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(54) Titre : ANTIMICROBIENS TRIPLE ACTION REFRACTAIRES AU DEVELOPPEMENT DE RESISTANCES
(54) Title: TRIPLE ACTING ANTIMICROBIALS THAT ARE REFRACTORY TO RESISTANCE DEVELOPMENT

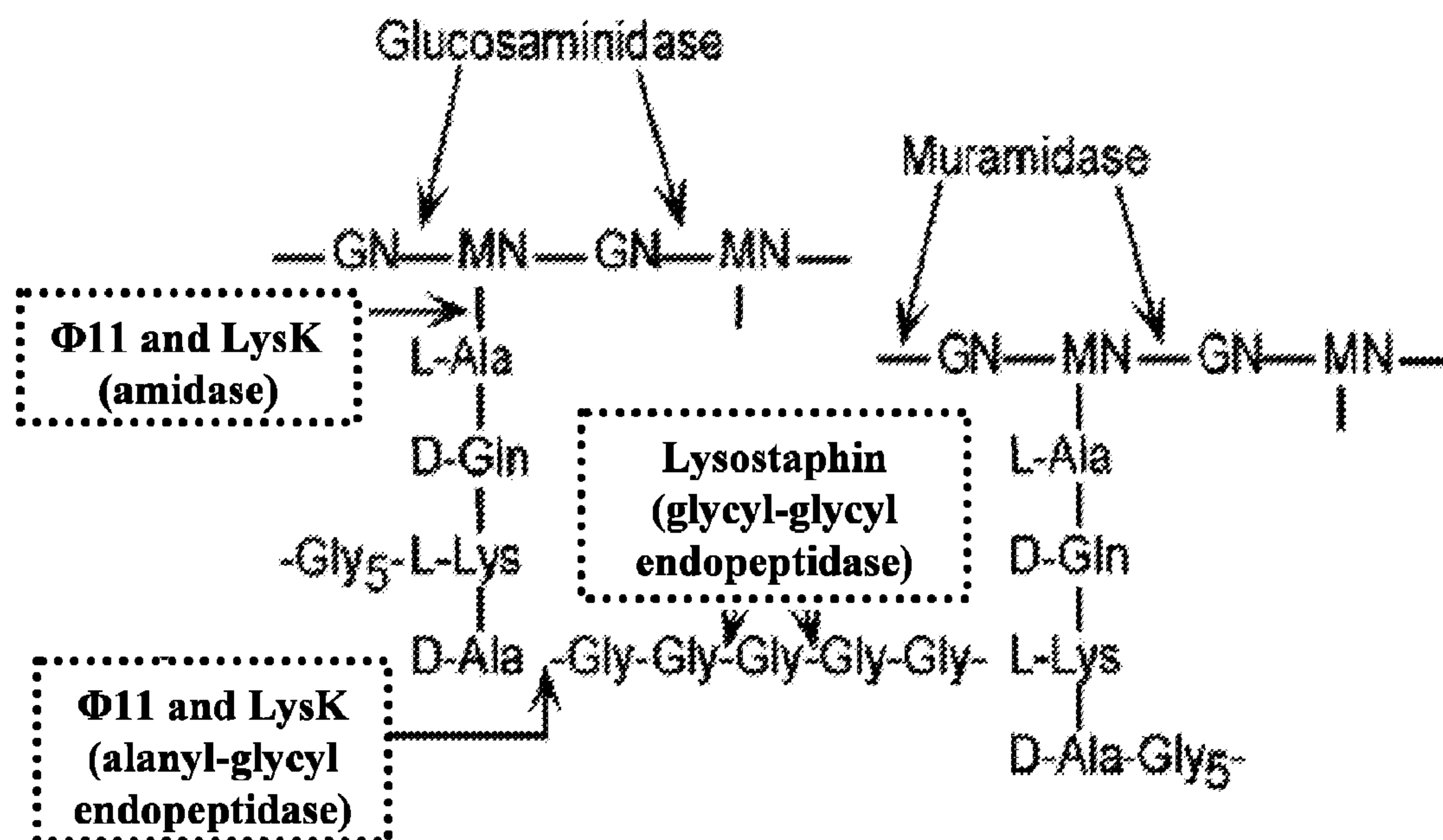


Figure 1

(57) Abrégé/Abstract:

Multi-drug resistant superbugs are a persistent problem in modern health care. This invention provides an antimicrobial endolysin - Lysostaphin triple fusion protein, comprising (1) an endolysin CHAP endopeptidase domain, (2) an endolysin amidase domain, and



(57) **Abrégé(suite)/Abstract(continued):**

(3) a Lysostaphin glycyl-glycine endopeptidase domain. The domains are derived from two proteins that show antimicrobial synergy when used in combination. The protein has specificity and exolytic activity for the peptidoglycan cell wall of untreated, live *Staphylococcus aureus* from many growth phases i.e. stationary, logarithmic and biofilm growth. The recombinant triple fusion protein comprising the three functional antimicrobial domains is designed to be refractory to resistance development.

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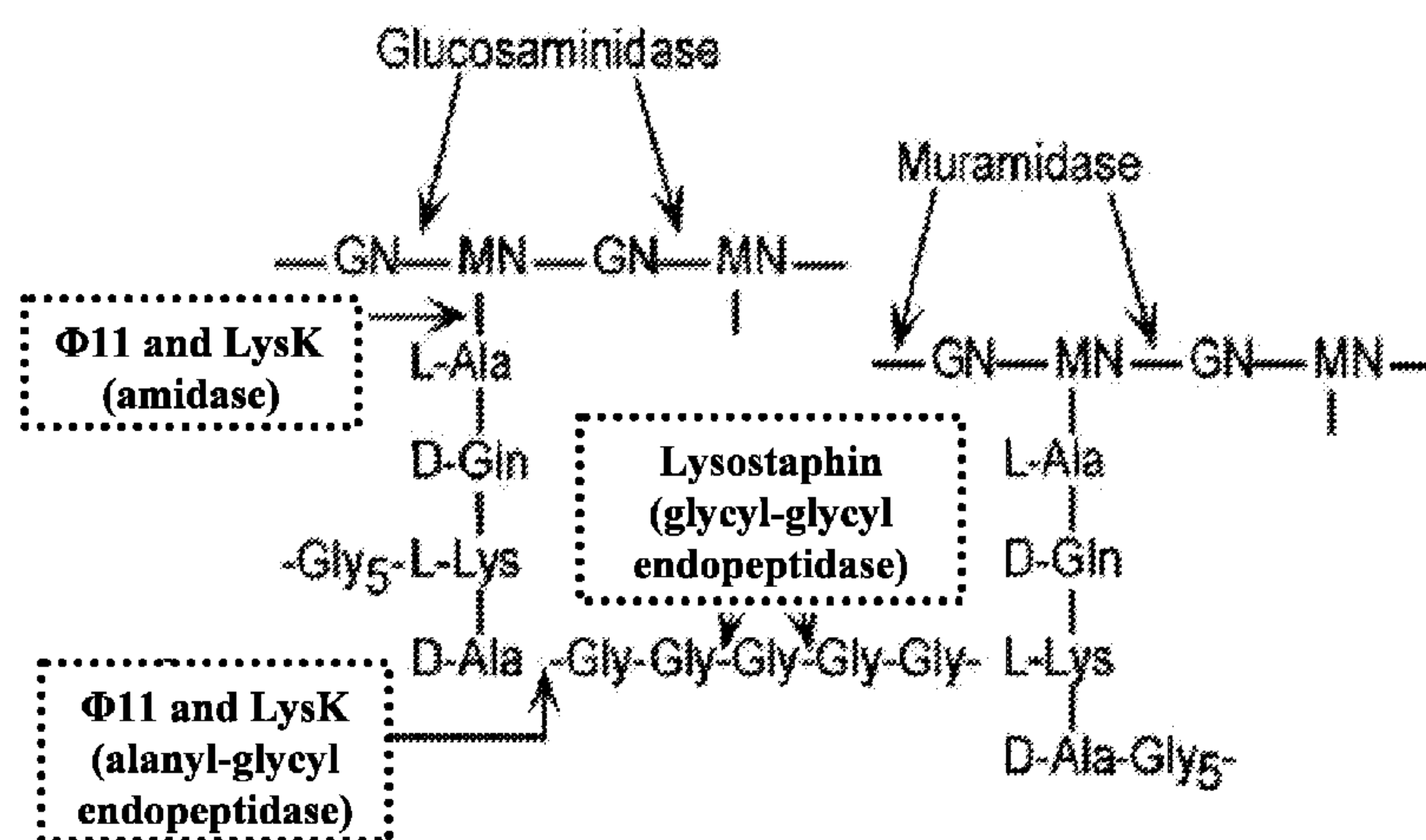


Figure 1

(57) Abstract: Multi-drug resistant superbugs are a persistent problem in modern health care. This invention provides an antimicrobial endolysin - Lysostaphin triple fusion protein, comprising (1) an endolysin CHAP endopeptidase domain, (2) an endolysin amidase domain, and (3) a Lysostaphin glycyl-glycine endopeptidase domain. The domains are derived from two proteins that show antimicrobial synergy when used in combination. The protein has specificity and exolytic activity for the peptidoglycan cell wall of untreated, live *Staphylococcus aureus* from many growth phases i.e. stationary, logarithmic and biofilm growth. The recombinant triple fusion protein comprising the three functional antimicrobial domains is designed to be refractory to resistance development.

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Triple Acting Antimicrobials That Are Refractory To Resistance Development

BACKGROUND OF THE INVENTION

Field of the Invention

[0001] This invention relates to constructs comprising three antimicrobial domains each harboring a unique lytic activity: a CHAP endopeptidase, an amidase, and a glycyl-glycine endopeptidase. In one embodiment, the CHAP endopeptidase and the amidase are provided by the peptidoglycan hydrolase, LysK endolysin, and the glycyl-glycine endopeptidase is provided by another peptidoglycan hydrolase, Lysostaphin generating a pathogen-specific triple fusion construct. The LysK endolysin specifically attacks the peptidoglycan cell wall of untreated, live staphylococci including *S. aureus* and methicillin-resistant *Staphylococcus aureus* (MRSA); Lysostaphin is a potent anti-staphylococcal bacteriocin. The constructs comprising the three functional antimicrobial domains are designed to be refractory to resistance development and can be used to treat staphylococcal pathogens including multi-drug resistant strains MRSA and USA300.

Description of the Relevant Art

[0002] *S. aureus* is an opportunistic bacterial pathogen responsible for a diverse spectrum of human and animal diseases. Although *S. aureus* may colonize mucosal surfaces of healthy humans, it is also a major cause of wound infections and has the invasive potential to induce severe infections, including osteomyelitis, endocarditis, and bacteremia with metastatic complications (Lowy, F.D. 1998. *New England J. Med.* 339: 520-532). Coagulase-negative staphylococci (CoNS) and *S. aureus* are the most common pathogens in nosocomial bacteremias and infections of implanted devices (Gordon *et al.* 2001. *Ann. Thorac. Surg.* 72: 725-730; Malani *et al.* 2002. *Clin. Infect. Dis.* 34: 1295-1300. Although methicillin-resistant *S. aureus* (MRSA) has classically been regarded as a nosocomial pathogen, it has emerged as a cause of community-acquired infections in hosts without predisposing risk factors. Superficial skin and soft tissue infections caused by MRSA are increasingly seen in clinical practice. There are limited treatment options available in terms of topical antimicrobial agents, and some strains of MRSA have developed resistance to topically applied antimicrobial agents. MRSA account for 40%-60% of nosocomial *S. aureus* infections in the U.S., and many of these strains are multi-drug resistant. Recent data indicate that more patients in U.S. hospitals die from MRSA (>18,000 per year) than AIDS (Klevens *et al.* 2007.

JAMA 298: 1763-1771). MRSA strains with reduced susceptibility or resistance to vancomycin have also been reported (Zhu *et al.* 2008. *Antimicrob. Agents Chemother.* 52: 452-457). Because *S. aureus* cannot always be controlled by antibiotics and because MRSA isolates are becoming increasingly prevalent in the community, additional control strategies are sorely needed.

[0003] Peptidoglycan is the major structural component of the bacterial cell wall and can be up to 40 layers thick. Bacteria have autolytic peptidoglycan hydrolases that allow the cell to grow and divide. Another well-studied group of peptidoglycan hydrolase enzymes are the bacteriophage (viruses that infect bacteria) endolysins. Endolysins allow the phage to escape from the bacterial cell during the phage lytic cycle. Some Gram-positive bacteria exposed to purified phage lysins externally undergo exolysis or "lysis from without." Use of phage endolysins as antimicrobials has not been reported for treatment of Gram-negative bacteria, presumably due to the presence of an outer membrane that prevents access to the peptidoglycan (Loessner, M. J. 2005. *Curr. Opin. Microbiol.* 8: 480-487). Peptidoglycan is unique to bacteria and has a complex structure (Loessner, *supra*) with a sugar backbone of alternating units of N-acetyl glucosamine (GN) and N-acetylmuramic acid (MN). Each MN residue is amide linked to a short pentapeptide chain. Characteristic of *S. aureus* is the pentaglycine bridge that connects the L-Lys of the stem peptide to the D-Ala at position 4 of a neighboring subunit (Fig. 1). Peptidoglycan hydrolases have evolved a modular design to deal with this complexity. Although single domain endolysins can lyse the target pathogen (Sanz *et al.* 1996. *Eur. J. Biochem.* 235: 601-605), endolysins can also harbor two short domains (~100-200 amino acids), each encoding a different peptidoglycan hydrolase activity.

[0004] Three classes of peptidoglycan hydrolase domains have been identified: endopeptidases, amidases, and glycosidases (includes glucosaminidase and lysozyme-like muramidases) (Lopez and Garcia. 2004. *FEMS Microbiol. Rev.* 28: 553-580; Fig. 1). Alignment of conserved domain sequences from multiple peptidoglycan hydrolase proteins has identified non-variant amino acid positions that, when mutated, can destroy the hydrolytic activity of the domain (Pritchard *et al.* 2004. *Microbiology* 150: 2079-2087; Huard *et al.* 2003. *Microbiology* 149: 695-705; Bateman and Rawlings. 2003. *Trends Biochem. Sci.* 28: 234-237; Rigden *et al.* 2003. *Trends Biochem. Sci.* 28: 230-234). Chimeric peptidoglycan hydrolases have been created by the exchange of cell wall binding domains of two lysins (Croux *et al.* 1993. *Mol. Microbiol.* 9: 1019-1025). Enzymatic activity was retained and regulatory properties exchanged when the cell wall binding domains of choline-binding

pneumococcal and clostridial lysins were swapped. Intra-generic chimeric fusion lysins are also functional (Diaz *et al.* 1990. *Proc. Natl. Acad. Sci. USA* 87: 8125-8129).

[0005] Lysostaphin is a bacteriocin secreted by *S. simulans*, that lyses *S. aureus* (Browder *et al.* 1965. *Biochem. Biophys. Res. Commun.* 19: 389). The endopeptidase activity is specific to the glycyl-glycyl bonds of the staphylococcal peptidoglycan inter-peptide bridge (Fig. 1). It is known that Lysostaphin can kill planktonic *S. aureus* (Walencka *et al.* 2005. *Pol. J. Microbiol.* 54: 191-200; Wu *et al.* 2003. *Antimicrob. Agents Chemother.* 47: 3407-3414), as well as MRSA (Dajcs *et al.* 2000. *Am. J. Ophthalmol.* 130: 544), vancomycin-intermediate *S. aureus* (Patron *et al.* 1999. *Antimicrob. Agents Chemother.* 43:1754-1755), and other antibiotic-resistant strains of *S. aureus* (Peterson *et al.* 1978. *J. Clin. Invest.* 61: 597-609). Lysostaphin can also kill *S. aureus* growing in a biofilm (Walencka, *supra*; Wu, *supra*), and it exhibits limited activity against CoNS (Cisani *et al.* 1982. *Antimicrob. Agents Chemother.* 21: 531-535); McCormick *et al.* 2006. *Curr. Eye Res.* 31: 225-230).

[0006] *S. simulans* produces Lysostaphin and avoids its lytic action by the product of the Lysostaphin immunity factor (*lif*) gene [same as endopeptidase resistance gene (*epr*) (DeHart *et al.* 1995. *Appl. Environ. Microbiol.* 61: 1475-1479) that resides on a native plasmid (pACK1) (Thumm and Gotz. 1997. *Mol. Microbiol.* 23: 1251-1265). The *lif* gene product functions by inserting serine residues into the peptidoglycan cross bridge, thus interfering with the ability of the glycyl-glycyl endopeptidase to recognize and cleave this structure. Similarly, mutations in the *S. aureus femA* gene (factor essential for methicillin resistance) (Sugai *et al.* 1997. *J. Bacteriol.* 179: 4311-4318) result in a reduction in the peptidoglycan interpeptide cross bridge from pentaglycine to a single glycine, rendering *S. aureus* resistant to the lytic action of Lysostaphin. MRSA have been shown to mutate *femA* when exposed *in vitro* or *in vivo* to sub-inhibitory doses of Lysostaphin (Climo *et al.* 2001. *Antimicrob. Agents Chemother.* 45: 1431-1437).

[0007] Grundling *et al.* identified *lyrA* (Lysostaphin resistance A) that, when mutated by a transposon insertion, reduced *S. aureus* susceptibility to Lysostaphin (Grundling *et al.* 2006. *J. Bacteriol.* 188: 6286-6297). Although some structural changes were noted in peptidoglycan purified from the mutant, the purified peptidoglycan was susceptible to Lysostaphin and the phl1 endolysin, suggesting that changes in accessibility of the enzyme to its substrate may have rendered the strain Lysostaphin resistant.

