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[Continued on next page]

(54) Title: ENHANCED STAPHYLOLYTIC ACTIVITY OF THE STAPHYLOCOCCUS AUREUS BACTERIOPHAGE VB_SAU-Phi-PLA88 VIRION-ASSOCIATED PEPTIDOGLYCAN HYDROLASE HYDH5: FUSIONS, DELETIONS AND SYNERGY WITH LYSH5

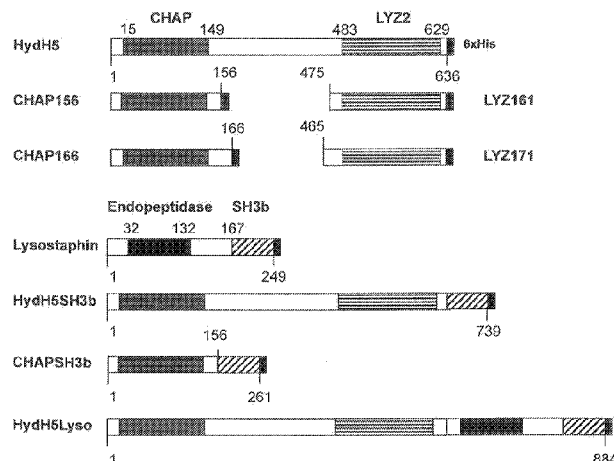


Fig. 1

(57) Abstract: Virion-associated peptidoglycan-hydrolases are potential antimicrobials due to their ability to lyse Gram-positive bacteria on contact. Full-length HydH5, a virion-associated peptidoglycan-hydrolase from the Staphylococcus aureus bacteriophage vB_SauS-phi-IPLA88, and two truncated derivatives, containing only the CHAP domain, exhibited high lytic activity against live *S. aureus* cells. Three different fusion proteins were created: HydH5 peptidoglycan hydrolase-lysostaphin, HydH5 peptidoglycan hydrolase-SH3b, or HydH5CHAP-SH3b. Each recombinant fusion polypeptide showed higher staphylolytic activity than the parental enzyme or its deletion construct. Both parental and fusion proteins lysed *S. aureus* cells in zymograms, plate-lysis and turbidity-reduction assays. In plate-lysis assays, HydH5 and its derivatives lysed bovine and human *S. aureus*, *S. aureus* MRSA N315 strain, and human Staphylococcus epidermidis strains. Several non-staphylococcal bacteria were not affected. HydH5 and its derivatives displayed antimicrobial synergy with the endolysin LysH5 in vitro, suggesting distinct cut sites for the two enzymes, which combined are potentially more efficient for eliminating staphylococcal infections.



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Enhanced Staphylytic Activity of the *Staphylococcus aureus* Bacteriophage
vB_SauS-phiPLA88 Virion-Associated Peptidoglycan Hydrolase HydH5:
Fusions, Deletions and Synergy with LysH5

BACKGROUND OF THE INVENTION

Field of the Invention

[0001] This invention relates to the field of polypeptides having antimicrobial activity and the polynucleotides encoding them. The invention also relates to nucleic acid constructs, vectors, and host cells comprising the nucleic acid constructs. The invention more specifically relates to an antimicrobial phage-associated HydH5 peptidoglycan hydrolase polypeptide, truncations of the HydH5 peptidoglycan hydrolase polypeptide, and fusion polypeptides comprising the HydH5 peptidoglycan hydrolase and truncated HydH5 polypeptides. This invention also relates to synergistic pathogen-specific compositions comprising the endolysin LysH5 together either with HydH5 or with fusion polypeptides comprising HydH5. The invention further relates to compositions and methods of making the polypeptides and methods of treating staphylococcal-associated diseases, including methicillin-resistant *Staphylococcus aureus* (MRSA).

Description of the Relevant Art

[0002] *Staphylococcus aureus* is a notorious pathogen that causes numerous pathologies including food poisoning, toxic shock syndrome, endocarditis, and skin and

wound infections, (Lowy, F. D. 1998. *N. Engl. J. Med.* 339:520-532). The emergence of multidrug-resistant strains, especially methicillin-resistant *S. aureus* (MRSA) and vancomycin-resistant *S. aureus* (VRSA) is raising serious concerns due to their high frequency in both nosocomial and community-acquired settings (Köck *et al.* 2010. *Euro Surveill.* 15(41):19688).

[0003] Recent results with phage therapy in animal models have driven much interest in phages and phage-encoded proteins to treat infections (O'Flaherty *et al.* 2009. *FEMS Microbiol. Rev.* 33(4):801-819; Fenton *et al.* 2010. *Bioeng. Bugs.* 1(1):9-16). These studies clearly show the efficacy of phages and lysins in killing human pathogenic bacteria in animal models (Matsuzaki *et al.* 2005. *J. Infect. Chemotherapy* 11:211-219; Fischetti, V.A. 2010. *Int. J. Med. Microbiol.* 300(6):357-362). Specifically, several assays have been performed against *S. aureus* bacteremia. The intraperitoneal administration of phages phiMR11 and phiMR25 rescued mice inoculated with a lethal dose of *S. aureus* (Matsuzaki *et al.* 2003. *J. Infect. Dis.* 187(4):613-624; Hoshiba *et al.* 2010. *Arch. Virol.* 155(4):545-552). Moreover, in a rabbit model of wound infection caused by *S. aureus*, phages prevented abscess formation (Wills *et al.* 2005. *Antimicrob. Agents Chemother.* 49(3):1220-1221). Phage lytic proteins also showed successful results. The intraperitoneal administration of the endolysin MV-L from phage phiMR11 protected mice against *S. aureus* MRSA septic death (Rashel *et al.* 2007. *J. Infect. Dis.* 196(8):1237-1247). In another animal model, bacteremia in unprotected mice reached colony counts of $\sim 10^7$ cfu/ml within 3.5 h after challenge, whereas the administration of the lytic enzyme LysGH15 1 h after MRSA injection was sufficient to protect mice with the mean colony count being less than 10^4 cfu/ml (Gu *et al.* 2011. *J. Clin. Microbiol.* 49(1):111-117). Furthermore, the activity of phage lytic proteins may be increased by using them in combination with other antimicrobials. Both *in vitro* (Becker *et al.* 2008. *FEMS Microbiol. Lett.* 287(2):185-191; Daniel *et al.* 2010. *Antimicrob. Agents Chemother.* 54(4):1603-1612; García *et al.* 2010. *Int. J. Food Microbiol.*

