The phage K lytic enzyme LysK and lysostaphin act synergistically to kill MRSA

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Abstract
LysK is the endolysin from the staphylococcal bacteriophage K, and can digest the cell wall of many staphylococci. Lysostaphin is a bacteriocin secreted by *Staphylococcus simulans* to kill *Staphylococcus aureus*. Both LysK and lysostaphin have been shown to lyse methicillin-resistant *S. aureus* (MRSA). This study describes optimal reaction conditions for the recombinant His-tagged LysK protein (pH range pH 6–10, and 0.3–0.5 M NaCl), and C-His-LysK MIC (32.85 ± 4.87 µg mL⁻¹). LysK and lysostaphin demonstrate antimicrobial synergy by the checkerboard assay.

Introduction
Antimicrobial resistance is a worldwide concern and new antimicrobials are sorely needed. Bacteriophage endolysins are peptidoglycan hydrolases that normally help nascent phage particles escape the host bacteria. They are also able to lyse Gram-positive cells when exposed externally. As antimicrobials, they are uniquely specific to the cell wall peptidoglycan of their host (or closely related species), thus reducing the risk of resistance development in nonrelated, commensal bacteria as often occurs with broad-range antibiotics (reviewed in Fischetti, 2005).

Many phage endolysins show promise in preclinical trials when used in animal models of human disease [e.g. endocarditis (Entenza et al., 2005), vaginal, and oropharynx infections (Fischetti, 2003; Loeffler et al., 2003a; Cheng et al., 2005), oral cavity infections (Nelson et al., 2001) and anthrax (Schuch et al., 2002)]. In vitro studies indicate serum antibodies to phage endolysins slowed but did not block in vitro killing of the target microbes *Bacillus anthracis*, *Streptococcus pyogenes*, or *Streptococcus pneumoniae* (Iado et al., 2003; Loeffler et al., 2003a; Fischetti, 2005). There is also a surge in recent patent applications utilizing peptidoglycan hydrolases as antimicrobials (for review see Donovan, 2007).

LysK, in plate lysis assays kills a wide range of staphylococci including methicillin-resistant *Staphylococcus aureus* (MRSA) (O’Flaherty et al., 2005). BLAST analysis of the LysK protein sequence reveals three domains, a cysteine, histidine-dependent amidohydrolase/peptidase (CHAP) domain, an amidase-2 domain, and a C-terminal SH3b cell wall-binding domain (O’Flaherty et al., 2005). It is common for phage endolysins to have an N-terminal lytic domain (or two) with a C-terminal cell wall-binding domain (Loessner, 2005).

Lysostaphin was discovered more than 50 years ago (Browder et al., 1965). It is a potent antistaphylococcal bacteriocin synthesized by *Staphylococcus simulans* that kills *S. aureus* through digestion of the peptidoglycan pentaglycine interpeptide bridge. Lysostaphin kills MRSA (Dajcs et al., 2000), planktonic (Wu et al., 2003; Walencka et al., 2005), vancomycin intermediate (Patron et al., 1999), and other antibiotic-resistant strains of *S. aureus* (Bhakta et al., 2003).
Lysostaphin has been shown to be synergistic with β-lactams against oxacillin-resistant *Staphylococcus epidermidis* (Kiri et al., 2002), with the catanionic peptide ranalexin against MRSA (Graham & Coote, 2007), and with β-lactam antibiotics (including benzylpenicillin, methicillin and cephalosporin B), bacitracin or polymyxin B, against five clinical *S. aureus* isolates including MRSA (Polak et al., 1993). A recent patent application (Kokai-Kun, 2003; US 20030211995) indicates synergy with lysostaphin and the phi11 endolysin or the antibiotic bacitracin against *S. aureus*. Similarly, the phage lytic enzyme Cpl-1 was synergistic with gentamycin, penicillin and with the phage endolysin Pal against several penicillin-resistant and -sensitive *S. pneumoniae* strains (Loeffler & Fischetti, 2003b; Djurkovic et al., 2005).

In this study, we examine some basic properties of the LysK enzyme (in order to optimize for storage), antimicrobial activity and the use of C-terminal His-tagged variants of LysK with lysostaphin to demonstrate synergy against the MRSA strain USA300.