[0008] Bacterial resistance to antibiotics usually involves the acquisition of enzymes that 1) inactivate the antibiotic; 2) reduce membrane permeability; 3) facilitate active efflux of the antimicrobial from the cell; 4) modify the target protein to a resistant form; or 5) produce higher quantities of the target protein. Alternatively, the original target protein can be 6) altered via a mutational or recombination event at the endogenous gene to an antibiotic-resistant form; or 7) the organism can be protected through the multi-faceted changes that accompany growth in a biofilm (Spratt, B. G. 1994. *Science* 264: 388-393).

[0009] The Gram-positive peptidoglycan is on the cell surface, outside of the cell membrane. Many mechanisms of resistance development take advantage of the ability to inactivate the antimicrobial inside the cell. Targets outside the cytoplasmic membrane reduce the possible mechanisms by which resistance can emerge (Spratt, *supra*). Although there have been no reports of extracellular inactivation of peptidoglycan hydrolase enzymes, *S. aureus* does secrete proteases that might degrade peptidoglycan hydrolases. A regulatory mutation that increases the activity, synthesis, regulation, or secretion of staphylococcal proteases (such as *sarA* (Karlsson *et al.* 2001. *Infect. Immun.* 69: 4742-4748) might confer some level of resistance. Similarly, although *phi11* and Lysostaphin could digest purified *lyrA* peptidoglycan, this mutant is slightly resistant to Lysostaphin, suggesting that resistance mechanisms could exist due to changes in surface structures that limit accessibility to the target peptidoglycan (Grundling, *supra*). O-acetylation of peptidoglycan N-acetyl muramic acid residues by an O-acetyltransferase (OatA) results in resistance to human lysozyme and correlates with heightened virulence of some *S. aureus* strains (Bera *et al.* 2006. *Infect. Immun.* 74: 4598-4604).

[0010] Bacteriophage endolysins are relatively new antimicrobials compared to Lysostaphin, which was described in the 1960's (Browder, *supra*). Despite repeated attempts, no strains of bacteria that can resist lysis by bacteriophage endolysins have been reported (Loeffler *et al.* 2001. *Science* 294: 2170-2172; Schuch *et al.* 2002. *Nature* 418: 884-889; Fischetti, V. A. 2005. *Trends. Microbiol.* 13: 491-496). Bacteriophages and bacteria may have evolved such that phages have selected immutable target peptidoglycan bonds for cleavage with the endolysin to guarantee escape from the bacterium.

[0011] The near-species specificity of phage lysins avoids many pitfalls associated with broad range antimicrobial treatments. Broad range antimicrobials lead to selection for resistant strains, not just in the target pathogen, but also in co-resident commensal bacteria exposed to the drug. The acquisition of antibiotic resistance is often accomplished by

transfer of DNA sequences from a resistant strain to a susceptible strain. This transfer is not necessarily species or genus limited, and can lead to commensal bacteria that are both antibiotic resistant and that can serve as carriers of these DNA elements for propagation to neighboring bacteria. Those neighboring strains (potential pathogens) with newly acquired resistance elements can emerge as antibiotic resistant strains during future treatment episodes. Thus, in order to reduce the spread of antibiotic resistance, it is recommended to avoid subjecting commensal bacterial communities to broad range antibiotics. Toward this end, FDA, USDA, and CDC promote the development of antimicrobials that reduce the risk of resistance development (CDC Action Plan: Retrieved from the Internet: <URL: www.cdc.gov/drug_resistance/actionplan/html/product.htm>).

[0012] Endolysins with two active domains are expected to be more refractory to resistance development since the cell will need to mutate or modify multiple target bonds to resist the lytic action of two activities (Fischetti, *supra*). The use of two bacteriophage endolysins has been reported to have a synergistic effect in the killing of streptococcal pathogens both *in vitro* (Loeffler *et al.* 2003. *Antimicrob. Agents Chemother.* 47: 375-377) and *in vivo* in a mouse sepsis model (Jado *et al.* 2003. *J. Antimicrob. Chemother.* 52: 967-973). This is consistent with synergy and better cure rates observed in models of *S. aureus* infections in which animals are treated with either antibiotics or Lysostaphin plus an antibiotic (Climo *et al.* 1998. *Antimicrob. Agents Chemother.* 42: 1355-1360; Climo *et al.* 2001, *supra*). Synergistic bactericidal activity has also been demonstrated with an endolysin and an antibiotic against *S. pneumoniae* (Djurkovic *et al.* 2005. *Antimicrob. Agents Chemother.* 49: 1225-1228). A recent patent application (Kokai-Kun, J.F. 2003. US 20030211995) indicates there is synergy with Lysostaphin and the phi 11 endolysin or the antibiotic bacitracin against *S. aureus*.

[0013] Lysostaphin or endolysin injections can cure bacterial infections and do not raise an adverse immune response. It has been reported that Lysostaphin was efficacious in treating *S. aureus* animal infections, but the preparation was likely contaminated with other bacterial antigens, and actual doses were probably less than those described in the 1960s (reviewed in (Climo *et al.* 1998, *supra*). Lysostaphin has also been used to treat bovine mastitis (Oldham and Daly, 1991. *J. Dairy Sci.* 74: 4175-4182). The treatment effectively cleared the milk of *S. aureus*, and no deleterious effects to the animals were reported. Nonetheless, the majority of Lysostaphin-treated quarters relapsed after treatment ceased.

[0014] Peptidoglycan hydrolases have been proposed for human antimicrobial applications (Fischetti, V. A. 2003. *Ann. N.Y. Acad. Sci.* 987: 207-214; Fischetti 2005, *supra*; Schuch *et al.*, *supra*), and they have demonstrated efficacy in animal models for eliminating Group B streptococcal colonization (Cheng *et al.* 2005. *Antimicrob. Agents Chemother.* 49: 111-117; Nelson *et al.* 2001. *Proc. Natl. Acad. Sci. USA* 98: 4107-4112), pneumococcal sepsis (Jado *et al.*, *supra*), and *S. aureus* infection of mammary glands in transgenic mice (Kerr *et al.* 2001. *Nat. Biotechnol.* 19: 66-70) and cows (Wall *et al.* 2005. *Nat. Biotechnol.* 23: 445-451). Lysostaphin significantly increased survival of neonatal rat pups when given intravenously (IV) at either 30 or 60 min post *S. aureus* challenge (Oluola *et al.* 2007. *Antimicrob. Agents Chemother.* 51: 2198-2200). In a recent catheter-induced *S. aureus* endocarditis model, Lysostaphin was tolerated by the systemic route with minimal adverse effects (Climo *et al.* 1998, *supra*). Rabbits injected weekly with Lysostaphin (15 mg/kg) for 9 wks by the IV route produced serum antibodies that resulted in an eight-fold reduction in its lytic activity, consistent with earlier work (Schaffner *et al.* 1967. *Yale J. Biol. Med.* 39: 230-244), but no adverse immune response. It is believed that high purity and the absence of Gram-negative lipopolysaccharide are essential for guaranteeing a minimal host immune response.

[0015] Serum antibodies raised to phage endolysins specific to *Bacillus anthracis*, *Streptococcus pyogenes*, or *Streptococcus pneumoniae* slowed but did not block *in vitro* killing of the organism *in vivo* (Fischetti 2005, *supra*; Loeffler *et al.* 2003, *supra*). Cpl-1, a *S. pneumoniae*-specific phage lysin, was injected IV 3 times per week into mice for 4 wks, and 5 of 6 mice tested positive for IgG antibodies to Cpl-1. Vaccinated and naive mice were then challenged IV with pneumococci, and the mice were treated IV with 200 µg Cpl-1 after 10 h. Bacteremia was reduced within 1 min to the same level in both mouse groups, indicating that the antibody did not neutralize the enzyme *in vivo* (Loeffler *et al.* 2003, *supra*). Western blot analysis revealed that Cpl-1 and Pal elicited antibodies 10 d after a 200-µg injection in mice, but the second injection (at 20 d) also reduced the bacteremia profile 2-3 log units, indicating that the antibodies were not neutralizing *in vivo*. All mice recovered fully with no apparent adverse side effects or anaphylaxis (Jado *et al.* 2003, *supra*). A bacteriophage lysin also cleared streptococci from the blood of rats in an experimental endocarditis model, although antibody production was not monitored in this study (Entenza *et al.* 2005. *Infect. Immun.* 73: 990-998). Similarly, aqueous preparations of phage lysins have been proposed for the control of pathogenic bacteria on human mucous membranes (Fischetti 2003, *supra*) and mucosal clearing has been obtained with phage lytic enzymes applied to the murine vagina,

oropharynx (Cheng *et al.* 2005, *supra*), and oral cavity (Nelson *et al.*, *supra*). The mucosal immune response to these enzymes was not monitored in any of these studies.

[0016] Thus, *S. aureus* is a significant pathogen in both agricultural and human disease. Multi-drug resistant strains have become more prevalent, especially nosocomial and community acquired strains, and current antibiotic treatments are often less than 50% effective. This increased incidence of bacterial antibiotic resistance has led to a renewed search for novel antimicrobials that are refractory to resistance development.

SUMMARY OF THE INVENTION

[0017] We have discovered that a triple fusion antimicrobial protein comprising three different peptidoglycan hydrolase domains each of which specifically attacks the peptidoglycan cell wall of live, untreated *S. aureus* from without, each of which cuts the peptidoglycan at a different, unique covalent bond of the peptidoglycan, and each of which is lytic in the presence of lysis by the others, is a novel antimicrobial polypeptide for the treatment of infections and disease caused by *S. aureus*.

[0018] In accordance with this discovery, it is an object of the invention to provide a triple fusion antimicrobial protein comprising three different peptidoglycan hydrolase domains each of which specifically attacks the peptidoglycan cell wall of live, untreated *S. aureus* from without, each of which cuts the peptidoglycan at a different, unique covalent bond of the peptidoglycan, and each of which is lytic in the presence of lysis by the others.

[0019] It is also an object of the invention to provide a recombinant nucleic acid encoding a triple fusion antimicrobial protein comprising three different peptidoglycan hydrolase domains each of which specifically attacks the peptidoglycan cell wall of live, untreated *S. aureus* from without, each of which cuts the peptidoglycan at a different, unique covalent bond of the peptidoglycan, and each of which is lytic in the presence of lysis by the others.

[0020] It is another object of the invention to provide a triple fusion antimicrobial protein comprising three different peptidoglycan hydrolase domains, the parental lysins of each having been shown to be synergistic in their antimicrobial activity, and the nucleic acid encoding the triple fusion protein.

[0021] It is a further object of the invention to provide an antimicrobial LysK endolysin – Lysostaphin triple fusion protein, comprising (1) a LysK CHAP endopeptidase, (2) a LysK amidase, and (3) a Lysostaphin glycyl-glycine endopeptidase domain, in which all three lytic

domains are functional, *i.e.*, degrades the peptidoglycan cell wall of untreated, live *Staphylococcus aureus*; and the nucleic acid encoding the protein.

[0022] It is another object of the invention to provide an antimicrobial phl11 endolysin – Lyso triple fusion protein, comprising a (1) phl11 CHAP endopeptidase, (2) a phl11 amidase, and (3) a Lysostaphin glycyl-glycine endopeptidase domain, in which all three lytic domains are functional, *i.e.*, degrades the peptidoglycan cell wall of untreated, live *Staphylococcus aureus*; and the nucleic acid encoding the protein.

[0023] An added object of the invention is to provide a pharmaceutical composition comprising the triple fusion polypeptides according to the invention, each which allows *Staphylococcus*-induced disease and infection to be treated.

[0024] An added object of the invention is to provide compositions useful for the treatment of diseases and infections caused by the bacteria for which the LysK endolysin and Lysostaphin are specific where the composition comprises a triple fusion polypeptide having three peptidoglycan hydrolase domains each of which retains its property of effectively lysing said bacteria.

[0025] An added object of the invention is method of treating diseases and infections with the triple fusion polypeptide of the invention, wherein said diseases and infections are caused by the bacteria for which the three peptidoglycan hydrolases of the triple fusion protein are specific.

[0026] A further object of the invention is method of using the triple fusion polypeptide of the invention to kill *S. aureus* in biofilms.

[0027] Also part of this invention is a kit, comprising a composition for treatment of disease caused by the bacteria for which the LysK endolysin and Lysostaphin are specific.

[0028] Other objects and advantages of this invention will become readily apparent from the ensuing description.

BRIEF DESCRIPTION OF THE DRAWINGS

[0029] The patent or application file contains at least one drawing executed in color. Copies of this patent or patent application publication with color drawing(s) will be provided by the U.S. Patent and Trademark Office upon request and payment of the necessary fee.

[0030] Figure 1 shows the peptidoglycan structure and sites of hydrolase cleavage. Phi11 hydrolase and Lysostaphin contain domains encoding peptidoglycan endopeptidases. The Phi11 hydrolase also has an amidase activity cleaving between the sugar backbone and the first alanine. LysK cleaves in the exact same locations as the phi11 endolysin (data described below Fig. 5). Glucosaminidase and muramidase are examples of glycosidases that cleave between N-acetyl glucosamine (GN) and N-acetyl muramic acid (MN). Amidases cleave between the MN and the first amino acid of the peptide. Gram positive cell walls can have up to 40 layers of this sugar-protein structure [adapted from (Navarre *et al.* 1999, *J. Biol. Chem.* 274: 15847-15856)].

[0031] Figures 2 is a schematic of five fusion construct preparations and the two parental peptidoglycan hydrolase enzymes from which some of the fusions were derived. Each protein is purified via nickel column chromatography that takes advantage of an engineered C-terminal 6 x His tag (white stripes). A pair of amino acids (LE) are introduced into each construct at the XhoI Restriction enzyme site immediately prior to the addition of the 6 x His tag. His-tagged wild type Lysostaphin (Lyso-His; SEQ ID NO:2) has just one (glycyl-glycine) endopeptidase domain (blue) and a SH3b cell wall binding domain (black). His-tagged wild type LysK (Wt LysK-His; SEQ ID NO:4) has a C-terminal SH3b cell wall binding domain (grey) and two lytic domains, a CHAP endopeptidase (red) and an amidase domain (green). Various fusions between LysK and Lysostaphin have been created, wherein the domain order is shuffled or deleted, and small restriction site sequences are inserted at some fusion junctions. LysK-Lyso (SEQ ID NO:6) is a fusion of both full length proteins with a C-terminal His-tag. 390LysK-Lyso (SEQ ID NO:8) is derived from the LysK-Lyso fusion but lacks the LysK SH3b domain. 221LysK-Lyso (SEQ ID NO:10) is lacking both the LysK SH3b domain and 122 amino acids of the LysK amidase domain. Lyso-390LysK (SEQ ID NO:12) is the reverse orientation as 390LysK-Lyso and is also lacking the LysK Sh3b domain. 155Lyso-390LysK-LysoSH3b is derived from an insertion of 390-LysK (less the initial methionine) into Lysostaphin at amino acid 156. SDS PAGE depicts 5µg of the nickel purified fusion proteins in each lane and 32 µg of Kaliedoscope prestained protein ladder sizing markers (Biorad).

[0032] Figure 3 depicts the SDS PAGE and zymogram of selected purified peptidoglycan hydrolases and fusion constructs. All samples were isolated from plasmid bearing *E. coli* cultures and purified via nickel chromatography. Zymogram analysis with *S. aureus* (ATCC 29740) cells embedded in the gel. SDS PAGE gel lanes are presented with corresponding

zymogram lanes (indicated as prime). Each well contains 5 μ g of purified protein. Lanes A and A', Lysostaphin; Lanes B and B', LysK endolysin; Lanes C and C', Φ 11; Lanes D and D', LysK-Lyso fusion; Lanes E and E', 390LysK-Lyso fusion; Lanes F and F', 221LysK-Lyso fusion; Lanes G and G', Lyso-390LysK fusion; Lanes H and H', 155Lyso-390LysK-LysoSh3b fusion; Lanes I and I', Φ 11-Lyso fusion. The size of the proteins is: Lysostaphin, 66 amino acids, MW=28.1 kD; LysK, 503 amino acids, MW = 55.8 kD; ϕ 11 endolysin, 489 amino acids, MW=55.1 kD; LysK-Lyso fusion, 751 amino acids, MW = 83.0 kD; 390LysK-Lyso, 673 amino acids, MW = 71.4 kD; 221LysK-Lyso, 477 amino acids, MW = 52.9 kD; Lyso-390LysK, 646 amino acids, MW=71.6 kD; 155Lyso-390LysK-LysoSH3b (SEQ ID NO:30), 646 amino acids, MW+71.6 kD; ϕ 11-Lyso fusion, 677 amino acids, MW = 75.4kD.

[0033] Figure 4 depicts results of turbidity reduction assays with four of the fusion proteins and two of the parental lysins. *S. aureus* Newman was grown to log phase (0.4-0.6 OD_{600nm}), pelleted, and suspended in 150 mM NaCl, 20 mM Tris pH 7.5 30% glycerol and frozen at -80 degrees Celsius until time of assay. At the time of assay, cells were thawed, washed twice with assay buffer, then resuspended in assay buffer. The assay buffers were either 150 mM NaCl, 20 mM Tris pH 7.5 (grey bars) or 300 mM NaCl, 20 mM Tris pH 7.5 (black bars). 100 μ l of the bacterial suspension was added to 5 μ g of enzyme in 100 μ l buffer in a 96 well plate for an initial OD_{600nm} of 1.0. The ODs were measured at 20 sec intervals over 5 min. The maximal activity in each assay for a 40 second interval is reported with error bars representing SEM across 3 experiments, each with 3 replicates. To make comparisons between molecules with different molecular weights, the specific activities of each enzyme (OD_{600nm}/min/ μ g protein) were corrected for the molarity of the enzyme solution (Δ OD_{600nm}/min/microMolarity).