141(3):151-155) and *in vivo* synergy (Daniel *et al.*, *supra*) between phage endolysin constructs and antibiotics or bacteriocins against *S. aureus* have been reported.

[0004] Bacterial cell walls of both Gram-positive and Gram-negative bacteria are composed of peptidoglycan, a complex molecule with a sugar backbone of alternating *N*-acetylglucosamine and *N*-acetyl muramic acid residues cross-linked with peptide bridges. Peptidoglycan prevents osmotic lysis of cell protoplast and confers rigidity and shape on cells. Peptidoglycan hydrolases are essential for modifying the peptidoglycan to allow the cell to grow and divide (Vollmer *et al.* 2008. *FEMS Microbiol. Rev.* 32(2):287-306). There are three major peptidoglycan hydrolase activities, namely, (i) glycosidase, (ii) amidase, and (iii) endopeptidase activities. Most peptidoglycan hydrolases are composed of a C-terminal cell wall-binding (CWB) domain and a N-terminal catalytic domain(s) (Fischetti, V.A. 2005. *Trends in Microbiol.* 13:491-496). Bacteriophages also encode peptidoglycan hydrolases (Hermoso *et al.* 2007. *Curr. Opin. Microbiol.* 10(5):461-472) that play essential roles in the phage life cycle to allow both entry (virion-associated peptidoglycan hydrolases) and release (endolysins) of the mature phage particles.

[0005] In addition to endolysins, other phage encoded proteins (virion-associated peptidoglycan hydrolases) have a potential as antimicrobials. Some bacteriophage virions harbor virion-associated peptidoglycan hydrolases that facilitate the entry of phage DNA across the bacterial cell envelope during infection (Moak and Molineux. 2004. *Mol. Microbiol.* 51:1169-1183). They are also responsible for the “lysis from without”, a phenomenon caused by some phages when adsorbed onto the host cell at very high numbers (Delbrück, M. 1940. *J. Gen. Physiol.* 23(5):643-660). This type of peptidoglycan hydrolase activity has been described from a variety of different phage particles infecting *S. aureus* (Moak and Molineux, *supra*; Rashel *et al.* 2008. *FEMS Microbiol. Lett.* 284:9-16; Takac and Blasi. 2005. *Antimicrob. Agents Chemother.* 49(7):2934-2940), *Lactococcus lactis* (Kenny *et al.* 2004. *J. Bacteriol.* 186:3480-3491),

E. coli (Molineux, I.J. 2001. *Mol. Microbiol.* 40:1-8; Kanamaru *et al.* 2002. *Nature* 415:553-557), and *Salmonella* (Steinbacher *et al.* 1997. *J. Mol. Biol.* 267:865-880).

[0006] Recently, a peptidoglycan hydrolase (HydH5) encoded by phage vB_SauS-philPLA88 (philPLA88) has been identified and characterized (Rodríguez *et al.* 2011, *BMC Microbiol.* 11: 138). HydH5 has a N-terminal CHAP (cysteine, histidine-dependent amidohydrolase/peptidase) lytic domain and a C-terminal LYZ2 (lysozyme subfamily 2) lytic domain. HydH5 does not have a recognized cell wall binding domain. The full-length 634 amino acid HydH5 and truncated version harboring just one lytic domain (and 6×His-tag) have been overproduced in *E. coli*. The nickel chromatography purified proteins are able to kill viable *S. aureus* cells. HydH5 is highly thermostable since it showed antimicrobial activity after heat treatment (100°C, 5 min) (Rodríguez *et al.*, *supra*).

[0007] 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. 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 coagulase-negative staphylococci (Cisani *et al.* 1982. *Antimicrob. Agents Chemother.* 21: 531-535); McCormick *et al.* 2006. *Curr. Eye Res.* 31: 225-230).

[0008] The endolysin LysH5 is encoded by phiPLA88 phage and has three putative domains: a cysteine, histidine-dependent amidohydrolases/peptidase (CHAP) domain, an amidase-2 domain, and a C-terminal SH3b cell wall-binding (CWB) domain. LysH5 is able to inhibit the *S. aureus* growth in milk (Obeso *et al.* 2008. *Int. J. Food Microbiol.* 128(2):212-218) and showed a synergistic antimicrobial effect with the bacteriocin nisin (García *et al.* 2010, *supra*).

[0009] Antibiotic resistance in combination with other important virulence determinants, such as surface-located binding proteins to facilitate adhesion to host tissue, as well as many mechanisms to evade attack by human host defenses makes *S. aureus* a threatening pathogen (Otto, M. 2010. *Ann. Rev. Microbiol.* 64:143-162). Novel therapeutic agents specific for staphylococcal species, including methicillin-resistant *Staphylococcus aureus* (MRSA), are sorely needed to counter the rise of drug resistant pathogenic bacteria.