**Materials and methods**

**Plasmids, constructs and strains**

Phage K genomic sequence has been published (AY176327) with the lysK gene having one intron. The lysK cDNA was provided by Paul Ross (O’Flaherty et al., 2005). The LysK protein sequence is also available (AA047477.2) through Genbank. Inducible vector constructs were created in pET21a (EMD Biosciences, San Diego, CA) for introduction of a C-terminal His-tag, or pQE2 (Qiagen, Valencia, CA) for introduction of an N-terminal His-tag. For cloning into pET21a, the lysK cDNA sequences were amplified with primers LysK Nde F (5′-GAG TTT GAA TAC TCC-3′) and LysK Xho R (5′-ACG TAC GTG ATG CTC GAG TTG GAA TAC TCC-3′) (engineered restriction enzyme sites are underlined). For cloning into pQE2, the lysK cDNA sequences were amplified with primers LysK pQE2 Not F (5′-ACG TAC GTG CAG CGG CCA GAA TGG CTA AGA CTC AAG C-3′) and LysK pQE2 Sal R (5′-ACG TAC GTG ATG CTC GAG TTG GAA TAC TCC-3′) (engineered restriction enzyme sites are underlined). For cloning into pQE2, the lysK cDNA sequences were amplified with primers LysK pQE2 Not F (5′-ACG TAC GTG CAG CGG CCA GAA TGG CTA AGA CTC AAG C-3′) and LysK pQE2 Sal R (5′-ACG TAC GTG ATG CTC GAG TTG GAA TAC TCC-3′) (engineered restriction enzyme sites are underlined). For cloning into pQE2, the lysK cDNA sequences were amplified with primers LysK pQE2 Not F (5′-ACG TAC GTG CAG CGG CCA GAA TGG CTA AGA CTC AAG C-3′) and LysK pQE2 Sal R (5′-ACG TAC GTG ATG CTC GAG TTG GAA TAC TCC-3′) (engineered restriction enzyme sites are underlined). For cloning into pQE2, the lysK cDNA sequences were amplified with primers LysK pQE2 Not F (5′-ACG TAC GTG CAG CGG CCA GAA TGG CTA AGA CTC AAG C-3′) and LysK pQE2 Sal R (5′-ACG TAC GTG ATG CTC GAG TTG GAA TAC TCC-3′) (engineered restriction enzyme sites are underlined). For cloning into pQE2, the lysK cDNA sequences were amplified with primers LysK pQE2 Not F (5′-ACG TAC GTG CAG CGG CCA GAA TGG CTA AGA CTC AAG C-3′) and LysK pQE2 Sal R (5′-ACG TAC GTG ATG CTC GAG TTG GAA TAC TCC-3′) (engineered restriction enzyme sites are underlined). For cloning into pQE2, the lysK cDNA sequences were amplified with primers LysK pQE2 Not F (5′-ACG TAC GTG CAG CGG CCA GAA TGG CTA AGA CTC AAG C-3′) and LysK pQE2 Sal R (5′-ACG TAC GTG ATG CTC GAG TTG GAA TAC TCC-3′) (engineered restriction enzyme sites are underlined).

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**Protein purification**

*Escherichia coli* cultures harboring pET21a or pQE2-derived LysK expression vectors were harvested after growth on selective media overnight at 19 °C with shaking for 18 h. *Escherichia coli* harvested from 100 mL cultures were sonicated and His-tagged proteins isolated using nickel chromatography Ni-NTA (Qiagen) as per the manufacturers’ instructions. Wash and elution profiles were empirically determined to be, 10 mL of 10 mM imidazole, 20 mL of 20 mM imidazole and elution with 1.2 mL of 250 mM imidazole in the same phosphate-buffered saline (PBS) (50 mM NaH₂PO₄, 300 mM NaCl, pH 8.0) with 1% glycerol to prevent precipitation of the purified protein. All samples were then converted to LysK storage buffer (400 mM NaCl, 20 mM Tris, 1% glycerol, pH 7.5) via the Micro Bio Spin P30 desalting column (BioRAD Inc.) or Zeba desalting column (Pierce) and then 0.22-μm filter sterilized for use in the MIC assays. Protein concentration determinations were via the BCA Protein kit (Pierce) and brought to 100 mL with dithiothreitol. Sterilized protein preparations were stored at −80 or 4 °C until the time of the assay. Purity of each preparation was determined using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Nontagged lysostaphin was purchased (recombinant, Sigma-Aldrich, L0761).