[0034] Figures 5A and B depict electrospray ionization mass spectra of *S. aureus* peptidoglycan fragments resulting from digestion with (A) LysK or (B) LysK-Lyso.

[0035] Figure 6 depicts the effect of salt and pH on lytic activity of endolysins, Lysostaphin and fusion constructs in the turbidity reduction assay. 10 μ g of each protein were added to freshly grown, untreated *S. aureus* Newman strain resuspended in buffers containing various salt concentrations at pH 7.5 or various pH buffers containing 150mM NaCl. The figure depicts actions of Lysostaphin, LysK, LysK-Lyso fusion, 390K-Lyso fusion, Φ 11 endolysin, and Φ 11 endolysin-Lyso fusion. 100 μ l of cell resuspension was added to 100 μ l of enzyme and buffer in a 96 well plate to reach an initial OD_{600nm} of 1.0. Each sample is

repeated in triplicate and the OD is measured in 20 second intervals for 5 minutes. The maximal activity in each assay, for a 40 second interval, is reported as $\Delta OD_{600nm}/min/\mu g$, with error bars representing standard deviation across experiments.

[0036] Figure 7 depicts the results of the fusion proteins in plate lysis assays with *S. aureus* strain Newman.

[0037] Figure 8 depicts the Minimum Inhibitory Concentration (MIC) of fusion proteins with *S. aureus* Newman.

[0038] Figure 9 shows bactericidal activity of fusion proteins in rat blood. CFUs were determined by serial dilution plating of rat blood following various incubation times with buffer alone (control), or various concentrations of each of six lytic enzyme constructs. Data is presented as percent buffer alone control CFUs at the zero minute time point. Note that the amount of enzyme is variable between samples. The figure depicts actions of Lysostaphin, LysK, LysK-Lyso fusion, 390LysK-Lyso fusion, 221LysK-Lyso fusion, and Lyso-390LysK fusion, while empty circles ($\circ$) represent the buffer only control. (see Fig. 4 for construct schematics). Error bars represent the standard deviation of three replicate experiments.

DETAILED DESCRIPTION OF THE INVENTION

[0039] Cell wall peptidoglycan is the major structural component of bacterial cell walls. Bacterial peptidoglycan has a complex structure; namely, a sugar backbone of alternating units of N-acetyl glucosamine (GN) and N-acetyl muramic acid (MN) residues, cross-linked by oligopeptide attachments at the MNs. Bacteriophage endolysins are peptidoglycan hydrolase enzymes synthesized by bacteriophage to help nascent phage escape the host at the end of the lytic cycle. Through digestion of the peptidoglycan, endolysins can lyse host bacterial cells with near species specificity, a characteristic that makes them an excellent source of new antimicrobial agents. It is believed that the phage and host have co-evolved, such that there has been no host identified that can develop resistance to their phage endolysin. Thus, these hydrolases are a novel source of new antimicrobials with an evolutionary proven track record in avoiding host resistance mechanisms. They function from outside the cell thus also reducing the potential resistance mechanisms that most bacterial cells employ. The endolysins digest the host cell walls with near-species specificity. A minimal pathogen target range is a preferred trait in new antimicrobials as a mechanism to reduce the risk of resistance development in non-pathogenic commensal strains as often occurs during broad range antibiotic treatment.

[0040] There are several advantages to the use of enzyme antimicrobials compared to conventional antibiotics. Phage endolysins have evolved a modular design to deal with the complex structure of the bacterial cell wall. One protein can harbor multiple domains, including both lytic and cell wall binding domains. Three classes of endolysin domains have been identified thus far: endopeptidase, glycosidase, and amidase. Any one of these domains is sufficient to lyse the bacterial target cell. Each has been localized to short protein domains (~100-200 amino acids). Here, we demonstrate that fusion constructs consisting of three lytic domains, with specificity to just one genus, *Staphylococcus*, maintain all three peptidoglycan digestion activities in the expressed triple fusion polypeptide. We show that bacteria cannot evade the effects of three unique peptidoglycan hydrolase lytic activities simultaneously. Thus, the triple fusion construct and the resulting triple fusion polypeptide of the invention represent the first class of Gram positive antimicrobials that are refractory to resistance development.

[0041] Phi11 hydrolase, LysK and Lysostaphin harbor endopeptidase domains that are examples of peptidoglycan hydrolase endopeptidases that cleave peptide bonds. Glucosaminidase and muramidase are examples of glycosidase that cleave between N-acetyl

glucoseamine (GN) and N-acetyl muramice acid (MN). The phi11 and LysK endolysins harbor amidase domains that cleave between the MN and the first amino acid of the peptide region of the peptidoglycan.

[0042] We have taken advantage of the modular nature of peptidoglycan hydrolase enzymes to create fusion proteins that harbor three lytic domains and at least one SH3b cell wall binding domain, each targeting the peptidoglycan bonds of a Gram positive pathogen (*S. aureus*). We have chosen three peptidoglycan hydrolase domains that are known to cleave the peptidoglycan at unique chemical bonds. The first triple fusion was created by the fusion of LysK (endolysin from the phage K) and Lysostaphin (a bacteriocin secreted by *Staphylococcus simulans* to kill *S. aureus*). The triple fusion protein, LysK-Lyso (SEQ ID NO: 6) harbors a CHAP endopeptidase, amidase and glycyl-glycine endopeptidase activity. It is generally accepted that no bacteria can avoid the effects of three antimicrobial domains simultaneously, thus we predict and demonstrate (data not shown) that our fusions will be refractory to resistance development. We have also created a second fusion protein, phi11 endolysin-Lysostaphin (phi11-Lyso; SEQ ID NO:14) and find a nearly identical set of results with all three lytic domains active in the final fusion (data not shown).

[0043] Peptidoglycan hydrolases are also important new antimicrobials because they have been shown to degrade staphylococcal biofilms. Biofilms are sessile forms of bacterial colonies that attach to a mechanical or prosthetic device or a layer of mammalian cells. NIH estimates that 80% of bacterial infections occur as biofilms (<http://grants.nih.gov/grants/guide/pa-files/PA-06-537.html>). Bacteria in biofilms can be orders of magnitude more resistant to antibiotic treatment than their planktonic (liquid culture) counterparts.

[0044] Several mechanisms are thought to contribute to the antimicrobial resistance associated with biofilms: 1) delayed or restricted penetration of antimicrobial agents through the biofilm exopolysaccharide matrix, which might serve as a barrier, an adsorbent, or a reactant; 2) decreased metabolism and growth rate of biofilm organisms which resist killing by compounds that only attack actively growing cells; 3) increased accumulation of antimicrobial-degrading enzymes; 4) enhanced exchange rates of genes encoding for resistance; 5) physiological changes due to the biofilm mode of growth, including "persister" cells which appear to have altered their physiology in such a way as to disable programmed cell death; and 6) increased antibiotic tolerance (as opposed to resistance) through expression of stress response genes, phase variation, and biofilm specific phenotype development. Most

of these mechanisms are avoided by our peptidoglycan hydrolases that lyse the cell from outside the cell.

[0045] Biofilms also show heightened resistance to host defense mechanisms. Cells grown in biofilms express a polymer of beta-1,6-linked N-acetylglucosamine (PNAG) in large amounts. Biofilm cultures are believed to exhibit reduced activation of complement (compared to planktonic cultures), and the aggregation of bacteria makes them less susceptible to phagocytosis. Altered gene expression of binding factors, cell surface peptidoglycan, glycoprotein synthesizing enzymes, and stress related proteins involved in the detoxification of formate, urea and reactive oxygen species, are likely factors involved in persistence and resistance of cells in a biofilm. Treatment with antibiotics, especially subinhibitory concentrations, can actually foster the formation of biofilms. There is clearly a current need for enzymes to break down biofilms for more efficient treatment of biofilm-associated staphylococcal infections.

[0046] It is known that Lysostaphin can kill cells in biofilms. Phil 1 endolysin was also recently reported to kill staphylococcal cells in biofilms (Sass and Bierbaum. 2007. *Appl. Environ. Microbiol.* 73 (1):347-52). We have also shown that LysK can kill cells in biofilms (data not shown). We anticipate that if all of the components of our triple fusions are known to kill cells in biofilms our triple fusion antimicrobials will be similarly effective.

[0047] According to the present invention, the terms "nucleic acid molecule", "nucleic acid sequence", "polynucleotide", "polynucleotide sequence", "nucleic acid fragment", "isolated nucleic acid fragment" are used interchangeably herein. These terms encompass nucleotide sequences and the like. A polynucleotide may be a polymer of RNA or DNA that is single- or double-stranded and that optionally contains synthetic, non-natural or altered nucleotide bases. A polynucleotide in the form of a polymer of DNA may be comprised of one or more segments of cDNA, genomic DNA, synthetic DNA, or mixtures thereof. This will also include a DNA sequence for which the codons encoding, for example, the LysK-Lyso fusion protein according to the invention will have been optimized according to the host organism in which it will be expressed, these optimization methods being well known to those skilled in the art.

[0048] The term "isolated" polynucleotide refers to a polynucleotide that is substantially free from other nucleic acid sequences, such as other chromosomal and extrachromosomal DNA and RNA, that normally accompany or interact with it as found in its naturally

occurring environment. However, isolated polynucleotides may contain polynucleotide sequences which may have originally existed as extrachromosomal DNA but exist as a nucleotide insertion within the isolated polynucleotide. Isolated polynucleotides may be purified from a host cell in which they naturally occur. Conventional nucleic acid purification methods known to skilled artisans may be used to obtain isolated polynucleotides. The term also embraces recombinant polynucleotides and chemically synthesized polynucleotides.

[0049] The term "construct" refers to a recombinant nucleic acid, generally recombinant DNA, that has been generated for the purpose of the expression of a specific nucleotide sequence(s), or is to be used in the construction of other recombinant nucleotide sequences. A "construct" or "chimeric gene construct" refers to a nucleic acid sequence encoding a protein, operably linked to a promoter and/or other regulatory sequences.

[0050] The term "operably linked" refers to the association of two or more nucleic acid fragments on a single nucleic acid fragment so that the function of one is affected by the other. For example, a promoter is operably linked with a coding sequence when it is capable of affecting the expression of that coding sequence (*i.e.*, that the coding sequence is under the transcriptional control of the promoter) or a DNA sequence and a regulatory sequence(s) are connected in such a way as to permit gene expression when the appropriate molecules (*e.g.*, transcriptional activator proteins) are bound to the regulatory sequence(s).

[0051] "Regulatory sequences" refer to nucleotide sequences located upstream (5' non-coding sequences), within, or downstream (3' non-coding sequences) of a coding sequence, and which influence the transcription, RNA processing or stability, or translation of the associated coding sequence.

[0052] "Promoter" refers to a nucleotide sequence capable of controlling the expression of a coding sequence or functional RNA. In general, a coding sequence is located 3' to a promoter sequence. The promoter sequence consists of proximal and more distal upstream elements, the latter elements often referred to as enhancers. Accordingly, an "enhancer" is a nucleotide sequence that can stimulate promoter activity and may be an innate element of the promoter or a heterologous element inserted to enhance the level or tissue-specificity of a promoter.

[0053] The term "cDNA" refers to all nucleic acids that share the arrangement of sequence elements found in native mature mRNA species, where sequence elements are exons and 3' and 5' non-coding regions. Normally mRNA species have contiguous exons, with the intervening introns removed by nuclear RNA splicing, to create a continuous open reading

frame encoding the protein. "cDNA" refers to a DNA that is complementary to and derived from an mRNA template.

[0054] As used herein, "recombinant" refers to a nucleic acid molecule which has been obtained by manipulation of genetic material using restriction enzymes, ligases, and similar genetic engineering techniques as described by, for example, Sambrook *et al.* 1989.

Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY or DNA Cloning: A Practical Approach, Vol. I and II (Ed. D. N. Glover), IRL Press, Oxford, 1985. "Recombinant," as used herein, does not refer to naturally occurring genetic recombinations.

[0055] As used herein, the terms "encoding", "coding", or "encoded" when used in the context of a specified nucleic acid mean that the nucleic acid comprises the requisite information to guide translation of the nucleotide sequence into a specified protein. The information by which a protein is encoded is specified by the use of codons. A nucleic acid encoding a protein may comprise non-translated sequences (*e.g.*, introns) within translated regions of the nucleic acid or may lack such intervening non-translated sequences (*e.g.*, as in cDNA).

[0056] A "protein" or "polypeptide" is a chain of amino acids arranged in a specific order determined by the coding sequence in a polynucleotide encoding the polypeptide. Each protein or polypeptide has a unique function.

[0057] The invention includes functional LysK-Lyso fusion protein, 390LysK-Lyso fusion protein, 221LysK-Lyso fusion protein, Lyso-390 LysK fusion protein, 155Lyso-390 LysK-LysoSH3b fusion protein, phil1-Lyso fusion protein, and functional fragments thereof, as well as mutants and variants having the same biological function or activity. As used herein, the terms "functional fragment", "mutant" and "variant" refers to a polypeptide which possesses biological function or activity identified through a defined functional assay and associated with a particular biologic, morphologic, or phenotypic alteration in the cell. The term "functional fragments" refers to all fragments of the lytic domains of the triple fusion polypeptide of the invention that retain lytic activity and function to lyse staphylococcal bacteria.

[0058] Modifications of the primary amino acid sequence of the lytic domains of the invention may result in further mutant or variant proteins having substantially equivalent activity to the fusion polypeptides described herein. Such modifications may be deliberate,

as by site-directed mutagenesis, or may occur by spontaneous changes in amino acid sequences where these changes produce modified polypeptides having substantially equivalent activity to the endolysin polypeptides of the triple fusion polypeptide. Any polypeptides produced by minor modifications of the endolysin primary amino acid sequence are included herein as long as the biological activity endolysin is present; *e.g.*, having a role in pathways leading to lysis of staphylococcal bacteria.

[0059] As used herein, "substantially similar" refers to nucleic acid fragments wherein changes in one or more nucleotide bases results in substitution of one or more amino acids, but do not affect the functional properties of the polypeptide encoded by the nucleotide sequence. "Substantially similar" also refers to modifications of the nucleic acid fragments of the instant invention such as deletion or insertion of nucleotides that do not substantially affect the functional properties of the resulting transcript. It is therefore understood that the invention encompasses more than the specific exemplary nucleotide or amino acid sequences and includes functional equivalents thereof. Alterations in a nucleic acid fragment that result in the production of a chemically equivalent amino acid at a given site, but do not affect the functional properties of the encoded polypeptide, are well known in the art. Thus, a codon for the amino acid alanine, a hydrophobic amino acid, may be substituted by a codon encoding another less hydrophobic residue, such as glycine, or a more hydrophobic residue, such as valine, leucine, or isoleucine. Similarly, changes which result in substitution of one negatively charged residue for another, such as aspartic acid for glutamic acid, or one positively charged residue for another, such as lysine for arginine, can also be expected to produce a functionally equivalent product. Nucleotide changes which result in alteration of the N-terminal and C-terminal portions of the polypeptide molecule would also not be expected to alter the activity of the polypeptide. Each of the proposed modifications is well within the routine skill in the art, as is determination of retention of biological activity of the encoded products.

[0060] Moreover, substantially similar nucleic acid fragments may also be characterized by their ability to hybridize. Estimates of such homology are provided by either DNA-DNA or DNA-RNA hybridization under conditions of stringency as is well understood by those skilled in the art (1985. *Nucleic Acid Hybridization*, Hames and Higgins, Eds., IRL Press, Oxford, U.K.). Stringency conditions can be adjusted to screen for moderately similar fragments, such as homologous sequences from distantly related organisms, to highly similar fragments, such as genes that duplicate functional enzymes from closely related organisms.

An indication that nucleotide sequences are substantially identical is if two molecules hybridize to each other under stringent conditions. Generally, stringent conditions are selected to be about 5°C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength and pH. However, stringent conditions encompass temperatures in the range of about 1° C to about 20°C, depending upon the desired degree of stringency as otherwise qualified herein. Thus, isolated sequences that encode a LysK-Lyso fusion polypeptide, 390 LysK-Lyso fusion polypeptide, 221 LysK-Lyso fusion polypeptide, Lyso-390 LysK fusion polypeptide, 155Lyso-390LysK-LysoSH3b fusion polypeptide, phi11-Lyso fusion polypeptide and which hybridize under stringent conditions to the LysK-Lyso fusion polypeptide, 390 LysK-Lyso fusion polypeptide, 221LysK-Lyso fusion polypeptide, Lyso-390 LysK fusion polypeptide, 155Lyso-390LysK-LysoSH3b fusion polypeptide, phi11-Lyso fusion polypeptide sequences disclosed herein, or to fragments thereof, are encompassed by the present invention.

[0061] Substantially similar nucleic acid fragments of the instant invention may also be characterized by the percent identity of the amino acid sequences that they encode to the amino acid sequences disclosed herein, as determined by algorithms commonly employed by those skilled in this art. Methods of alignment of sequences for comparison are well known in the art. Thus, the determination of percent identity between any two sequences can be accomplished using a mathematical algorithm. Non-limiting examples of such mathematical algorithms are the algorithm of Myers and Miller (1988. *CABIOS* 4:11-17), the local homology algorithm of Smith *et al.* (1981. *Adv. Appl. Math.* 2:482); the homology alignment algorithm of Needleman and Wunsch (1970. *J. Mol. Biol.* 48:443-453); the search-for-similarity-method of Pearson and Lipman (1988. *Proc. Natl. Acad. Sci.* 85:2444-2448; the algorithm of Karlin and Altschul (1990. *Proc. Natl. Acad. Sci. USA* 87:2264), modified as in Karlin and Altschul (1993. *Proc. Natl. Acad. Sci. USA* 90:5873-5877).