SUMMARY OF THE INVENTION

[0010] We have discovered that the nucleic acid encoding the vB_SauS-phiPLA88 virion-associated peptidoglycan hydrolase HydH5, a protein which specifically attacks the peptidoglycan cell wall of untreated live staphylococci can be truncated, that truncations encoding the CHAP (cysteine, histidine-dependent amidohydrolase/peptidase) lytic domain of the HydH5 peptidoglycan hydrolase result in polypeptides capable of exolysis, *i.e.*, "lysis from without" lytic activity, and that truncations of HydH5 and fusion polypeptides comprising HydH5 or truncations of HydH5 can be used as an antimicrobial treatment for *Staphylococcal*-induced infection and diseases, including infection and disease caused by multidrug-resistant strains.

[0011] In accordance with this discovery, it is an object of the invention to provide

nucleic acid molecules encoding the truncated HydH5 polypeptides and fusion proteins comprising the HydH5 peptidoglycan hydrolase polypeptide and truncations of HydH5.

[0012] It is also an object of the invention to provide an antimicrobial truncated HydH5 peptidoglycan hydrolase polypeptide which is functional in that it comprises the CHAP domain and retains its properties for exolysis of the peptidoglycan cell wall of staphylococcal bacteria.

[0013] It is a further object of the invention to provide polynucleotides encoding antimicrobial fusion proteins formed from a nucleic acid encoding a non-truncated HydH5 peptidoglycan hydrolase or encoding a truncated HydH5, *i.e.*, a functioning CHAP domain, in combination with a nucleic acid encoding lysostaphin or one or more of the SH3 cell wall-binding domain(s) of native lysostaphin.

[0014] It is a further object of the invention to provide antimicrobial fusion proteins comprising either a non-truncated HydH5 peptidoglycan hydrolase or a functioning HydH5 CHAP domain from truncated HydH5 in combination with either lysostaphin or one or more of the SH3b cell wall-binding (CWB) domain(s) of native lysostaphin, *i.e.*, HydH5 peptidoglycan hydrolase- lysostaphin, HydH5 peptidoglycan hydrolase-SH3b, and HydH5CHAP-SH3b.

[0015] It is another object of the invention to provide a method of using antimicrobial fusion proteins comprising the CHAP domain from truncated HydH5 peptidoglycan hydrolase in combination with lysostaphin or a SH3b cell wall-binding domain from lysostaphin to enhance the exolysis of *Staphylococcus* strains and also extend the targets that can be “lysed from without” to additional bovine and human strains of *Staphylococcus* over and above the lysis observed with functional native HydH5 peptidoglycan hydrolase comprising the native N-terminal CHAP domain in combination with the native C-terminal LYZ2 (lysozyme subfamily 2) lytic domain.

[0016] An added object of the invention is to provide a nucleic acid sequence encoding an antimicrobial HydH5 fusion polypeptide comprising the nucleic acid encoding the HydH5 peptidoglycan hydrolase or the truncated HydH5 peptidoglycan hydrolase, *i.e.*, the CHAP domain, in combination with nucleic acid encoding lysostaphin or a SH3b cell wall-binding domain from lysostaphin according to the invention as an encoding sequence which allows disease resistance to be imparted to the organism. It is well understood that this sequence can also be used in combination with another sequence, or sequences, encoding one or more disease resistant properties.

[0017] An additional object of the invention is to provide nucleic acid constructs, vectors, and host cells comprising the nucleic acid constructs encoding the fusion polypeptides of the invention.

[0018] An added object of the invention is to provide compositions useful for the treatment of disease caused by the *Staphylococcus* strains for which the fusion proteins of the invention are specific and effective.

[0019] Another object of the invention is to provide compositions comprising the LysH5 endolysin together with HydH5 peptidoglycan hydrolase or with an HydH5-derived fusion protein selected from HydH5 peptidoglycan hydrolase- lysostaphin, HydH5 peptidoglycan hydrolase-SH3b, or HydH5CHAP-SH3b for treatment of diseases and infections caused by *Staphylococcus* strains where the composition comprises LysH5 and a particular HydH5 fusion protein each in amounts that are ineffective alone but act synergistically together to effectively treat said diseases and infections.

[0020] Also part of this invention is a kit, comprising a composition for treatment of disease caused by the *Staphylococcus* strains for which the CHAP domain of the truncated HydH5 peptidoglycan hydrolase and fusions comprising the truncated HydH5 peptidoglycan hydrolase CHAP domain are specific and effective.

[0021] Other objects and advantages of this invention will become readily apparent from the ensuing description.

BRIEF DESCRIPTION OF THE DRAWINGS

[0022] Figure 1 is a schematic representation of HydH5 deletion and fusion constructs. The numbers indicate the initial and final amino acids in the domains as determined by Pfam domain database. Grey box: CHAP domain; horizontal stripes: LYZ2 domain; diagonal stripes = SH3b domain; large black box: endopeptidase domain; small black box: 6×His-tag.

[0023] Figures 2A, 2B, and 2C depict the antimicrobial activity of the HydH5, deletion and fusion constructs. Figure 2A shows the SDS-PAGE and zymogram analyses of 5 µg nickel affinity-purified proteins. Lane M: Standard molecular mass marker in kDa (Prestained SDS-PAGE Standards, broad range; BioRad Laboratories); lane 1, CHAP (19.6 kDa); 2, HydH5 (73.6 kDa); 3, HydH5SH3b (85.1 kDa); 4, HydH5Lyso (100.8 kDa); 5, CHAPSH3b (30.4 kDa); 6, Lysostaphin (28.1 kDa). Figure 2B shows a plate lysis assay of HydH5 and derived proteins using mid-log phase growing cells of *S. aureus* Sa9 and MRSA strain N315. Figure 2C shows turbidity reduction assay results using 1 µM of each protein. Specific activity is expressed as $\Delta OD_{600 \text{ nm}} \text{ min}^{-1} \mu\text{M}^{-1}$. Error bars are the means $\pm$ standard deviations of three independent assays.