**SDS-PAGE and zymogram**

The purified LysK protein constructs and Kaleidoscope protein standards (Bio-Rad) were analyzed using 15% SDS-PAGE, with or without 300 mL equivalent of mid log phase *S. aureus* 305 cells (OD₆₀₀nm of 0.4–0.6). SDS gels were Coomassie stained and zymograms were washed in excess water for 1 h and incubated at room temperature in water, resulting in areas of clearing in the turbid gel where a lytic protein had localized.

**Turbidity reduction assays**

The turbidity assay is performed in a Molecular Devices, Spectra Max 340 plate reader. The assay was modified from the cuvette method reported previously (Donovan et al., 2006). *Staphylococcus aureus* is grown to logarithmic phase (OD₆₀₀nm = 0.4–0.6) at 37 °C in growth media (TSB, BHI
broth, or Mueller–Hinton Broth) with shaking, harvested at 4 °C by centrifugation, and stored on ice until just before the assay when the cells are resuspended to OD_{600 nm} = 1.0 in 400 mM NaCl, 20 mM Tris HCl, 1% glycerol, pH 7.5 unless otherwise stated. Enzyme samples are added to three wells of a 96-well dish in 100 μL of buffer. All conditions are performed in triplicate wells. The assay is started by the addition of 100 μL of cells in buffer at OD_{600 nm} c. 1.0 using a multichannel pipettor. A ‘no enzyme control’ of buffer and cells, but no enzyme is included. OD_{600 nm} readings are taken automatically every 20 s. The readings for each well are transferred electronically to an Excel spreadsheet where they are analyzed in a sliding 40 s window over each group of three consecutive time points during the 5-min period, to identify the highest instantaneous change in OD_{600 nm} for each well. The absolute values of ΔOD_{600 nm} for each group of three time points is ranked for the entire 5 min period. A plot of these values vs. time is examined for consistency (bubbles in the well cause high variability) and the highest consistent value is chosen. The highest cells alone control value (obtained in the same way) is then subtracted from the highest ranked ΔOD_{600 nm} value for each experimental sample, and the 40 s values for the triplicate samples (wells) are averaged and multiplied by 1.5 to give a ΔOD_{600 nm} min^{-1}. This value is then divided by the microgram of enzyme protein in the sample to yield a specific activity ΔOD_{600 nm} μg^{-1} min^{-1}.

**pH buffers**

pH buffers were as follows: 10 mM sodium acetate buffer pH 5, 10 mM sodium acetate buffer pH 6, 10 mM Tris HCl buffer pH 7, 10 mM Tris HCl buffer pH 8, 10 mM Tris HCl buffer pH 9 and 10 mM carbonate buffer pH 10.

**Salt buffers**

Salt buffers were composed of 1% glycerol, 20 mM Tris pH 7.5 with varying NaCl from 0 to 500 mM.

**Storage buffers**

Storage buffers were composed of 400 mM NaCl, 1% glycerol, 10 mM dithiothreitol, 20 mM Tris HCl pH 7.5 or with the addition of 1 M trehalose, 2 M proline, or 25% (final concentration) glycerol.

**Minimum inhibitory concentration**

A classical microdilution broth method for determination of the minimal inhibitory concentration (MIC) was used (Jones et al., 1985).

**Synergy**

Modified checkerboard assay experiments were performed according to Graham & Coote (2007). MRSA USA300 cells at 1 × 10^6 CFU mL^{-1} were added to wells containing varying concentrations of both LysK and lysostaphin diluted in TSB. The plate was incubated at 37 °C for 20 h, and then analyzed for clear vs. turbid wells. The fractional inhibitory concentration (FIC) is calculated as the MIC of the antimicrobial in combination divided by the MIC of the antimicrobial acting alone. Strong synergy exists if the sum of the two FICs [\( \sum \text{FIC} = \text{FIC}_A + \text{FIC}_B \)] is < 0.5 (Hall et al., 1983).