[0062] Computer implementations of these mathematical algorithms can be utilized for comparison of sequences to determine sequence identity. Such implementations include, but are not limited to: CLUSTAL in the PC/Gene program (available from Intelligenetics, Mountain View, Calif.); the ALIGN program (Version 2.0) and GAP, BESTFIT, BLAST, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Version 8 (available from Genetics Computer Group (GCG), 575 Science Drive, Madison, Wis., USA). Alignments using these programs can be performed using the default parameters.

[0063] As used herein, "sequence identity" or "identity" in the context of two nucleic acid or polypeptide sequences makes reference to the residues in the two sequences that are the same when aligned for maximum correspondence over a specified comparison window. When percentage of sequence identity is used in reference to proteins, it is recognized that residue positions which are not identical often differ by conservative amino acid substitutions, where amino acid residues are substituted for other amino acid residues with similar chemical properties (*e.g.*, charge or hydrophobicity) and therefore do not change the functional properties of the molecule.

[0064] As used herein, "percentage of sequence identity" means the value determined by comparing two optimally aligned sequences over a comparison window, wherein the portion of the polynucleotide sequence in the comparison window may comprise additions or deletions (*i.e.*, gaps) as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical nucleic acid base or amino acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison, and multiplying the result by 100 to yield the percentage of sequence identity.

[0065] As used herein, "reference sequence" is a defined sequence used as a basis for sequence comparison. A reference sequence may be a subset or the entirety of a specified sequence; for example, as a segment of a full-length cDNA or gene sequence, or the complete cDNA or gene sequence.

[0066] The term "substantial identity" of polynucleotide sequences means that a polynucleotide comprises a sequence that has at least 80% sequence identity, preferably at least 85%, more preferably at least 90%, most preferably at least 95% sequence identity compared to a reference sequence using one of the alignment programs described using standard parameters. One of skill in the art will recognize that these values can be appropriately adjusted to determine corresponding identity of proteins encoded by two nucleotide sequences by taking into account codon degeneracy, amino acid similarity, reading frame positioning, and the like. Substantial identity of amino acid sequences for these purposes normally means sequence identity of at least 80%, preferably at least 85%, more preferably at least 90%, and most preferably at least 95%. Preferably, optimal alignment is conducted using the homology alignment algorithm of Needleman *et al.* (1970. *J. Mol. Biol.* 48:443).

[0067] A "substantial portion" of an amino acid or nucleotide sequence comprises an amino acid or a nucleotide sequence that is sufficient to afford putative identification of the protein or gene that the amino acid or nucleotide sequence comprises. Amino acid and nucleotide sequences can be evaluated either manually by one skilled in the art, or by using computer-based sequence comparison and identification tools that employ algorithms such as BLAST. In general, a sequence of ten or more contiguous amino acids or thirty or more contiguous nucleotides is necessary in order to putatively identify a polypeptide or nucleic acid sequence as homologous to a known protein or gene. Moreover, with respect to nucleotide sequences, gene-specific oligonucleotide probes comprising 30 or more contiguous nucleotides may be used in sequence-dependent methods of gene identification and isolation. In addition, short oligonucleotides of 12 or more nucleotides may be used as amplification primers in PCR in order to obtain a particular nucleic acid fragment comprising the primers. Accordingly, a "substantial portion" of a nucleotide sequence comprises a nucleotide sequence that will afford specific identification and/or isolation of a nucleic acid fragment comprising the sequence. The instant specification teaches amino acid and nucleotide sequences encoding polypeptides that comprise a particular plant protein. The skilled artisan, having the benefit of the sequences as reported herein, may now use all or a substantial portion of the disclosed sequences for purposes known to those skilled in this art. Thus, such a portion represents a "substantial portion" and can be used to establish "substantial identity", *i.e.*, sequence identity of at least 80%, compared to the reference sequence. Accordingly, the instant invention comprises the complete sequences as reported in the accompanying Sequence Listing, as well as substantial portions at those sequences as defined above.

[0068] Fragments and variants of the disclosed nucleotide sequences and proteins encoded thereby are also encompassed by the present invention. By "fragment" a portion of the nucleotide sequence or a portion of the amino acid sequence and hence protein encoded thereby is intended. Fragments of a nucleotide sequence may encode protein fragments that retain the biological activity of the native protein and hence have LysK-Lyso fusion polypeptide-, 390 LysK-Lyso fusion polypeptide-, 221 LysK-Lyso fusion polypeptide-, Lyso-390 LysK fusion polypeptide-, 155Lyso-390LysK-LysoSH3b fusion polypeptide-, and phi11-Lyso fusion polypeptide-like activity. Alternatively, fragments of a nucleotide sequence that are useful as hybridization probes may not encode fragment proteins retaining biological activity.

[0069] By "variants" substantially similar sequences are intended. For nucleotide sequences, conservative variants include those sequences that, because of the degeneracy of the genetic code, encode the amino acid sequence of one of the LysK-Lyso fusion polypeptides, 390 LysK-Lyso fusion polypeptides, 221 LysK-Lyso fusion polypeptides, Lyso-390LysK fusion polypeptides, 155Lyso-390LysK-LysoSH3b fusion polypeptide or phil1-Lyso fusion polypeptides of the invention. Naturally occurring allelic variants such as these can be identified with the use of well-known molecular biology techniques, as, for example, with polymerase chain reaction (PCR), a technique used for the amplification of specific DNA segments. Generally, variants of a particular nucleotide sequence of the invention will have generally at least about 90%, preferably at least about 95% and more preferably at least about 98% sequence identity to that particular nucleotide sequence as determined by sequence alignment programs described elsewhere herein.

[0070] By "variant protein" a protein derived from the native protein by deletion (so-called truncation) or addition of one or more amino acids to the N-terminal and/or C-terminal end of the native protein; deletion or addition of one or more amino acids at one or more sites in the native protein; or substitution of one or more amino acids at one or more sites in the native protein is intended. Variant proteins encompassed by the present invention are biologically active, that is they possess the desired biological activity, that is, LysK-Lyso fusion protein, 390 LysK-Lyso fusion protein, 221 LysK-Lyso fusion protein, Lyso-390LysK fusion protein, 155Lyso-390LysK-LysoSH3b fusion polypeptide, phil1-Lyso fusion protein activity as described herein. Such variants may result from, for example, genetic polymorphism or from human manipulation. Biologically active variants of a LysK-Lyso fusion polypeptide, 390LysK-Lyso fusion polypeptide, 221LysK-Lyso fusion polypeptide, Lyso-390LysK fusion polypeptide, 155Lyso-390LysK-LysoSH3b fusion polypeptide or phil1-Lyso fusion polypeptide of the invention will have at least about 90%, preferably at least about 95%, and more preferably at least about 98% sequence identity to the amino acid sequence for the protein of the invention as determined by sequence alignment programs described elsewhere herein. A biologically active variant of a protein of the invention may differ from that protein by as few as 1-15 amino acid residues, or even 1 amino acid residue.

[0071] The polypeptides of the invention may be altered in various ways including amino acid substitutions, deletions, truncations, and insertions. Novel proteins having properties of interest may be created by combining elements and fragments of proteins of the present invention, as well as with other proteins. Methods for such manipulations are generally

known in the art. Thus, the genes and nucleotide sequences of the invention include both the naturally occurring sequences as well as mutant forms. Likewise, the proteins of the invention encompass naturally occurring proteins as well as variations and modified forms thereof. Such variants will continue to possess the desired LysK-Lyso fusion protein, 390LysK-Lyso fusion protein, 221 LysK-Lyso fusion protein, Lyso-390LysK fusion protein, 155Lyso-390LysK-LysoSH3b fusion polypeptide, and/or phl1-Lyso fusion protein activity. Obviously, the mutations that will be made in the DNA encoding the variant must not place the sequence out of reading frame and preferably will not create complementary regions that could produce secondary mRNA structure.

[0072] The deletions, insertions, and substitutions of the protein sequences encompassed herein are not expected to produce radical changes in the characteristics of the protein. However, when it is difficult to predict the exact effect of the substitution, deletion, or insertion in advance of doing so, one skilled in the art will appreciate that the effect will be evaluated by routine screening assays where the effects of LysK-Lyso fusion protein, 390LysK-Lyso fusion protein, 221LysK-Lyso fusion protein, Lyso-390LysK fusion protein, 155Lyso-390LysK-LysoSH3b fusion polypeptide, and/or phl1-Lyso fusion protein can be observed.

[0073] "Codon degeneracy" refers to divergence in the genetic code permitting variation of the nucleotide sequence without affecting the amino acid sequence of an encoded polypeptide. Accordingly, the instant invention relates to any nucleic acid fragment comprising a nucleotide sequence that encodes all or a substantial portion of the amino acid sequences set forth herein.

[0074] The staphylococcal control compositions of the invention comprise the antimicrobial composition of the invention dissolved or suspended in an aqueous carrier or medium. The composition may further generally comprise an acidulant or admixture, a rheology modifier or admixture, a film-forming agent or admixture, a buffer system, a hydrotrope or admixture, an emollient or admixture, a surfactant or surfactant admixture, a chromophore or colorant, and optional adjuvants. The preferred compositions of this invention comprise ingredients which are generally regarded as safe, and are not of themselves or in admixture incompatible with milk or milk by-products or human and veterinary applications. Likewise, ingredients may be selected for any given composition which are cooperative in their combined effects whether incorporated for antimicrobial efficacy, physical integrity of the formulation or to facilitate healing and health in medical and veterinary applications, including for example in

the case of mastitis, healing and health of the teat or other human or animal body part. Generally, the composition comprises a carrier which functions to dilute the active ingredients and facilitates stability and application to the intended surface. The carrier is generally an aqueous medium such as water, or an organic liquid such as an oil, a surfactant, an alcohol, an ester, an ether, or an organic or aqueous mixture of any of these, or attached to a solid stratum such as colloidal gold. Water is preferred as a carrier or diluent in compositions of this invention because of its universal availability and unquestionable economic advantages over other liquid diluents.

[0075] Avoiding the generalized use of broad range antimicrobials and using highly specific antimicrobials for just the target organisms involved, should help reduce the ever-increasing incidence of antibiotic resistance.

EXAMPLES

[0076] Having now generally described this invention, the same will be better understood by reference to certain specific examples, which are included herein only to further illustrate the invention and are not intended to limit the scope of the invention as defined by the claims.

EXAMPLE 1

Plasmids, Constructs and Strains

[0077] The LysK cDNA was kindly provided by Paul Ross (O'Flaherty *et al.* 2005. *J. Bacteriol.* 187: 7161-7164). Phage K genomic sequence has been published (AY176327) and 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. For cloning into pET21a, the LysK sequences were amplified with primers LysK Nde F (5'-GAGAAATTACCATATGGCTAAG ACTC-3'; SEQ ID NO:17) and LysK Xho R (5'-ATGGTGATGCTCGAGTTTGAATACTC C-3'; SEQ ID NO:18, Table 1) (engineered restriction enzyme sites are underlined). [All primers utilized in construct preparation are described in Table I.] PCR subcloning is performed when PCR products are gel purified and digested appropriately with Restriction Enzymes (RE) that recognize and cleave at the engineered sites. The resultant gene fragments are purified over a Micro Bio Spin P30 desalting column (BioRAD, Inc.) and introduced into similarly digested, dephosphorylated and gel purified vector pET21a, via conventional means. At the C-terminus of the C-tagged LysK, there are an additional 2 amino acids corresponding to the

XhoI site (Leu-Glu) followed by 6 His residues. The resulting plasmid was termed p3514 (listed in Table 2).

[0078] A plasmid harboring the Mature Lysostaphin (gi|153046|gb|M15686.1|STALYS) cDNA was a gift from David Kerr, Univ. Vermont. The entire mature protein coding sequence was amplified using the primers Lyso AA1 NdeI F (5'-ACGTACGTCATATGGCTGCAACACATGAAC ATTCAGCAC; SEQ ID NO:19) and RlysoXhoI (5'-GCGCTACTCGAGACCACCTGCT TTTCCATATC; SEQ ID NO:20) and introduced into pET21a via PCR cloning similar to that described for LysK. The resulting plasmid was termed p5301 with the NdeI site contributing the new ATG translational start site for the protein coding sequences.

[0079] LysK-Lyso was generated by amplification of Lysostaphin gene sequences, using plasmid p5301 as template and the primers FlysoSal I (5'-ATCATC GTCGACGCTGCAACACATGAACATTCAGCAC; SEQ ID NO: 21) and RlysoXhoI (5'-GC GCTACTCGAGACCACCTGCTTTTCCATATC; SEQ ID NO: 20). The Lysostaphin fragment was PCR subcloned into the XhoI linearized LysK expression plasmid p3514. The ligation of XhoI to SalI destroys both RE sites and adds two amino acids to the fusion joint, LD (Leu-Asp) which is present in all fusion constructs. The resulting plasmid was termed p5031.

[0080] 390LysK-Lyso was created by amplification of the LysK fragment with the primers R lysKCA 390 (5'-GTGGTGCTCGAGACTTGCGCTACTTGTTTTACC; SEQ ID NO: 22) (Xho I site) and pET21a XbaI F (5'-GGATAACAATTCCCCTTCTAG; SEQ ID NO: 23), using plasmid p3514 as template. The amplified fragment was RE digested and PCR subcloned into XbaI and XhoI cut pET21a similar to the methods described previously, generating plasmid p5404. The Lysostaphin fragment was amplified with primers FlysoSalI and RlysoXhoI and the amplified fragment was RE digested and then introduced into plasmid p5404 that had been linearized with XhoI generating plasmid pSB1101.

[0081] 221lysK-Lyso was created by first PCR subcloning the lysK fragment encoding amino acids 1-221 into pET21a. The fragment was generated with the template plasmid p3514 and the primers lysK Chap 221S R (5'-GTATTGCTCGAGTGA AGAACGACCTGC; SEQ ID NO:24) and pET21a XbaI F (5'-GGATAACAATTCCCCTTCTAG-3'; SEQ ID NO:23). The resultant fragment was RE digested with XhoI and XbaI and PCR subcloned into pET21a to create plasmid pSB0201. The Lysostaphin fragment was

then PCR amplified, with FlysoSall and RlysoXhoI and the amplified fragment was then PCR subcloned into the XhoI linearized plasmid SB0201, generating plasmid pSB0408.

[0082] Lyso-390lysK was generated by introducing the PCR product generated by amplification of the template p3514 with the primers LysK aa1 Sal F (5'-GATATA GTCGACGCTAAGACTC; SEQ ID NO: 25) pET21a Sty I - R (5'-CGTTTAGAGGCCCC AAGGGGTTATG; SEQ ID NO:26) into the XhoI StyI digested p5301. The resulting plasmid was termed pSB1501

[0083] The phi11-Lyso fusion was created first by amplifying the phi11 endolysin gene from the template plasmid pTZ18R (a gift from R. Jayaswal containing the phi11 endolysin on a 3 kb EcoRI fragment) with the primers LytA NdeF (5'-GTGGCGCAT ATGCAAGCAAAATTAAC; SEQ ID NO:27) and LytA XhoI 481 R (5'-TGACTATGTC CTCGAGACTGATTTC; SEQ ID NO:28). The resultant PCR product was PCR subcloned into NdeI and XhoI digested pET21a to generate the plasmid pLytA481. (Donovan *et al.* 2006. *FEMS Microbiol Lett.* 265(1):133-239). The Lysostaphin gene was then PCR amplified with the primers FlysoSall and RlysoXhoI and was PCR subcloned into plasmid pLytA481 via the XhoI site to generate the plasmid p5809. This fusion is a direct fusion of the phi11 endolysin (481 amino acids) and mature Lysostaphin (246 amino acids) open reading frames, in a head to tail, head to tail fusion.

[0084] The 155Lyso-390LysK-LysoSH3b fusion was created by first PCR-amplifying the Lysostaphin gene from the template 5301 with the primers LysoAD 155 XHO R (5'GTTTGTCTCGAGACCTGTATTCGG-3' SEQ ID: 31) and Lyso AA1 NdeI F (5'-ACGTACGTCATATGGCTGCAACACATGAACATTCAGCAC-3', SEQ ID NO: 19) and introducing this fragment into NdeI XhoI digested pET21a generating pSB1701. A second intermediate was produced by introducing the Lysostaphin SH3b domain to the construct p5404 by amplification of the template 5301 with the primers LysoSH3b Sall F GCGCATCTCGAGACAGTAACTCCAACGCCG, SEQ ID NO: 32) pET21a Sty I - R (5'-CGTTTAGAGGCCCC AAGGGGTTATG; SEQ ID NO: 26) and introducing the fragment into XhoI Styl linearized p5404 generating plasmid pSB1001. The final construct 155Lyso-390LysK-LysoSH3b was generated by introducing the PCR product generated by amplification of the template pSB1001 with the primers LysK aa1 Sal F (5'-GATATA GTCGACGCTAAGACTC; SEQ ID NO: 25) pET21a Sty I - R (5'-CGTTTAGAGGCCCC AAGGGGTTATG; SEQ ID NO: 26) into the XhoI Styl digested pSB1701 generating pSB1801.

[0085] All subcloning was performed in *E. coli* DH5 α (Invitrogen, Carlsbad, CA) for plasmid DNA isolation. All constructs were sequence verified. All constructs were DNA sequence verified. pET21a constructs were induced in *E. coli* BL21 (DE3) (EMD Biosciences, San Diego, CA).