[0024] Figure 3 shows the synergistic effect in checkerboard assay between endolysin LysH5 and HydH5 fusion constructs. Minimum Lytic Concentration (MLC) of each protein in the presence of subinhibitory concentrations of endolysin LysH5 is indicated. A (HydH5, 56.7 µg/ml to 0.06 µg/ml, $\Sigma\text{FIC } 0.065 \pm 0.001$), B (HydH5SH3b, 13.5 µg/ml to 0.01 µg/ml, $\Sigma\text{FIC } 0.130 \pm 0.000$), C (HydH5Lyso, 25.1 µg/ml to 0.02 µg/ml, $\Sigma\text{FIC } 0.049 \pm 0.023$), and D (CHAPSH3b, 17.34 µg/ml to 0.03 µg/ml, $\Sigma\text{FIC } 0.063 \pm 0.022$).

DETAILED DESCRIPTION OF THE INVENTION

[0025] In order to enhance the native antimicrobial activity of HydH5 peptidoglycan hydrolase against target staphylococcal species and strains, in this work we have fused HydH5 to both full length and truncated fragments of lysostaphin, a staphylolytic bacteriocin produced by *Staphylococcus simulans*. A chimeric protein made up of complete HydH5 and lysostaphin fused in a head to tail configuration was created. Similarly, both the complete HydH5 protein and just the HydH5 CHAP domain were fused to the SH3b CWB domain of lysostaphin in two separate constructs. The enzybiotic activity of all these fusion proteins towards live *S. aureus* and numerous non-staphylococcal bacteria was assessed. The list of pathogen species effectively lysed by proteins encoded by the constructs of invention has been extended to include both bovine and human staphylococcal strains. Finally, we have also evaluated possible synergistic effects between HydH5 and the endolysin LysH5 with the purpose to explore new enzybiotic-based strategies to fight *S. aureus* infections.

[0026] In our previous work (Rodríguez *et al.* 2011, *supra*), we demonstrated the antimicrobial activity of HydH5 peptidoglycan hydrolase against *S. aureus* Sa9 strain. With the goal to increase the HydH5 lytic activity we performed deletion analysis and created fusion proteins with the most active deletion constructs. Through deletion analysis we had identified in HydH5 the protein domains that are essential for its activity and showed that both CHAP and LYZ2 domains possess lytic activity against *S. aureus* cells (Rodríguez *et al.* 2011, *supra*). Previous to this work several reports have shown that endolysins can be truncated and some individual catalytic domains retain lytic activity with some of them showing higher specific activity than the full length protein (Cheng *et al.* 2007. *Appl. Microbiol. Biotechnol.* 74(6):1284-1291; Horgan *et al.* 2009. *Appl. Environ. Microbiol.* 75(3):872-874; Donovan *et al.* 2006. *Appl. Environ. Microbiol.* 72:5108-5112; Becker *et al.* 2008, *supra*). However, as was previously shown, some endolysin catalytic domains are almost inactive, such as the glycosidase domains from

lambdaSa2 prophage endolysin (Donovan and Foster-Frey. 2008. *FEMS Microbiol. Lett.* 287(1):22-33), or the B30 endolysin (Donovan *et al.* 2006, *supra*) and the amidase domain from LysK (Becker *et al.* 2008, *supra*). Although there might be protein stability or aberrant protein folding influencing our results, the HydH5 CHAP domain construct has approximately 1.4-fold lower specific activity compared to the activity of the full length HydH5, suggesting that LYZ2 domain also contributes to the complete protein activity.

[0027] We and others have shown previously that virion-associated peptidoglycan hydrolases lacking a defined CWB domain can also bind to the cell surface (Rashel *et al.* 2008, *supra*; Rodríguez *et al.* 2011, *supra*). Known CWB domains have been shown for some peptidoglycan hydrolases to be necessary for accurate cell wall recognition and subsequent lytic activity such as lysostaphin (Baba and Schneewind. 1996. *EMBO J.* 15(18):4789-4797), ALE-1 (Lu *et al.* 2006. *J. Biol. Chem.* 281(1):549-558), *L. monocytogenes* endolysins Ply118 and Ply500 (Loessner *et al.* 2002. *Mol. Microbiol.* 44(2):335-349) and *S. pneumoniae* CPL-1 (Pérez-Dorado *et al.* 2007. *J. Biol. Chem.* 282(34):24990-24999). However, there are numerous reports of C-terminally deleted lysin constructs with deleted CWB domains where the N-terminal lytic domain maintains its activity in the absence of its CWB domain (Cheng *et al.*, *supra*; Sass and Bierbaum. 2007. *Appl. Environ. Microbiol.* 73(1):347-352; Horgan *et al.*, *supra*; Donovan *et al.* 2006, *supra*; Becker *et al.* 2008, *supra*). Thus a defined CWB domain is not a necessity for full lytic activity, and this binding does not rule out the possibility that the inter-lytic domain region might harbour a heretofore unidentified CWB domain as suggested previously for the B30 endolysin (Donovan *et al.* 2006, *supra*; lysostaphin/B30 fusion).

