities ( $\Delta OD_{600nm}/\mu\text{mol}/\text{min}$ ). The assay volume was 200 µl. Error bars represent SEM for three or more independent experiments. **C.** Representative MIC of LysK and purified Ply187 derivatives. The MIC assays were repeated four times with 3 replicates in each assay.“



**Figure 3. Fusion of LysK SH3b domain enhances Ply187AN antimicrobial activity in pasteurized cow milk**

At five time points over a time period of 3 hours, CFU counts increased dramatically in inoculated milk samples following the addition of enzyme storage buffer [■], Ply187AN [◆], and LysK [▲]. However, following the addition of Ply187AN-KSH3b [✕], the CFU counts were reduced to, and stayed, at 0% of the enzyme storage buffer control at time point zero, indicating effective killing. Error bars = SEM.

Table 1

Susceptibility of multiple bacterial strains to lysis by the parental enzyme LysK and the fusion protein Ply187AN-KSH3b

Strain	Source	Susceptibility <sup>a</sup>	
		LysK	Ply187AN-KSH3b
<i>S. aureus</i> Newman	<i>b</i>	++	++(+)
<i>S. aureus</i> MN8	<i>b</i>	++(+)	+++
<i>S. aureus</i> SA113	<i>b</i>	++	+++
<i>S. aureus</i> Reynolds CP5	<i>b</i>	++(+)	+++
<i>S. aureus</i> Newbould (305)	ATCC 29740	++(+)	+++
<i>S. aureus</i> SA019	<i>c</i>	++(+)	+++
<i>S. aureus</i> SA020	<i>c</i>	++(+)	++(+)
<i>S. aureus</i> SA021	<i>c</i>	++	++
<i>S. aureus</i> SA026	<i>c</i>	++	++(+)
<i>S. aureus</i> NRS382 (MRSA)	NRS 382	+(+)	++(+)
<i>S. aureus</i> NRS383 (MRSA)	NRS 383	++	++(+)
<i>S. aureus</i> NRS384 (MRSA)	NRS 384	++	+++
<i>S. aureus</i> NRS385 (MRSA)	NRS 385	++	++(+)
<i>Staphylococcus chromogenes</i>	<i>d</i>	++(+)	++(+)
<i>Staphylococcus epidermidis</i>	<i>d</i>	++	+++
<i>Staphylococcus hyicus</i>	<i>d</i>	+++	+++
<i>Staphylococcus simulans</i>	<i>d</i>	++(+)	+++
<i>Staphylococcus warneri</i>	<i>d</i>	++(+)	+++
<i>Staphylococcus xylosum</i>	<i>d</i>	+++(+)	+++(+)
<i>Streptococcus agalactiae</i>	ATCC 27541	-	-
<i>Streptococcus dysgalactiae</i>	<i>e</i>	-	+
<i>Streptococcus uberis</i>	<i>f</i>	-	-
<i>Listeria monocytogenes</i> Petite ScottA	ATCC 49594	-	-
<i>Rhodococcus equi</i>	<i>g</i>	-	-
<i>Lactobacillus amylovorus</i> 4540	<i>h</i>	-	-
<i>Lactobacillus reuteri</i> 14171	<i>h</i>	-	-
<i>E. coli</i> H5	<i>i</i>	-	-
<i>E. coli</i> DH5 $\alpha$	Invitrogen	-	-
<i>Salmonella</i> enteritidis	ATCC13076	-	-
<i>Klebsiella pneumoniae</i>	<i>j</i>	-	-

<sup>a</sup> smallest amount of protein in a volume of 10  $\mu$ l causing a lysis zone after overnight incubation: +++, 0.1 pmol; ++, 1 pmol; +, 10 pmol; +, 100 pmol. “(+)” represents a faint lysis zone; “-”, no lysis zone at the highest amount tested (100 pmol). Scores represent averaged results from two separate experiments.

<sup>b</sup> Jean C. Lee, Channing Laboratory, Brigham and Women’s Hospital, Boston, MA, USA

<sup>c</sup> Yasunori Tanji, Tokyo Institute of Technology, Yokohama, Japan; bovine mastitis isolates

<sup>d</sup> Max Paape, ABBL, ANRI, ARS, USDA, Beltsville, MD, USA; bovine mastitis isolates

<sup>e</sup>W.D. Schultze, BARC Dairy, Beltsville, MD, USA; isolated from a clinical case

<sup>f</sup>Strain 0140; Dr. A.J. Bramley, Compton Laboratory, Newbury, United Kingdom; isolated from a clinical case

<sup>g</sup>Strain 33701; Steve Giguere, College of Veterinary Medicine, University of Georgia, Athens, GA, USA

<sup>h</sup>Ken Bischoff, ARS, Peoria, Ill, USA.

<sup>i</sup>Manan Sharma, EMFSL, ANRI, ARS, USDA, Beltsville, MD, USA

<sup>j</sup>Strain K-6; E.J. Carroll, Dept. Vet Med, University of California, Davis, CA, USA; cow 2612 clinical case