Table 1. PCR primers utilized to create the fusion constructs.

Name	Sequence	SEQ ID NO:
Lyso AA1 NdeI F	ACGTACGTC ATATGGCTGCAACACATGAACATTCAGCAC	19
RlysoXhoI	GCGCTACT CGAGACCACCTGCTTTTCCATATC	20
Lyso Sal I - F	ATCATCG TCGACGCTGCAACACATGAACATTCAGCAC	21
LysK Nde F	GAGAAATTAC ATATGGCTAAGACTC	17
LysK Xho R	ATGGTGATG CTCGAGTTTGAATACTCC	18
lysK Chap 221SR	GTATTG CTCGAGTGAAGAACGACCTGC	24
R lysKCA 390	GTGGTG CTCGAGACTTGCGCTACTTGTTTTACC	22
pET21a xbaI F	GGATAACAATTCCC CTCTAG	23
LysK aa1 Sal F	GATATAG TCGACGCTAAGACTC	25
pET21a Sty I - R	CGTTTAGAGGCC CCAAGGGGGTTATG	26
LytA NdeF	GTGGCGC ATATGCAAGCAAAATTAAC	27
LytA XhoI 481 R	TGACTATGTC CTCGAGACTGATTTC	28
LysoSH3b Sall F	GCGCAT CTCGAGACAGTAACTCCAACGCCG	32

Bolded sequences represent Restriction Enzyme sequences utilized in the cloning protocol.

Table 2. Inducible plasmids used during construction of, and expression of, fusion constructs.

Plasmid	Construct
p3514	LysK
p5301	Lysostaphin
p5031	LysK-Lyso
p5404	390LysK
pSB1101	390LysK-Lyso
pSB0201	221LysK
pSB0408	221LysK-Lyso
pSB1501	Lyso-390LysK
pSB1801	155Lyso-390LysK-LysoSH3b
pLytA481	Phil1
p5809	Phil1-Lyso

[0086] *Staphylococcus aureus* Newbolt 305 capsular polysaccharide serotype 5 (ATCC 29740) and *Staphylococcus* Newman (gift from Jean Lee, Harvard Univ.) were grown at 37°C in Brain Heart Infusion broth (BD, Sparks, MD) or Tryptic Soy Broth (BD, Sparks, MD).

EXAMPLE 2

Protein Purification

[0087] *E. coli* cultures harboring pET21a derived Lysostaphin expression vectors were grown under ampicillin selection to mid log phase (OD_{600nm} of 0.4-0.6), chilled on ice for 30 min, induced with 1 mM IPTG(isopropyl-beta-D-thiogalactopyranoside), and incubated at 19°C with shaking for 18 h. *E. coli* harvested from 100 ml cultures were suspended in 2 ml lysis buffer (50 mM NaH_2PO_4 , 300 mM NaCl, 10 mM imidazole, pH 8), sonicated on ice for 15 x 5 sec pulses separated by 15 sec. rests, and centrifuged at 11,000 x g for 20 minutes at 4°C. The cleared lysate was transferred to microfuge tubes and centrifuged at 16,000 x g for 30 min at 4°C. The cleared supernatant was applied to 1 ml Ni-NTA (nickel matrix) in a slurry and mixed gently for 1 hour at 4°C (Qiagen). The slurry was loaded into a polypropylene column (Qiagen # 34964) where wash and elution buffer profiles were empirically determined for the LysK constructs 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 (50 mM NaH_2PO_4 , 300 mM NaCl, pH 8.0). Immediately after purification from the nickel column, all samples were brought to 1% glycerol to prevent precipitation of the purified protein. Addition of 1% glycerol has become a routine practice in this lab when isolating His-tagged proteins in order to help resolve solubility issues faced with other His-tagged proteins. All samples were then converted to LysK storage buffer (400 mM NaCl, 20mM Tris HCl, 1% glycerol, pH 7.5) via Micro Bio Spin P30 desalting column (BioRAD, Inc.) or Zeba desalting column (Pierce) that had been converted to LysK Storage buffer. All samples were then 0.22 micron filter sterilized for use in the MIC assays. After filtration, protein concentration determinations were made via BCA Protein kit (Pierce) and DTT was added to 10mM after protein concentration determination. Sterilized protein preparations were stored at -80°C or 4°C until the time of the assay. Purity of each preparation was determined via SDS-PAGE (Fig. 2). Non-tagged Lysostaphin was purchased (recombinant, Sigma-Aldrich, L0761).

EXAMPLE 3

SDS PAGE and Zymogram

[0088] The purified fusion proteins and Kaleidoscope protein standards (Invitrogen, Carlsbad, CA) were analyzed with 15% SDS-PAGE, with or without 300 ml equivalent of mid log phase *S. aureus* 305 cells (OD_{600nm} of 0.4-0.6). Gels were prepared and electrophoresed in Tris-Glycine buffer at 100 volts for 1.5 hours in the BioRad Mini-

PROTEAN 3 gel apparatus, according to manufacturer's instructions. SDS gels were stained in BioSafe Coomassie stain (BioRad, Hercules, Ca.) for one hour and then rinsed in distilled water overnight. Zymograms were washed in excess water for 1 hour to remove the SDS and incubated at room temperature in water, resulting in areas of clearing in the turbid gel wherever a lytic protein was localized.

[0089] Zymogram analysis was performed with a 50x concentrated suspension of log phase *S. aureus* cells added to the SDS PAGE gel mixture prior to polymerization. The SDS PAGE and zymogram gels were made identically, loaded with identical samples, and electrophoresed for the same period of time. The gel results indicate that the protein preparations are >95% pure.

[0090] As shown in Fig. 3, 5 µg of the phi11 endolysin, LysK endolysins, LysK-Lysostaphin fusions and the phi11 endolysin-Lyso fusion or 5 µg Lysostaphin produced cleared regions in the zymogram (representing lysis of the *S. aureus* cells embedded in the gel). The position of these cleared zones corresponds to the observed (and predicted) position of the peptidoglycan hydrolase proteins seen in the Coomassie blue-stained SDS-PAGE gel.

EXAMPLE 4

Catalytic Activity of the Three Domains of the Triple Fusion Lysins

[0091] We assessed the relative lytic contributions of the three different domains of the triple fusion constructs to determine the activity of all the domains and also to determine that the products resulting from a given domain's activity were suitable substrates for the catalytic activities of the other two domains. Our approach to identifying active lytic domains in the lysin constructs is to analyze the cell wall digestion products. An increase in reducing activity of the reaction products during digestion indicates glycosidase activity. Analysis of the sodium borohydride reduction products of the digests allows us to determine if the glycosidase is an N-acetylglucosaminidase or an N-acetylmuramidase. Using such a procedure previously, indicated that the B30 lysin possesses N-acetylmuramidase activity (Baker *et al.* 2006. *Appl. Environ. Microbiol.* 72: 6825-6828; Pritchard *et al.* 2004, *supra*) and the LambdaSa2 lysin has N-acetyl-glucosaminidase activity (Pritchard *et al.* 2007. *Appl. Environ. Microbiol.* 73: 7150-7154). We analyzed the LysK, the LysK-Lyso, and 390LysK-Lyso enzyme digestion products with electrospray ionization mass spectrometry (ESI-MS) (sometimes coupled to HPLC) to detect amidase and endopeptidase activities. In addition, we used synthetic peptide substrates that mimic the stem peptide and cross bridges of

peptidoglycan to confirm the endopeptidase cleavage sites. For example, using synthetic peptides, we were able to show that the endopeptidase of the B30 lysin cleaves between the D-Ala of the stem peptide and the L-Ala of the cross bridge (Baker *et al.*, *supra*; Pritchard *et al.* 2004, *supra*). Similar methods were used to demonstrate gamma-D-glutaminy-L-lysine activity in the LambdaSa2 lysin (Pritchard *et al.* 2007, *supra*). We have also determined the peptidoglycan cut sites for LysK, which are identical to the phi11 endolysin (Fig. 1).

[0092] When the full length phi11-Lyso (data not shown), LysK-Lyso (Fig. 5B), and 390LysK-Lyso (data not shown) triple fusions were tested using ESI-MS for cut site determination, all 3 domains were active in all three constructs. Characterization of the peptide products in cell wall digests of LysK resulted in the identification of the two enzymatically active peptidoglycan lytic domains. One is an amidase that cleaves between N-acetylmuramic acid residues and L-alanine of the stem peptide, and the other is an endopeptidase that cleaves between a D-alanine in the stem peptide and a glycine in the cross-bridge peptide. Similar activities have been reported for the phi11 lysin (Navarre *et al.* 1999. J. Biol. Chem. 274: 15847-15856). The primary product of LysK digestion was A₂QKG₅, which in positive-ion ESI-MS gives a peak with a m/z = 702 (Fig. 5A).

Lysostaphin cleaves staphylococcal peptidoglycan between the second and third, and third and fourth, glycine residues of the cross-bridges. ESI-MS analysis of the peptide digestion products of a LysK-Lyso fusion protein shows that all three lytic domains are active. In Fig. 5B the presence of the m/z 702 peak shows that both LysK domains are active. However, the peaks at m/z 645 (A₂QKG₄), 588 (A₂QKG₃), and 531 (A₂QKG₂) are the result of Gly-Gly cleavages by the Lysostaphin component of the fusion. Digestion with 390LysK-Lyso gives similar spectrums of peaks, with a less predominant peak at m/z702. The peak at m/z 702 indicates that the predominant enzyme activity in LysK-Lyso construct is due to the LysK domains; the more evenly distributed peaks in the 390LysK-Lyso construct suggests the Lysostaphin endopeptidase domain is more active in this fusion.

EXAMPLE 5

Turbidity Reduction Assay

[0093] The turbidity assay measures the drop in optical density (OD_{600 nm}) resulting from lysis of the target bacteria with the phage endolysin-derived protein. The 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.* 2006b. *FEMS Microbiol. Lett.* 265: 133-139). *S. aureus* is grown to logarithmic phase (OD_{600 nm} = 0.4 - 0.6) at 37°C in growth media

(Tryptic Soy Broth, Brain Heart Infusion broth (BHI), 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 $OD_{600\text{ nm}} = 1.0$ in 150 mM NaCl, 10mM Tris HCl, pH 7.5 unless otherwise stated. Enzyme samples are added to three wells of a 96 well dish in 100 μ l of buffer. All conditions are performed in triplicate wells. The assay is started by the addition, via multi channel pipettor, of 100 μ l of cells resuspended in buffer. The cell suspensions are at sufficient concentration to reach an $OD_{600\text{ nm}} \sim 1.0$ when combined with the 100 μ l of buffer/enzyme in the well. The “no enzyme control” contains buffer and cells, but no enzyme is included. $OD_{600\text{ nm}}$ readings are taken automatically every 20 seconds. The readings for each well are transferred electronically to an Excel spreadsheet where they are analyzed in a sliding 40 second window over each group of 3 consecutive time points during the five minute period, to identify the highest instantaneous change in $OD_{600\text{ nm}}$ for each well. The absolute values of $\Delta OD_{600\text{ nm}}$ for each group of 3 time points is ranked for the entire 5 minute 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 value representing changes in the $OD_{600\text{ nm}}$ in the control sample (cells alone) obtained the same way is then subtracted from the highest ranked $\Delta OD_{600\text{ nm}}$ value for each experimental sample, and the 40 second values for the triplicate samples (wells) are averaged and multiplied by 1.5 to give a $\Delta OD_{600\text{ nm}}/\text{minute}$. This value is then divided by the μ g of enzyme protein in the sample to yield a specific activity $\Delta OD_{600\text{ nm}}/\mu\text{g}/\text{min}$. pH Buffers: pH buffers were as follows: 10mM sodium acetate buffer pH 5, 10mM sodium acetate buffer pH 6, 10mM Tris HCl buffer pH 7, 10 mM Tris HCl buffer pH 8, 10mM Tris HCl buffer pH 9, and 10mM Carbonate buffer pH 10. Salt Buffers: Salt buffers were composed of 1% glycerol, 20mM Tris pH 7.5 with varying NaCl from 0-500 mM. Storage Buffers: Storage buffers were composed of 400 mM NaCl, 1% glycerol, 20mM Tris HCl pH 7.5 or with the addition of 1M trehalose, 2M proline, or 25% (final concentration) Glycerol.

[0094] Turbidity reduction assays were also performed with frozen cells. Live cells were grown to mid logarithmic phase ($OD_{600\text{ nm}} = 0.4 - 0.6$) at 37°C in BHI with shaking, harvested at 4°C by centrifugation, and stored on ice for 30 min to arrest growth. Cells were resuspended in 5 ml of buffer (150 mM NaCl, 10 mM Tris HCl, pH 7.5) per 250ml of liquid culture. Glycerol was added to 20% (1.25 ml of 100% glycerol per 5 ml). The suspension was then separated into 1 ml aliquots and stored at -80°C until needed. For turbidity assays, aliquots of cells were rapidly thawed by agitation in a room temperature water bath, pelleted

by 16,000g centrifugation, washed twice to remove residual glycerol, then resuspended in 150 mM NaCl, 10 mM Tris HCl, pH 7.5 or 300 mM NaCl, 10 mM Tris HCl, pH 7.5. Cell suspensions were then adjusted to concentration and used as in a standard turbidity reduction assay.

[0095] In the turbidity reduction assay (Fig. 4), the LysK-Lyso fusion is less active than Lysostaphin or LysK alone. However, removal of the LysK SH3b domain improves activity of the triple fusion lysin ~4 fold. This finding suggests that a phage endolysin's binding domain might inhibit antimicrobial activity, whereas the binding domain from a bacteriocin might make a better antimicrobial. Lysostaphin is a *S. simulans* bacteriocin; by definition, it is designed to kill all neighboring *S. aureus*. In contrast, LysK is predicted to have a strong binding constant in order to achieve lysis of just the host bacterial cell. Reversing the orientation of the LysK and Lysostaphin components of this triple domain fusion or inserting the 390LysK peptide into Lysostaphin does not appear to enhance the lytic activity in the turbidity reduction assay (390K-Lyso or 155Lyso-390LysK-LysoSH3b vs. Lyso-390K). Similarly, creating a hybrid dual domain lysine by removing the functional amidase domain (221K-Lyso) does not improve the level of lytic activity in this assay, over the 390K-Lyso fusion. Regardless of the levels of activity, it is important to note that all are functional fusions and are lytic for live cells.

[0096] All of the turbidity reduction assays were performed under optimal salt and pH conditions. To determine the optimal conditions for high antimicrobial activity, LysK-Lysostaphin had been tested in the turbidity reduction assay against live cells for pH optimum and NaCl concentrations. *S. aureus* 305 cells were resuspended in 20mM Tris pH 7.5 containing 1% glycerol and NaCl concentrations ranging from 0-500 mM. The cells were treated with 10 µg of either C-His-LysK or Lysostaphin for 5 minutes in the turbidity reduction assay (Fig. 6). Lysostaphin activity is relatively unaffected by salt concentrations between 200 mM to 500 mM whereas LysK shows increasing activity from 150mM with maximal activity at concentrations approaching 400 mM. LysK has a higher specific activity than Lysostaphin at NaCl concentrations greater than 150 mM. To determine the optimal pH, *S. aureus* 305 cells were resuspended in buffers ranging from pH 5 to pH 10 and treated with C-His-LysK or Lysostaphin for 5 minutes (Fig. 6). LysK and Lysostaphin show strong activity over a broad pH range from pH 6 to pH 9 (similar to previous reports for Lysostaphin (Schindler and Schuhardt. 1965. *Biochim. Biophys. Acta* 97: 242-250)).

[0097] Each peptidoglycan hydrolase has unique salt and pH optima. When combined these optima are sometimes shifted with respect to their components. Removing the LysK SH3b cell wall binding domain from the LysK-Lyso fusion increases the activity of the fusion protein.

EXAMPLE 6

Plate Lysis Assay

[0098] Due to the fact that antimicrobial assays for peptidoglycan hydrolases do not yield the same quantitative results between assays (Kusuma and Kokai-Kun. 2005. *Antimicrob. Agents Chemother.* 49(8): 3256-63), it was decided to test a second assay, namely, the plate lysis assay, with the fusion and parental proteins. Purified fusion enzymes were serially diluted into 150mM NaCl, 10mM tris, pH 7.5 buffer to yield concentrations of 10, 1, 0.1, and 0.01 µg/10 µl. 10µl of each dilution was spotted onto TSB agar plates which were previously irrigated with 2 mL of mid-log (0.4-0.6 OD_{600nm}) *S. aureus* strain Newman, excess culture removed, and plates allowed to air dry at room temperature for ~30 minutes in a laminar flow hood. Enzyme spots are allowed to air dry and incubated overnight at 37°C.

[0099] The results of the plate lysis assay are shown in Fig. 7. As shown above in the turbidity reduction assay, all of the fusion constructs are able to kill live *S. aureus* in the plate lysis assay. As expected from the work of Kusuma and Kokai-Kun (*supra*), the relative activity levels differ from those observed in the turbidity reduction assay. The bacteriocin, Lysostaphin, shows the highest activity in the plate lysis assay, lysing cells at 0.01 µg. All other enzymes analyzed show a weaker but similar activity, requiring 0.1 µg to lyse the *S. aureus*. It should be noted that Lysostaphin is much smaller than the other proteins so in molar equivalents, there are ~3 x as many Lysostaphin molecules as some of the other fusions *e.g.* LysK-Lyso (see Figs. 2 and 3 for SDS gels and molecular weight comparisons). This might be contributing to the lack of quantitative identity between assays.