[0028] Our hypothesis to justify the addition of a CWB domain considers the fact that a phage structural protein, HydH5, is likely brought into close proximity of the cell wall and the peptidoglycan substrate by the intact virion, meaning that a CWB domain is likely not a strict requirement. However, as a soluble single protein antimicrobial, the scenario

is quite different and the addition of a CWB domain might improve its lytic activity. Our SDS PAGE and zymogram results show that for all constructs, we are working with >95% pure proteins. In the HydH5-SH3b construct, the lysostaphin SH3b domain was fused to full-length HydH5 increasing the lytic activity 1.7-fold, suggesting that this CWB domain helps the lytic domain to degrade the peptidoglycan substrate. A similar result was obtained when the native Cpl-7 cell wall binding domains of the streptococcal LambdaSa2 endolysin was replaced by staphylococcal SH3b domain from lysostaphin or LysK resulting in a 5× increase in staphylolytic activity (Becker *et al.* 2009. *FEMS Microbiol. Lett.* 294(1):52-60). In the HydH5CHAP-SH3b construct, the lysostaphin SH3b domain was fused to the CHAP domain of HydH5 resulting in a 4.8-fold increase in the lytic activity of the CHAP domain alone construct (CHAP) and a 3.3-fold increase compared to full length HydH5. This result indicates the importance of the SH3b CWB domain in recognizing the bacteria cell wall, taking into account that only one catalytic domain (CHAP) fused to the SH3b domain was sufficient to obtain the highest specific activity of the various constructs.

[0029] Moreover, addition of the full length lysostaphin to HydH5 (HydH5-lysostaphin) also achieved greater activity levels than the parental protein (HydH5). This result further indicates the modular nature of these protein domains and that the addition of new catalytic domains to HydH5 (*i.e.*, the glycyl-glycine endopeptidase of lysostaphin) can also improve its lytic activity. The three different catalytic domains are believed to each attack a different peptidoglycan bond. The presence of multiple unique domains theoretically decreases the likelihood of bacterial resistance development (Fischetti, V.A. 2005, *supra*). The increased activity also suggests that each domain is modular, as expected (Garcia *et al.* 1990. *Gene* 86(1):81-88), and although the activity of the final construct is not the sum of parental lytic activities, each domain likely achieves a near-native conformation.

[0030] HydH5 and its derivatives fusions were assessed for their ability to lyse a number of staphylococcal and non-staphylococcal strains. No previous data have been reported regarding the lytic spectrum of *S. aureus* virion-associated peptidoglycan hydrolases. All proteins were active against staphylococcal strains but no other genus was lysed at detectable levels. Our results indicate a similar lytic spectrum for both HydH5 and its derivative fusions. These results are in agreement with *S. aureus* endolysins described to date that demonstrate a lytic spectrum limited to staphylococci. However, a different level of the lytic activity was observed for each species/strain. In general, *S. aureus* strains were more sensitive than *S. epidermidis* strains. Within the *S. aureus* strains, we observed that bovine strains were more sensitive than clinical strains. This could be due to the shared origin of these strains and the phage from which HydH5 originated; both share a source from a dairy environment. A similar result was previously observed for endolysin LysH5 (Obeso *et al.*, *supra*). Although we do not yet have direct biochemical proof that all three lytic domains are functional in the HydH5-lysostaphin construct, the reduced activity against *S. epidermidis* compared to *S. aureus* is consistent with the lytic range of lysostaphin and suggests that the lysostaphin domain is functional in this construct, given that lysostaphin is known to have a reduced activity against *S. epidermidis* compared to *S. aureus* (Zygmunt *et al.* 1968. *Appl. Microbiol.* 16(8):1168-1173).

[0031] Synergistic interactions between antimicrobial compounds have the potential to be exploited in order to increase effectiveness against the target bacteria, thereby reducing the dose required of each while decreasing the likelihood of antimicrobial-resistance. The combination of the two lytic bacteriophage enzymes HydH5 and LysH5 appears to have synergistic activity on *S. aureus* Sa9 strain. The HydH5-derived fusions also showed this positive interaction. The basis of synergy between peptidoglycan hydrolases might be explained by the hydrolytic activity of one enzyme loosening the peptidoglycan structure, thus facilitating better access of the second enzyme to its target. It is encouraging that the strongest *in vitro* synergy was observed between

LysH5 and HydH5-lysostaphin, suggesting that this combination might be protective *in vivo*. However, in the absence of biochemical data to indicate which domains are active in the fusion and the parental LysH5, we cannot identify the particular domains that are responsible for the observed antimicrobial synergy. However, these results are in agreement with previous studies indicating synergy between Pal and Cpl-1 lysins against *S. pneumoniae* (Loeffler and Fischetti. 2003. *Antimicrob. Agents Chemother.* 47(1):375-377), and the synergy between endolysin LysK and the bacteriocin lysostaphin against *S. aureus*. A 33% reduction in LysK concentration was obtained in the presence of lysostaphin (Becker *et al.* 2008, *supra*). Another bacteriocin, nisin, enhanced 8-fold the lytic activity of LysH5 on *S. aureus* cell suspensions (García *et al.* 2010, *supra*). Comparable results were obtained by combination with antibiotics (Manoharadas *et al.* 2009. *J. Biotechnol.* 139:118-123; Daniel *et al.*, *supra*). However, the effect of a second antimicrobial compound on reducing the development of resistant staphylococcal strains is also a consideration.