**Results**

**Purification of C- and N-His-tagged LysK**

A recombinant His-tag was added to the LysK N-terminus via subcloning into the pQE2 vector (N-His-LysK). However, it was decided to also produce the C-terminal His-tag version of LysK (C-His-LysK) in pET21a. The wild-type lysostaphin and both N- and C-terminal His-tag fusions to the LysK protein are depicted in Fig. 1. The recombinant lysostaphin protein (Sigma-Aldrich Chem Co.) used throughout this work contains only the 246-amino acid (aa) mature peptide (MW = 26.8 kD) consisting of a 154 aa LAS metalloprotease domain (Bochtler et al., 2004) and a 63-aa SH3b cell wall-binding domain (Pfam domain database: http://www.sanger.ac.uk/Software/Pfam/). LysK is a 495-aa endolysin from *S. aureus* phage K described previously (O’Flaherty et al., 2005).

Coomassie-stained SDS-PAGE of each nickel chromatography purified protein C-His-LysK, and the N-His-LysK and a zymogram (embedded with mid log phase *S. aureus* 305 cells) ran in parallel indicates the quality of the nickel column purification (Fig. 1). The full-length N-His-LysK co-purifies with multiple smaller proteins (c. 25 kD) that are absent from the C-His-LysK preparation and one of these smaller proteins shows lytic activity in the zymogram. Attempts to purify the full-length N-His-LysK protein away from these smaller proteins were unsuccessful and thus presumed to be truncations of LysK that harbor the N-terminal His-tag. Owing to the presence of lytic activity in these smaller bands, all synergy experiments were performed with the C-His-LysK protein.

**LysK lyses live staphylococci in the turbidity reduction assay**

To determine the optimal conditions for high antimicrobial activity, LysK and lysostaphin were tested in the turbidity reduction assay with varying salt, pH and protein concentrations against live cells. Lysostaphin activity is relatively
unaffected by salt concentrations between 200 and 500 mM whereas LysK shows increasing activity from 150 mM with maximal activity at concentrations approaching 400 mM (Fig. 2). LysK has a higher specific activity than lysostaphin at NaCl concentrations > 150 mM. LysK and lysostaphin show strong activity over a broad pH range from pH 6 to 9 (Fig. 2), similar to previous reports for lysostaphin (Schindler & Schuhardt, 1965). The antimicrobial effects of these lysins are near maximal at physiological pH. Both LysK and lysostaphin demonstrate a linear relationship in the range of 1 to c. 5 µg total protein in the turbidity reduction assay (Fig. 2).

Storage optimization of LysK

With the goal to use LysK as an antimicrobial, it is important to optimize for long-term storage. Various osmolytes were screened for their ability to increase the stability of stored LysK at −80 and 4 °C. Purified His-tagged-LysK was stored in any of four buffers and tested weekly. The four buffers were: (1) 400 mM NaCl, 1% glycerol, 10 mM dithiothreitol, 20 mM Tris-HCl pH 7.5; (2) 400 mM NaCl, 25% glycerol, 10 mM dithiothreitol, 20 mM Tris-HCl pH 7.5; (3) 2 M proline, 1% glycerol, 10 mM dithiothreitol, 20 mM Tris-HCl pH 7.5 (4) 1 M trehalose, 1% glycerol, 10 mM
dithiothreitol, 20 mM Tris-HCl pH 7.5. Fifty microgram aliquots of enzyme in each buffer were stored at −80 or 4°C and removed from storage immediately before a turbidity reduction assay where 10 μg were used to examine the effect of storage on enzyme activity. One percent glycerol was optimal for maintenance of high activity at either 4 or −80°C for up to 60 days with both the C- and N-terminally His-tagged proteins (data not shown).

**MIC determinations for MRSA strain USA 300**

Lysostaphin inhibits growth of MRSA USA300 at a concentration of 0.096 ± 0.018 μg mL⁻¹. The C-His LysK was found to be more active in the MIC assay than the N-His-LysK construct with the C-His LysK yielding an MIC of 3.285 ± 4.87 μg mL⁻¹ and the N-His-LysK with an MIC of 32.85 ± 28.66 μg mL⁻¹. A similar effect was observed in the turbidity reduction assay with the specific activities of these proteins being 0.034 ± 0.0030 and 0.018 ± 0.0015 ΔOD₆₀₀ nm μg⁻¹ min⁻¹ for C-His-LysK and N-His-LysK, respectively. As expected, neither LysK storage buffer nor PBS had a noticeable effect on the MRSA USA300 cell growth (data not shown).