EXAMPLE 7

Minimum Inhibitory Concentration

[0100] The Minimal Inhibitory Concentration (MIC) of fusion proteins with *S. aureus* Newman was determined. Enzymes are first serially diluted two fold across a 96 well plate from the first well containing 100 µl of buffer (150 mM NaCl, 10 mM tris, pH 7.5) + enzyme and 100 µl of 2x sterile Tryptic Soy Broth (TSB). 100 µl of these dilutions are then transferred to duplicate 96 well plates to which 100µl of *S. aureus* Newman in TSB is added

to each well. The CFU of the inoculating culture is $\sim 5 \times 10^5$ cells/ml. Plates are incubated 20 hours at 37°C, at which time plates are read with a 96 well plate reader and photographed. Plate reader OD_{600nm} values are used to determine the MIC. Wells that have less than 50% OD_{600nm} of the full growth (bright wells) are considered growth inhibited (red lines). Lysostaphin is serially diluted from 25 µg/ml, all other proteins are serially diluted from 125 µg/ml in the first well. Each well in the final assay contains 200 µl of 1xTSB with the buffer contributing 37.5 mM NaCl and 2.5 mM Tris.

[0101] MIC determinations for the fusion and parental lysins were performed in a 96 well microtiter plate (Fig. 8). Again, all of the fusion and parental lysins demonstrate the ability to inhibit *S. aureus* growth. Lysostaphin is again more active than the fusion proteins. Among the fusion proteins, the 221K-lyso construct is most active in the MIC assay, inhibiting culture growth at 5 µg/ml concentration.

EXAMPLE 8

Bactericidal Blood Assays

[0102] Blood was taken from euthanized rats aseptically and immediately added to conical tubes containing heparin (5U/ml). Heparinized rat blood was then stored rocking at room temperature until used. *S. aureus* mastitis strain 305 was grown to mid-log phase (OD_{600 nm} = 0.4 - 0.6) in Tryptic Soy Broth to ~ 100 cfu/µl. 1 µl of the diluted bacterial culture was added per 90 µls of heparinized rat blood en masse at a total volume sufficient to include for all samples. 455 µl of inoculated blood was then added to tubes containing 45 µl of each enzyme or buffer only (400 mM NaCl, 20mM Tris HCl, 1% glycerol, pH 7.5). The final volume of enzyme and TSB inoculum were 9% and 1% of the final reaction volume, respectively. Reactions were incubated in a shaker at 37°C between time points. Upon addition of blood, and at 90 and 180 minutes, aliquots were removed, diluted, and immediately plated onto TSB agar in triplicate. Plates were incubated at 37°C overnight; colonies were then counted to determine the number of colony forming units per ml.

[0103] The results in Fig. 9 are presented as the % CFUs of the buffer alone control (no lysin added) and indicate that all of the LysK-Lyso fusions kill *S. aureus* in heparinized whole rat blood; therefore, the fusions should be active when applied systemically to cure septicemia and other tissue infections.

EXAMPLE 9

Plate Lysis Assay for Testing Resistance Development

[0104] Cells were repeatedly exposed to peptidoglycan hydrolases over night on a tryptic soy agar (TSA) plate over a period of up to 20 days, similar to Loeffler *et al.* (2001, *supra*) with the following modifications. Lawns were prepared by diluting a mid log phase culture (OD_{600nm} 0.4-0.6) of *S. aureus* Newman cells 1/20 in TSB, flooding a TSA plate with 2 mL of the culture dilution, incubating at room temperature for 1 minute, and removing the excess culture. The plates (with lids removed) were allowed to air dry in a laminar flow hood (~30 minutes). Serial 10 fold dilutions of each enzyme in 150 mM NaCl, 10 mM Tris, pH 7.5 were prepared yielding five solutions ranging between 10 μ g to 0.1ng in 10 μ l of each enzyme to be tested. Dilutions were spotted onto the lawn, allowed to air dry for 30 minutes, and incubated overnight at 37°C. Cells were scraped from the spot with the lowest concentration of enzyme where there was only partial clearing (some obvious lysis had occurred as indicated by partial clearing of the spot on the plate). These 'exposed' cells were used to inoculate 5 mL of TSB and grown for several hours to generate a new culture and subsequent lawn. Cells were exposed consecutively for up to 20 days, at which time cells were prepared and tested in turbidity reduction assays, as described previously. Each strain that resulted from repeated exposure to the peptidoglycan hydrolase construct was then tested against the hydrolase used in the selection procedure (and if a fusion, the parental hydrolases used to make the construct) to ascertain if any resistance to either the test construct or the hydrolases of origin had occurred as a result of repeated exposure.

[0105] The *S. aureus* strain Newman isolates resulting from the 10 day "resistant strain selection protocol" demonstrated nearly identical susceptibility, in the turbidity reduction assay, to both the parental lysins and the fusion constructs as the *S. aureus* strain Newman used to initiate the selection protocol. We conclude that there was no resistance development following repeated exposure to the lytic proteins.

[0106] All publications and patents mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication or patent was specifically and individually indicated to be incorporated by reference.

[0107] The foregoing description and certain representative embodiments and details of the invention have been presented for purposes of illustration and description of the invention. It is not intended to be exhaustive or to limit the invention to the precise forms disclosed. It

will be apparent to practitioners skilled in this art that modifications and variations may be made therein without departing from the scope of the invention.

Claims

We claim:

1. A recombinant nucleic acid encoding an antimicrobial *Staphylococcus* -specific endolysin – Lysostaphin triple fusion protein, comprising (1) an endolysin CHAP endopeptidase domain, (2) an endolysin amidase domain, and (3) a Lysostaphin glycyl-glycine endopeptidase domain, said protein having specificity and exolytic activity for the peptidoglycan cell wall of untreated, live *Staphylococcus aureus*.
2. The recombinant nucleic acid of Claim 1 wherein each domain of said protein cuts the peptidoglycan at a different, unique covalent bond of the peptidoglycan, and each is lytic in the presence of lysis by the others.
3. The recombinant nucleic acid of Claim 1 wherein the parental lysin of each domain of the triple fusion protein is synergistic in antimicrobial activity when the parental lysins of said domains are used in combination.
4. The recombinant nucleic acid of Claim 1 wherein the endolysin is a LysK endolysin-derived peptidoglycan hydrolase.
5. The recombinant nucleic acid of Claim 1 wherein the endolysin is a phi11 endolysin-derived peptidoglycan hydrolase.
6. The recombinant nucleic acid of Claim 4 wherein said nucleic acid encodes an endolysin-derived peptidoglycan hydrolase molecule comprising a nucleic acid identified by SEQ ID NO: 5.
7. The recombinant nucleic acid of Claim 5 wherein said nucleic acid encodes an endolysin-derived peptidoglycan hydrolase molecule comprising a nucleic acid identified by SEQ ID NO: 13.
8. A recombinant nucleic acid encoding an antimicrobial *Staphylococcus* -specific endolysin – Lysostaphin triple fusion protein, comprising (1) an endolysin-derived CHAP endopeptidase and (2) an endolysin-derived amidase, and (3) a Lysostaphin glycyl-glycine

endopeptidase domain, said protein having specificity and exolytic activity for the peptidoglycan cell wall of untreated, live *S. aureus*, wherein said nucleic acid encodes a truncated endolysin-derived peptidoglycan hydrolase molecule.

9. The recombinant nucleic acid of Claim 8 wherein said nucleic acid comprises a nucleic acid identified by SEQ ID NO: 7, SEQ ID NO:9, or SEQ ID NO:11.

10. A construct comprising the nucleic acid of claim 1, wherein said nucleic acid is in operable linkage to a promoter that drives expression in a host cell.

11. A cloning vector comprising the construct of Claim 10.

12. An expression vector comprising the construct of Claim 10.

13. A process for transforming a host cell, comprising stably integrating the nucleic acid of Claim 1 or the construct of Claim 10 into the host cell.

14. An isolated host cell transformed with the nucleic acid according to Claim 1.

15. An isolated host cell transformed with the construct according to Claim 10.

16. The host cell of Claim 14 or 15, wherein said host cell is a single-celled or lower or higher multi-celled organism into which the construct according to the invention can be introduced so as to produce an antimicrobial *Staphylococcus* -specific endolysin – Lysostaphin triple fusion protein.

17. A method of making a recombinant antimicrobial *Staphylococcus* -specific endolysin – Lysostaphin triple fusion protein, said method comprising steps:

- a. introducing into a host cell a nucleic acid or construct encoding an antimicrobial *Staphylococcus* -specific endolysin – Lysostaphin triple fusion protein;
- b. culturing said cell under conditions suitable for expression of said protein;

c. recovering the protein so expressed.

18. A recombinant antimicrobial *Staphylococcus* -specific endolysin – Lysostaphin triple fusion protein, comprising (1) an endolysin CHAP endopeptidase domain, (2) an endolysin amidase domain, and (3) a Lysostaphin glycyl-glycine endopeptidase domain, said protein having specificity and exolytic activity for the peptidoglycan cell wall of untreated, live *Staphylococcus aureus*.

19. The recombinant triple fusion protein of Claim 18 wherein each domain of said protein cuts the peptidoglycan at a different, unique covalent bond of the peptidoglycan, and each is lytic in the presence of lysis by the others.

20. The recombinant triple fusion protein of Claim 18 wherein the parental lysin of each domain of the triple fusion protein is synergistic in antimicrobial activity when the parental lysins of said domains are used in combination.

21. The protein of claim 18, wherein the endolysin is a LysK endolysin-derived peptidoglycan hydrolase.

22. The protein of claim 18, wherein the endolysin is a phi11 endolysin-derived peptidoglycan hydrolase.

23. The protein of Claim 21 wherein said triple fusion protein comprises a polypeptide identified by SEQ ID NO:6.

24. The protein of Claim 22 wherein said triple fusion protein comprises a polypeptide identified by SEQ ID NO:14.

25. A recombinant antimicrobial *Staphylococcus* -specific endolysin – Lysostaphin triple fusion protein, comprising (1) an endolysin-derived CHAP endopeptidase domain and (2) an endolysin-derived amidase domain, and (3) a Lysostaphin glycyl-glycine endopeptidase domain, said protein having specificity and exolytic activity for the peptidoglycan cell wall of untreated, live *S. aureus*, wherein an endolysin-derived domain is truncated.

26. The protein of Claim 25, wherein the fusion protein has endopeptidase and amidase activity and does not require a SH3b binding domain.
27. The protein of Claim 25, wherein the fusion protein has endopeptidase activity and does not require a SH3b binding domain.
28. The recombinant antimicrobial *Staphylococcus* -specific endolysin – Lysostaphin triple fusion protein of Claim 25 wherein said protein comprises a polypeptide identified by SEQ ID NO: 8, SEQ ID NO:10, or SEQ ID NO:12.
29. A composition useful for the treatment of a disease caused by multidrug-resistant staphylococci, wherein said composition comprises the protein of Claim 18 and a pharmaceutically acceptable carrier.
30. A composition useful for the treatment of a disease caused by staphylococci, wherein said composition comprises the protein of Claim 25 and a pharmaceutically acceptable carrier.
31. A method of treating infection and disease caused by multidrug-resistant staphylococci in an individual comprising:
- administering to said individual an effective dosage of a composition of any one of Claims 16-18, wherein said composition comprises a recombinant antimicrobial *Staphylococcus* -specific endolysin – Lysostaphin triple fusion protein, comprising (1) an endolysin CHAP endopeptidase domain, (2) an endolysin amidase domain, and (3) a Lysostaphin glycyl-glycine endopeptidase domain, said protein having specificity and exolytic activity for the peptidoglycan cell wall of untreated, live *Staphylococcus aureus*, wherein said administration is effective for the treatment of said multidrug-resistant staphylococci.
32. A method of treating mastitis in an animal comprising:
- administering to said animal an effective dosage of a composition of Claims 16-18, wherein said composition comprises a recombinant antimicrobial *Staphylococcus* -specific endolysin – Lysostaphin triple fusion protein, comprising (1) an endolysin CHAP endopeptidase domain, (2) an endolysin amidase domain, and (3) a Lysostaphin

glycyl-glycine endopeptidase domain, said protein having specificity and exolytic activity for the peptidoglycan cell wall of untreated, live *Staphylococcus aureus*, wherein said administration is effective for reducing the severity of said mastitis.

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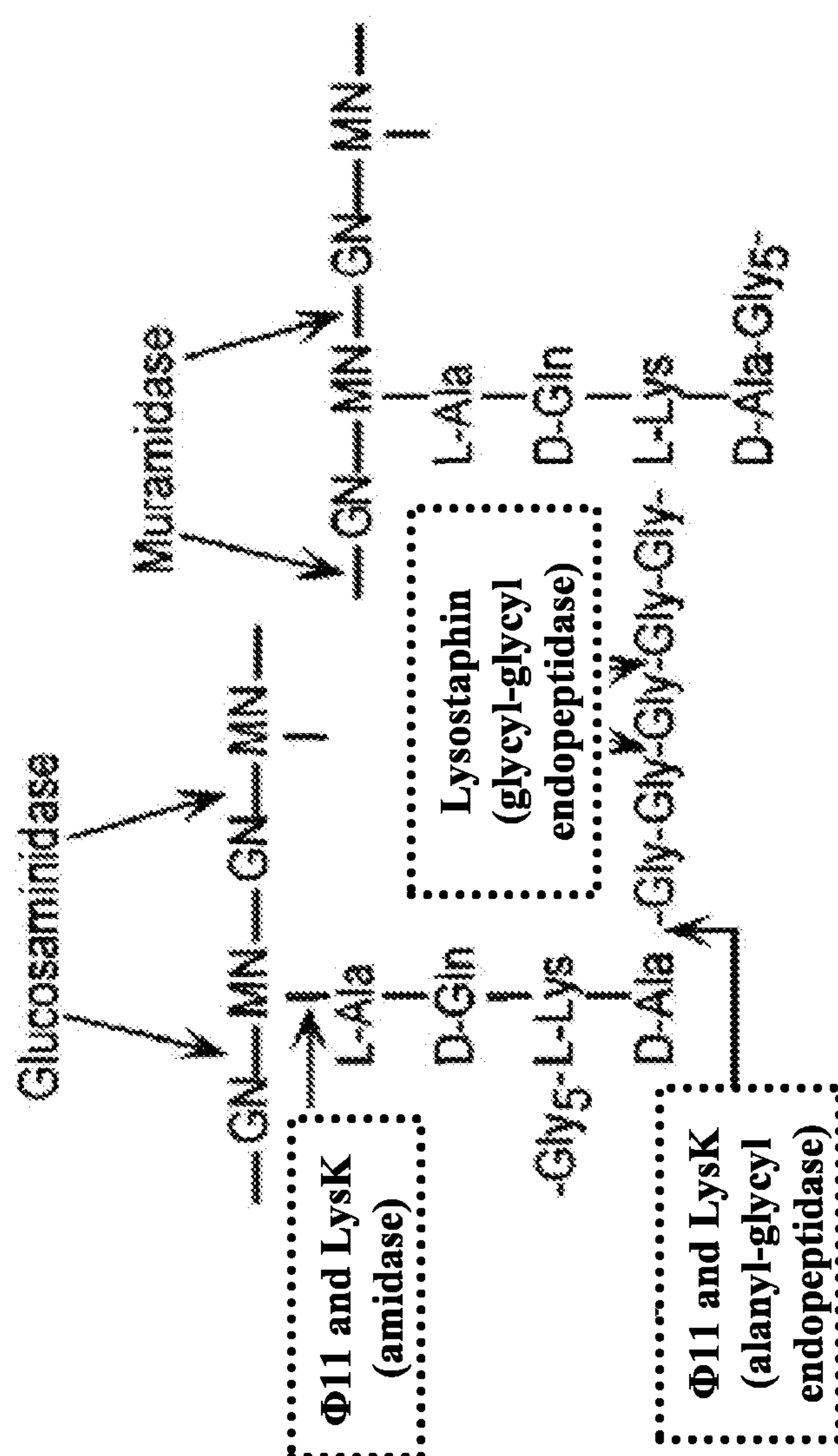
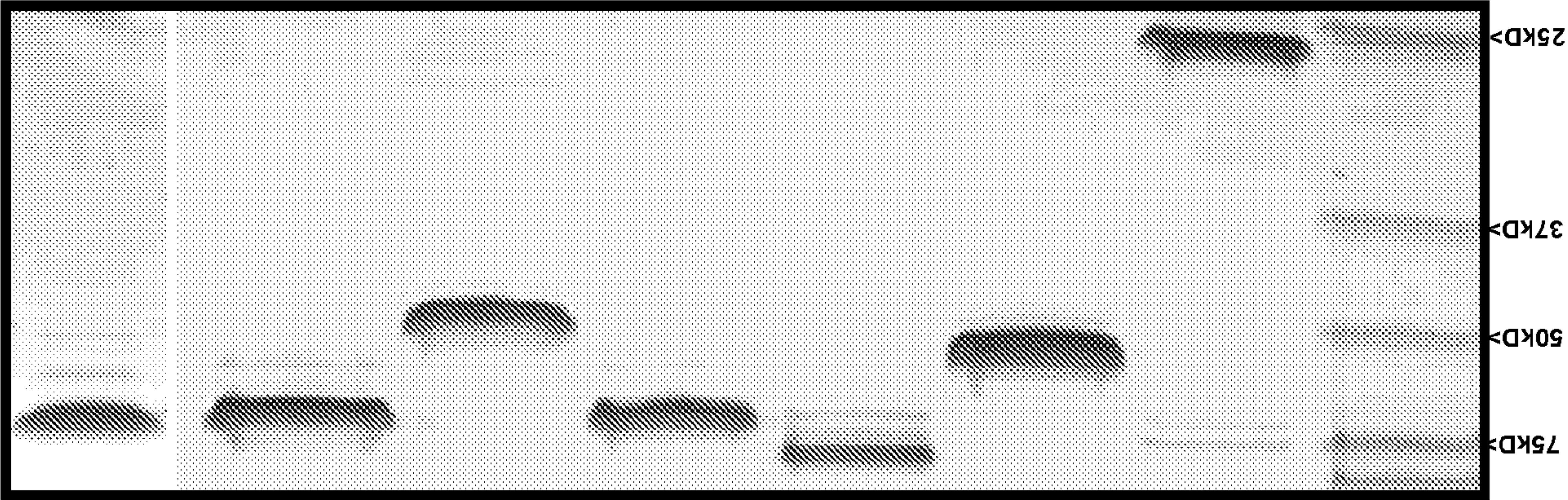
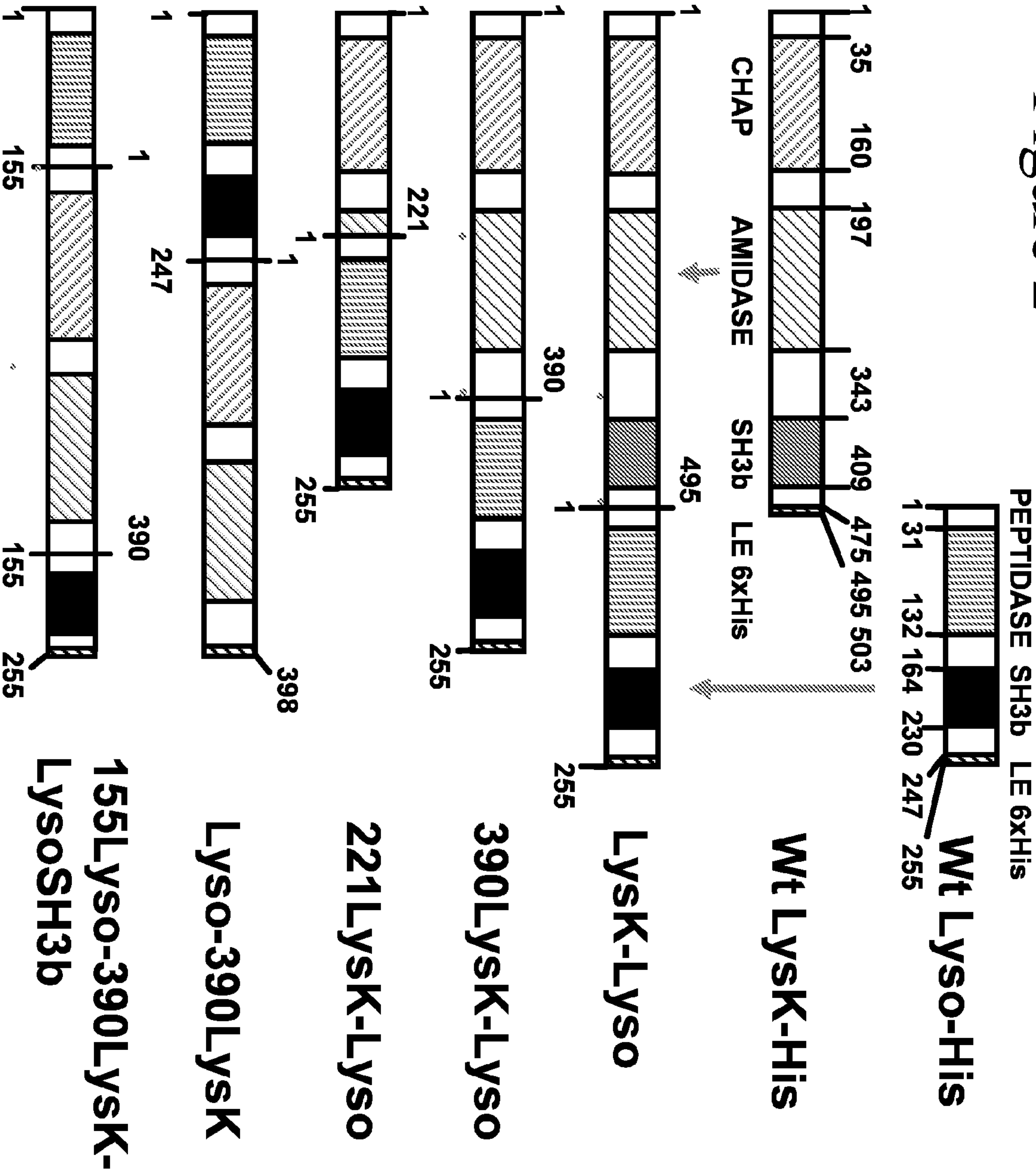