[0032] In summary, we report the development of novel chimeric peptidoglycan hydrolases with improved lytic activity against *S. aureus* and *S. epidermidis*, including MRSA N315 strain. The effectiveness of HydH5 and its derivative fusions when used in combination with LysH5, another dairy-derived protein were remarkable. We expect that these constructs will provide new weapons to combat multidrug-resistant *S. aureus* infections in both dairy and clinical environments.

[0033] The present invention also relates to a chimeric gene (or expression cassette) comprising an encoding sequence as well as heterologous regulatory elements in positions 5' and 3' which can function in a host organism, the encoding sequence comprising at least one nucleic acid sequence encoding an isolated truncated HydH5 peptidoglycan hydrolase related protein (truncation or fusion) as defined above. By host organism there is to be understood any single-celled or lower or higher non-human multi-celled organism into which HydH5 peptidoglycan hydrolase gene according to the

invention can be introduced. The regulatory elements required for expressing the nucleic acid sequence encoding a truncated HydH5 peptidoglycan hydrolase are well known to those skilled in the art and depend on the host organism. The means and methods for identifying and choosing the regulatory elements are well known to those skilled in the art and widely described in the literature.

[0034] The present invention also relates to a cloning and/or expression vector for transforming a host organism containing at least the truncated HydH5 peptidoglycan hydrolase gene as defined hereinabove. This vector comprises, in addition, to the above truncated HydH5 peptidoglycan hydrolase gene, at least one replication origin. This vector can be constituted by a plasmid, a cosmid, a bacteriophage or a virus which is transformed by introducing the chimeric gene according to the invention. Such transformation vectors according to the host organism to be transformed are well known to those skilled in the art and widely described in the literature.

[0035] A further subject of the invention is a process for the transformation of host organisms, by integrating a least one nucleic acid sequence or chimeric gene as defined hereinabove, which transformation may be carried out by any suitable known means which have been widely described in the specialist literature and in particular in the references cited in the present application, more particularly by the vector according to the invention.

[0036] 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 the HydH5 peptidoglycan hydrolase 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.

[0037] 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 occur. Conventional nucleic acid methods known to skilled artisans may be used to obtain isolated polynucleotides. The term embraces cDNA, recombinant polynucleotides and chemically synthesized polynucleotides.

[0038] The term "transformation" refers to a permanent or transient genetic change induced in a cell following the incorporation of new DNA (*i.e.* DNA exogenous to the cell). Where the cell is a mammalian cell, a permanent genetic change is generally achieved by introduction of the DNA into the genome of the cell. When the cell is a bacterial cell, the term usually refers to an extrachromosomal, self-replicating vector which harbors a selectable antibiotic resistance. Thus, isolated polynucleotides of the present invention can be incorporated into recombinant constructs, typically DNA constructs, capable of introduction into and replication in a host cell. Such a construct can be a vector that includes a replication system and sequences that are capable of transcription and translation of a polypeptide-encoding sequence in a given host cell.

[0039] 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.

[0040] 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).

[0041] "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.

[0042] "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.

[0043] 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.

[0044] The term "genomic sequence" refers to a sequence having non-contiguous open reading frames, where introns interrupt the protein coding regions. It may further include the 3' and 5' untranslated regions found in the mature mRNA. It may further include specific transcriptional and translational regulatory sequences, such as promoters, enhancers, etc., including about 1 kb, but possibly more, of flanking genomic DNA at either the 5' or 3' end of the transcribed region. The genomic DNA may be isolated as a fragment of 100 kbp or smaller; and substantially free of flanking chromosomal sequence.

[0045] 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.

[0046] As used herein, the term "chimeric" refers to two or more DNA molecules which are derived from different sources, strains, or species, which do not recombine under natural conditions, or to two or more DNA molecules from the same species, which are linked in a manner that does not occur in the native genome.

[0047] 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).

[0048] 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.

[0049] The invention includes functional fragments of the HydH5 peptidoglycan hydrolase polypeptide and functional fusion polypeptides encompassing a functional HydH5 peptidoglycan hydrolase 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 of HydH5 peptidoglycan hydrolase" refers to all fragments of HydH5 peptidoglycan hydrolase that retain HydH5 peptidoglycan hydrolase activity and function to lyse staphylococcal bacteria.

[0050] Modifications of the HydH5 peptidoglycan hydrolase primary amino acid sequence may result in further mutant or variant proteins having substantially equivalent activity to the HydH5 peptidoglycan hydrolase 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 HydH5 peptidoglycan hydrolase polypeptide. Any polypeptides produced by minor modifications of the HydH5 peptidoglycan hydrolase primary amino acid sequence are included herein as long as

the biological activity of HydH5 peptidoglycan hydrolase is present; *e.g.*, having a role in pathways leading to lysis of staphylococcal bacteria.

[0051] 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. A method of selecting an isolated polynucleotide that affects the level of expression of a polypeptide in a host cell may comprise the steps of: constructing an isolated polynucleotide of the present invention or an isolated chimeric gene of the present invention; introducing the isolated polynucleotide or the isolated chimeric gene into a host cell; measuring the level of a polypeptide in the host cell containing the isolated polynucleotide; and comparing the

level of a polypeptide in the host cell containing the isolated polynucleotide with the level of a polypeptide in a host cell that does not contain the isolated polynucleotide.

[0052] 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 HydH5 peptidoglycan hydrolase polypeptide and which hybridize under stringent conditions to the HydH5 peptidoglycan hydrolase sequences disclosed herein, or to fragments thereof, are encompassed by the present invention.

[0053] 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).

[0054] 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.

[0055] 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.

[0056] 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.

[0057] 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.

[0058] 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).

[0059] 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.