**Synergy between LysK and lysostaphin**

The pattern of cleared wells demonstrates that the growth inhibition of the two enzymes in combination is greater than either enzyme alone (Fig. 3). The two most effective conditions identified in this assay were at concentrations of 0.027 and 5.71 μg mL⁻¹, and 0.018 and 11.43 μg mL⁻¹ for lysostaphin and LysK, respectively, representing concentrations that correspond to 30%, 18% and 16%, 33%, respectively, of each compounds MIC. The ∑ FIC was 0.45 ± 0.07 (three independent experiments), indicating strong synergistic activity (Hall et al., 1983), including one assay with *S. aureus* 305 (data not shown).

**Discussion**

Lysostaphin and LysK are potent antimicrobials. LysK was shown to have an optimal salt concentration of c. 400 mM, and maintain this high activity across a broad pH range, including physiological pH 7.5. Storage at −80°C with the addition of 1% glycerol maintains LysK lytic activity against live staphylococci for 30 days or more. In our assays, lysostaphin has an MIC of 0.096 μg mL⁻¹ ±0.018, C-His-LysK has an MIC of 32.85 ± 4.87 μg mL⁻¹, N-His-LysK has an MIC of 80 ± 28.66 μg mL⁻¹, and in combination C-His-LysK and lysostaphin show strong synergy against the MRSA USA300.

The MIC for lysostaphin is within the range of previously reported values (Gisani et al., 1982; Kusuma & Kokai-Kun, 2005; Graham & Coote, 2007). The LysK MIC is within the range of MICs reported for the Pal and Cpl-1 endolysins of streptococcal phage origin (Loeffler & Fischetti, 2003b). On a molar basis, these MICs translate into 650 nM LysK, similar to the c. 1 μM MIC for ampicillin (data not shown). Thus, LysK is demonstrating a very similar activity on a molar basis to other lysins.

The C-tagged LysK achieves a specific activity of 0.034 ± 0.003 ΔOD₆₀₀ nm min⁻¹ in the turbidity reduction assays and an MIC of 32.85 ± 4.87 μg mL⁻¹. In both the MIC and turbidity reduction assays, the N-tag version shows approximately two- to threefold reduced activity. It should be noted that that the assays to measure peptidoglycan hydrolase activities do not always agree quantitatively but do usually agree qualitatively (Kusuma & Kokai-Kun, 2005). It is with this understanding that we present these results and have attempted to avoid drawing conclusions that dictate direct quantitative comparisons between assay types.

His tags have been shown to alter protein properties. We find that the addition of 1% glycerol helps to reduce the solubility problems we faced with His-tagged LysK, and other endolysins (data not shown), but does not significantly alter the antimicrobial activity.

The finding that a second antimicrobial can reduce or alleviate the development of resistant staphylococcal strains is a strong argument for the use of dual agents in treating multi-drug-resistant infections. Synergy with lysostaphin has been reported previously (see Introduction). We demonstrate synergy in the checkerboard assay between lysostaphin and LysK on the MRSA strain USA300 and the mastitis-causing strain *S. aureus* 305. Two effective conditions were identified in Fig. 3, with lysostaphin and LysK clearing microtiter wells at concentrations that were
< 30%, 18% and 16%, respectively, of each agents' MIC. The \( \frac{\Sigma FIC}{3} \) was 0.449 ± 0.069 suggesting strong synergistic activity (Hall et al., 1983).

A potentially increased antimicrobial value might be achieved in the use of LysK through the fact that LysK has two lytic domains (CHAP endopeptidase and amidase). Thus, so far only peptidoglycan hydrolases (e.g. bacteriophage endolysins, lysozyme) harboring single lytic domains have been tested for synergy in vitro or in animal models. The lytic domains of LysK have not been sufficiently characterized to know if both domains are functional, but much like predictions that dual activity lysins should reduce the potential for resistance development in sensitive strains (Fischetti, 2005), it is feasible to expect that there could be added efficacy and/or synergy when using lysins with dual lytic activities. Thus, a more thorough characterization of the LysK domain activities is necessary to address whether or not dual domain lysins are more efficacious than single lytic domain lysins in tests of antimicrobial synergy.

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**References**