Figure 1

Figure 2



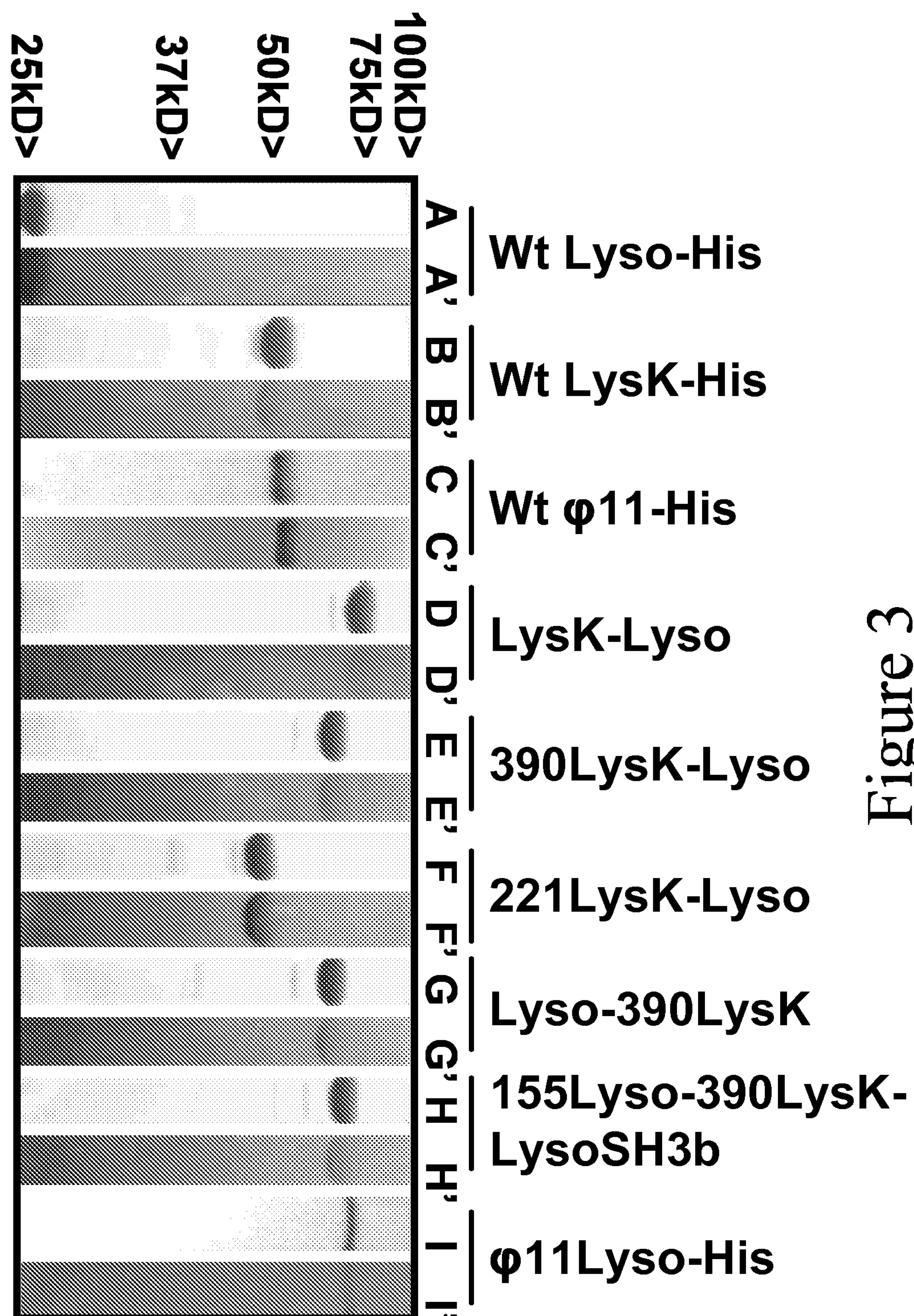


Figure 3

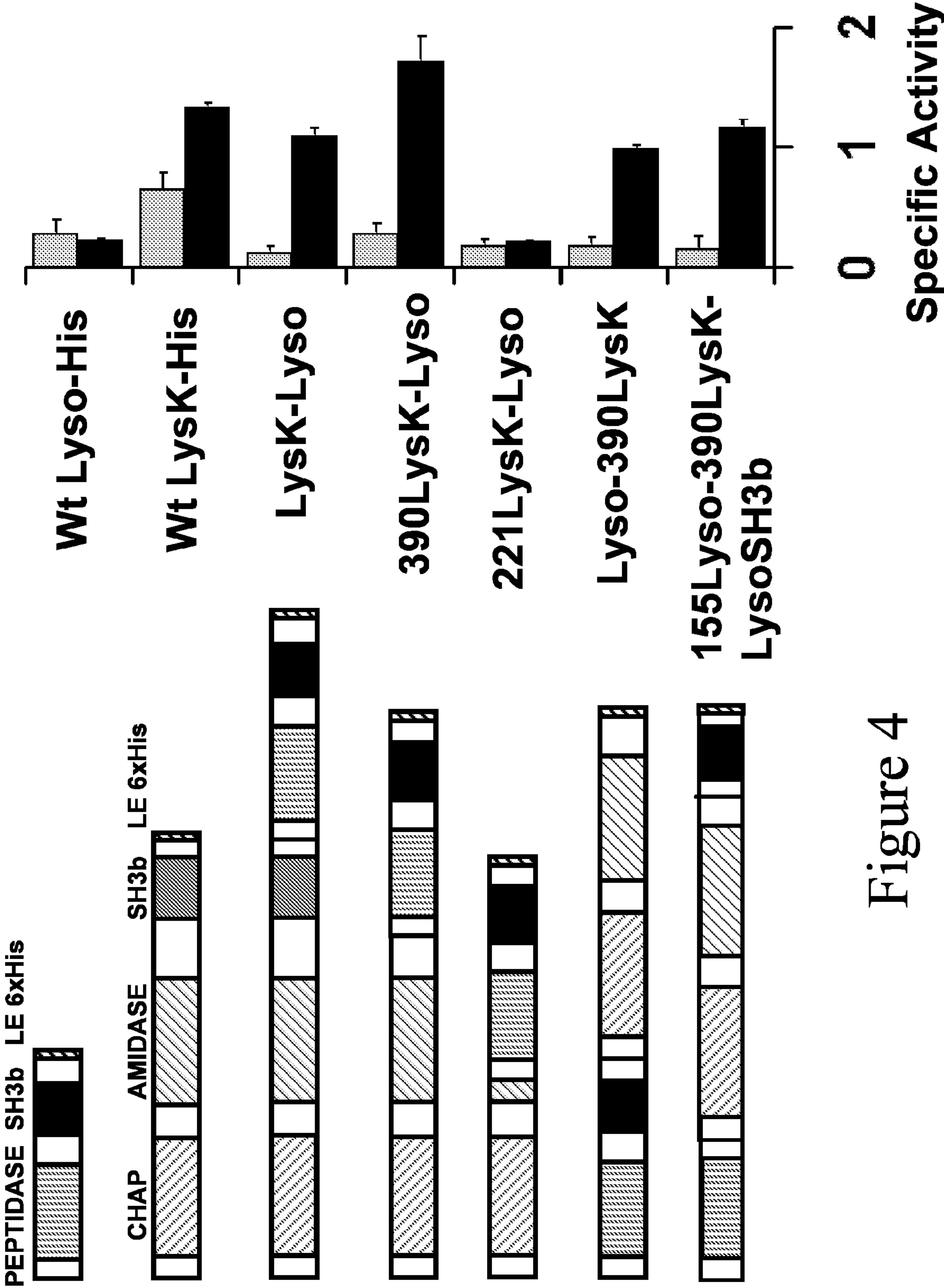


Figure 4

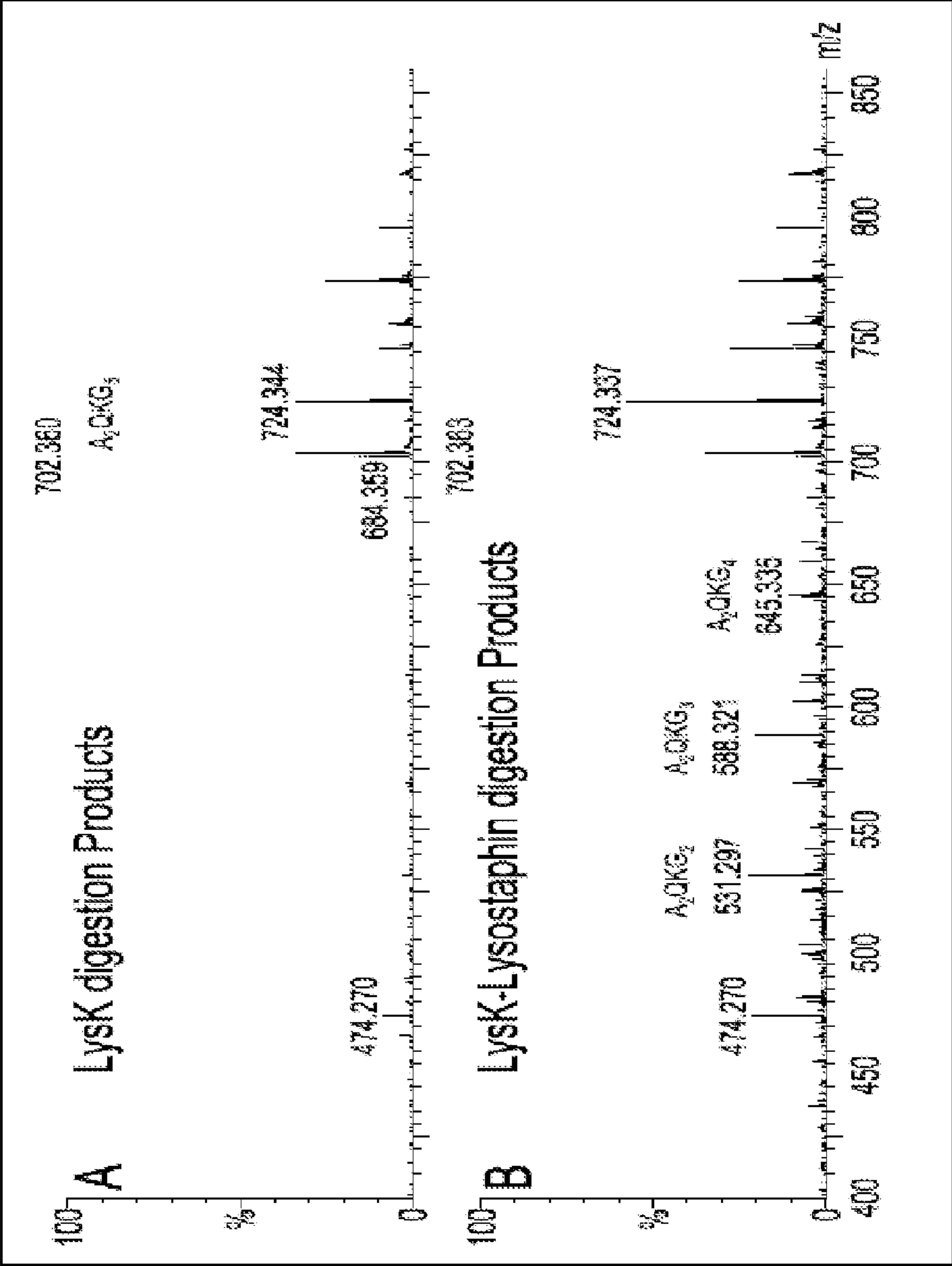


Figure 5

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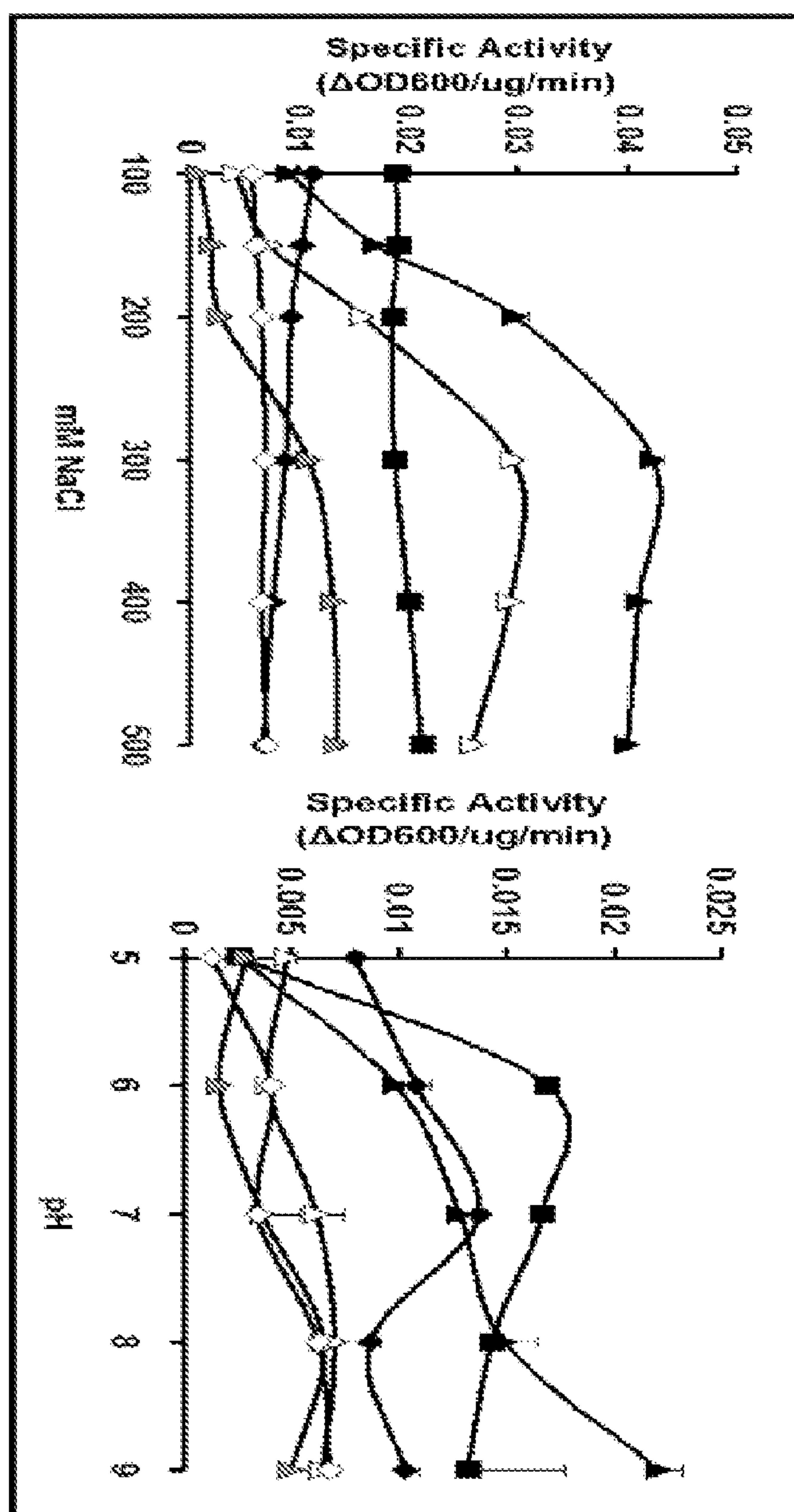