[0060] 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 HydH5 peptidoglycan hydrolase-like activity. Alternatively, fragments of a nucleotide sequence that are useful as hybridization probes may not encode fragment proteins retaining biological activity.

[0061] 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 HydH5 peptidoglycan hydrolase 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.

[0062] 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, HydH5 peptidoglycan hydrolase activity as described herein. Such variants may result from, for example, genetic polymorphism or from human manipulation. Biologically active variants of a native HydH5 peptidoglycan hydrolase protein 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 native protein 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.

[0063] 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 HydH5 peptidoglycan hydrolase 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.

[0064] 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 HydH5 peptidoglycan hydrolase protein can be observed.

[0065] "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.

[0066] 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. 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.

[0067] 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

[0068] 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

Bacterial Strains and Culture Conditions

[0069] Bacterial strains used to determine lytic spectrum of proteins are listed in Table 1. *S. aureus* Sa9 was used as the indicator strain for lytic activity (Obeso *et al.*, *supra*). The *S. aureus* bovine isolates were from in house stocks (IPLA-CSIC), the *S. aureus* clinical isolates by Dr. Suárez (University of Oviedo, Spain), the *S. epidermidis* by Dr. Delgado (IPLA-CSIC) and the *S. aureus* MRSA strain N315 by Prof. Gabi Bierbaum (University of Bonn, Germany). All *Staphylococcus* strains were grown in TSB broth (Tryptic Soy Broth, DIFCO, Franklin Lakes, NJ) at 37°C with shaking or in TSB plates containing 2% (w/v) bacteriological agar (TSA). Other bacteria strains belonging to several different genera such as *Bacillus*, *Streptococcus*, *Listeria*, *Enterococcus*, *Clostridium*, *Lactococcus*, *Leuconostoc* and *Lactobacillus*, were insensitive to the lytic proteins (data not shown). *Bacillus*, *Streptococcus*, *Listeria* and *Enterococcus* strains were grown in 2 × YT at 37°C with shaking. *Clostridium* strain was grown in BHI (Brain Heart Infusion, Scharlau, Barcelona, Spain) at 30°C under static conditions and anaerobiosis (Anaerocult A, Merck, Darmstadt, Germany). *Lactococcus* strain was grown in lactose-M17 (Scharlau, Barcelona, Spain) at 30°C and static. *Leuconostoc* and *Lactobacillus* strains were grown in MRS (Scharlau, Barcelona, Spain) at 30°C and static.

EXAMPLE 2

Plasmid Constructs; DNA Manipulation

[0070] Bacteriophage vB_SauS-philPLA88 gene *orf58* encoding HydH5 (Acc. Number ACJ64586) was codon-optimized (*orf58-opt*) based on the *E. coli* codon usage (Laird *et*

al. 2005. *Protein Expr. Purif.* 39(2):237-246) and commercially synthesized (Genescript, Piscataway, NJ, USA). Then *orf58-opt* was synthesized with a 5' *NdeI* and a 3' *XhoI* restriction enzyme sites and the resultant fragment was subcloned between the *NdeI* and *XhoI* sites in the multi cloning site of the inducible expression vector pET21a (EMD Biosciences, San Diego, CA) which introduces a C-terminal 6 x His-tag. All constructions in this work were created in this vector and thus have two additional amino acids residues introduced at the C-terminus corresponding to *XhoI* site (Leu-Glu) followed by the 6×His-tag. Truncated versions of HydH5 were constructed via PCR amplification by introducing cloning sites via the PCR primers: *XhoI* sites (underlined) after aa codon 156 for CHAP156 domain (CHAP156R: 5'- ACTGACTGGCTCGAGTTT GTCCGGGTG- 3'; SEQ ID NO: 1) or after aa codon 166 for CHAP166 domain (CHAP166R: 5' –ATCGACTGGCTCGAGTTTCGGCACCGG- 3'; SEQ ID NO: 2) and a *NdeI* site before aa codon 475 for LYZ161 domain (LYZ161F: 5'- ACTGACTGCATATG GTTAGCGTCTCC- 3'; SEQ ID NO: 3) or before aa codon 465 for LYZ171 domain (LYZ171F: 5' –ACTGACTGCATATGCAAATGCTGAAC- 3'; SEQ ID NO: 4). Forward primer for CHAP domains was pET21aBgIII-F: 5'- CGTAGA GGATCGAGATCTCGATC- 3' (SEQ ID NO: 5) and reverse primer for LYZ2 domains was pET21aStyl-R: 5'- CGT TTAGAGGCCCCAAGGGGTTATG- 3' (SEQ ID NO: 6). The full-length lysostaphin gene was amplified with PCR pairs Full Lyso Sall-F (5'- ATC ATCGTCGACGCTGCAACAC ATGAACATTCAGCAC- 3'; SEQ ID NO: 7) and pET21aStyl-R. The lysostaphin fragment encoding the SH3b domain was amplified with PCR primer pairs Lyso Sall144- F (5'-GGAAAAGCAGTCGACACAGTAACTCC-3'; SEQ ID NO: 8) and pET21aStyl-R. These amplified fragments have an N-terminal *Sall* restriction site (underlined) designed to allow in-frame gene fusions of full- length lysostaphin or its SH3b domain individually to the C-terminus of HydH5 or its catalytic domain CHAP156. Vector constructs were performed in *E. coli* DH5α cells (Invitrogen, Carlsbad, CA) and induced in *E. coli* BL21(DE3) (EMD Biosciences, San Diego, CA).