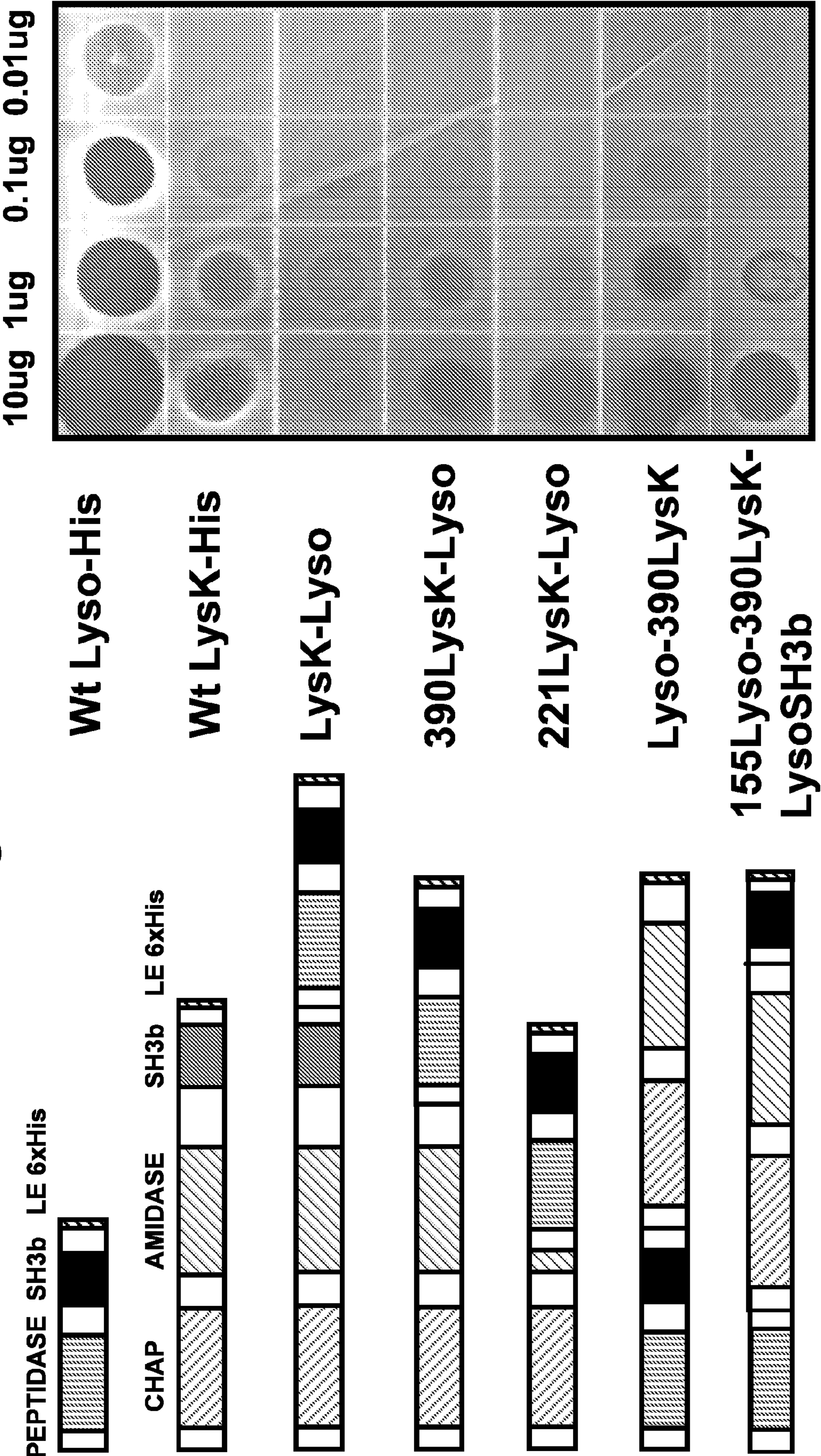
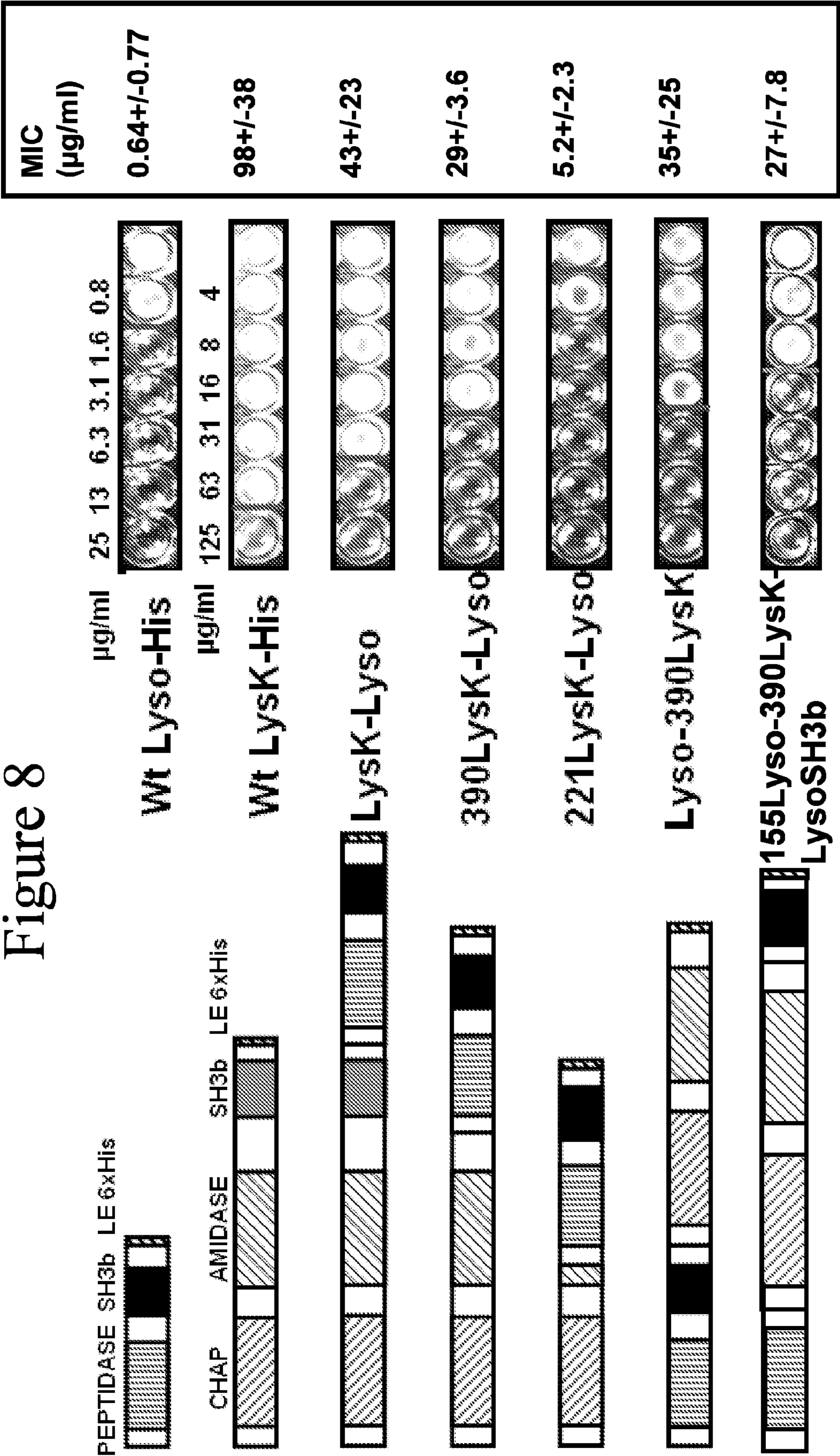


Figure 6

Figure 7





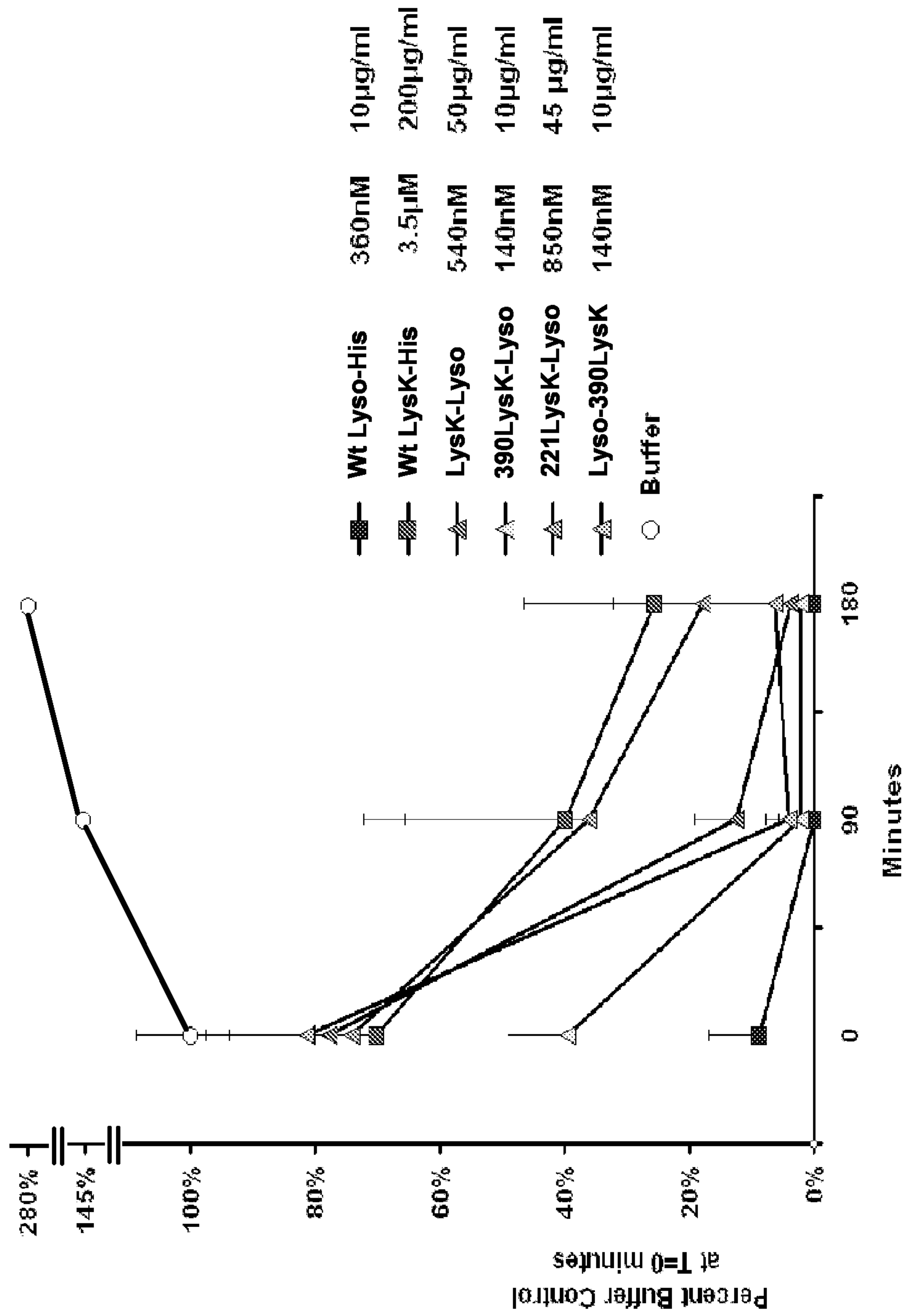


Figure 9

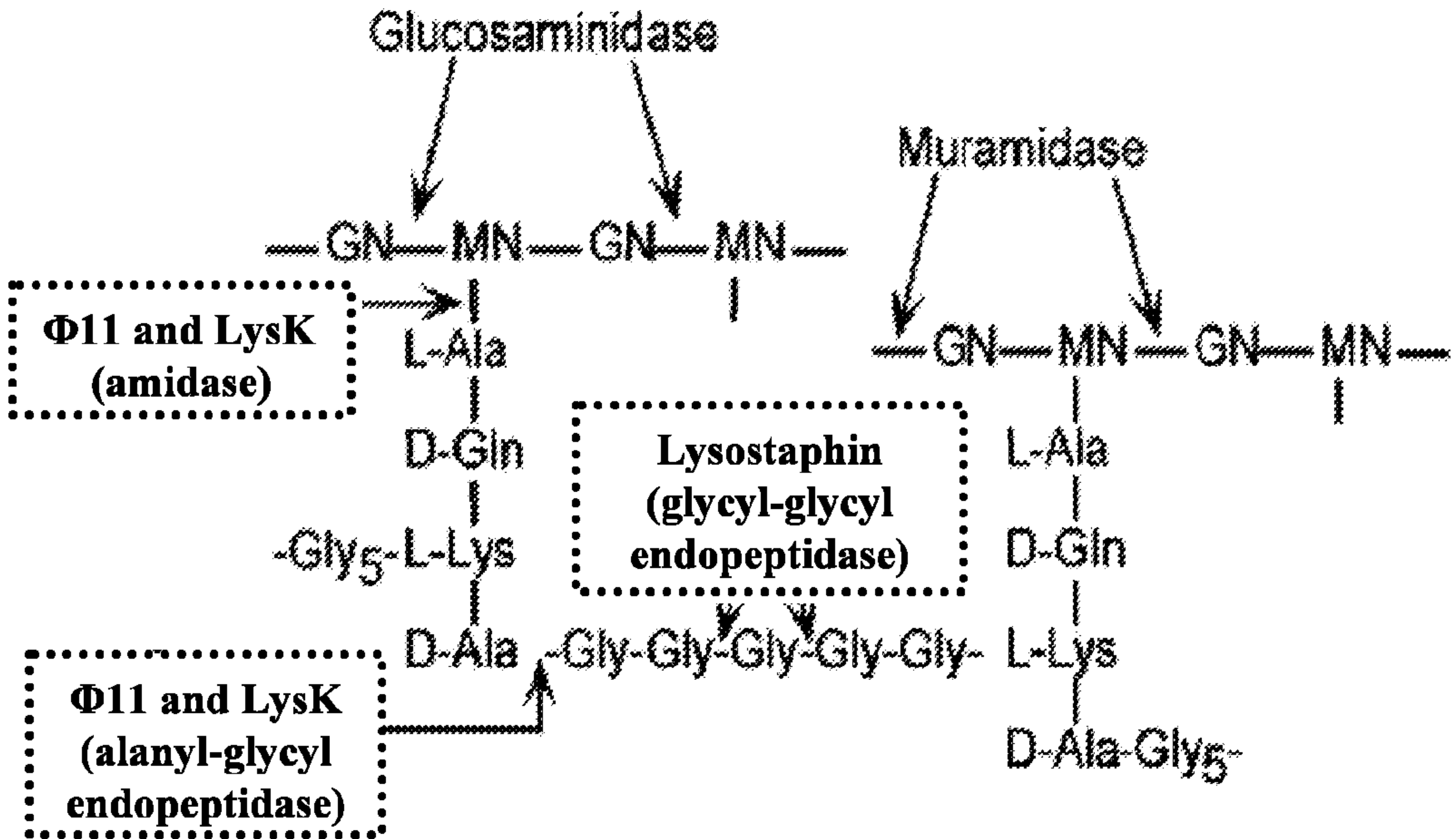


Figure 1