[0071] To determine whether both the CHAP and LYZ2 as single domains showed increased lytic activity toward *S. aureus* Sa9 over those previously obtained (Rodríguez *et al.* 2011.) four deletion constructs were generated. A schematic representation of all constructs is presented in Fig. 1. In order to ensure proper folding, each construct encoded a single lytic domain with an additional sequence of either 7 or 17 amino acids surrounding the catalytic domain (CHAP156, CHAP166, LYZ161 and LYZ171). In addition, due to the lack of a known cell wall binding domain in HydH5, we proceeded to determine whether the addition of a cell wall binding domain might increase the lytic activity of HydH5 and CHAP. Therefore, three different fusion proteins were created between lysostaphin and HydH5 (Fig. 1). Initially, the nucleic acid encoding the lysostaphin binding domain SH3b was fused to the nucleic acid encoding the full-length HydH5 protein, resulting in a protein with two catalytic domains and one cell wall binding domain, *i.e.*, the protein HydH5-SH3b (SEQ ID NO: 10). A second construct was obtained by fusion of the nucleic acid encoding the CHAP156 domain to the nucleic acid encoding the lysostaphin SH3b domain resulting in the protein HydH5CHAP-SH3b (SEQ ID NO: 12). Finally, the nucleic acid encoding the full-length lysostaphin and the full-length HydH5 were fused in order to obtain the protein HydH5-Lysostaphin (SEQ ID NO: 14) with three catalytic domains and a cell wall binding domain. All the fusion proteins could be detected and purified with the exception of LYZ161 and LYZ171 whose products could not be detected after nickel column purification of the *E. coli* cultures.

[0072] The nucleic acid molecules encoding the constructs HydH5-SH3b, HydH5CHAP-SH3b, and HydH5-Lysostaphin, comprise nucleotides encoding C-terminal Leu-Glu-His-His-His-His-His residues and are identified by SEQ ID NOs: 9, 11, and 13, respectively. The expressed proteins are His-tagged with eight additional amino acid residues introduced at the C-terminus corresponding to the XhoI site (Leu-Glu) followed by six His residues. The proteins encoded by these nucleic acid sequences are identified by SEQ ID NOs: 10, 12 and 14, respectively. The constructs encoding

CHAP156, CHAP166, LYZ161 and LYZ171 are identified by SEQ ID NOs: 15, 17, 19 and 21, respectively, and the resulting proteins, by SEQ ID NOs: 16, 18, 20 and 22, respectively.

EXAMPLE 3

Protein Purification and Analysis

[0073] Protein purification was performed as previously described (Donovan and Foster-Frey, *supra*) with the following modifications: Exponential growing cultures induced by IPTG (1 mM, final concentration) were incubated at 10°C for 20 h. Then 500 ml pellets were sonicated for 5 min using an automatic pulsing sonication (Bronson Sonifier; Bronson Sonic Power Co., Danbury, CT, USA). Protein purification was carried out by NiNTA nickel column chromatography (Qiagen). Wash and elution profiles were empirically determined to be 20 ml of 10 mM imidazole, 40 ml of 20 mM imidazole and elution with 1 ml of 250 mM imidazole in phosphate buffered saline (50 mM NaH₂PO₄, 300 mM NaCl, pH 8.0) with 30% glycerol to prevent precipitation of the purified protein. Then all samples were converted to HydH5 activity buffer (HEPES 50 mM, NDSB-201 0.5 M, CaCl₂ 0.25 mM, MnCl₂ 0.25 mM, MgCl₂ 0.25 mM, TCEP 1 mM, NaCl 24 mM, KCl 1 mM pH 7.5)(Rodriguez *et al.* 2011.) containing 30% glycerol using pre-equilibrated Zeba Desalting Columns (Thermo Fisher Scientific, Rockford, IL).

[0074] Purity of HydH5 constructs were evaluated in a 15% (v/w) SDS-PAGE in Tris-Glycine buffer at 150 V for 1.5 h using Criterion Precast gels (Bio-Rad, Inc., Hercules, CA). The proteins purified on the nickel column retained the His tags, thus the His tags were present in the various assays. His tags can be removed by methods well known and practiced in the art.

EXAMPLE 4

Zymogram, Plate Lysis and Turbidity Reduction Assays

[0075] Due to reports that peptidoglycan hydrolase lytic activity is not always quantitatively comparable between multiple assays although it usually agrees qualitatively (Kusuma and Kokai-Kun. 2005. *Antimicrob. Agents Chemother.* 49(8): 3256-3263), the lytic activities of the other proteins (HydH5, CHAP156, CHAP166, HydH5SH3b, CHAPSH3b and HydH5Lyso) were determined in three antimicrobial assays: zymogram, plate lysis and turbidity reduction assays. The turbidity reduction assay requires a robust enzyme in order to lyse large numbers of cells (starting concentration is $>10^9$ cells/ml), while the plate lysis assay can define relative activity with much lower amounts of bacteria, approximately 10^6 cells.

[0076] Zymogram assays were performed as previously described (Rodríguez *et al.* 2011, *supra*). SDS gels were stained via conventional Coomassie staining and zymograms were soaked for 30 min in distilled water to remove SDS and then incubated at room temperature in water for 15 min to detect areas of clearing in the turbid gel where a lytic protein is localized.

[0077] As shown in Fig. 2A, all fusion proteins showed a single zone of clearing in the zymogram consistent with the predicted molecular mass and positions of the purified proteins in the SDS-PAGE. In addition, the truncation constructs (CHAP156 and CHAP166) also had the ability to lyse *S. aureus*. The activity of both truncated constructs (CHAP156 and CHAP166) turned out to be the same regardless of the length of the lytic domain. Therefore, only the results obtained with CHAP156 are shown.

[0078] Plate lysis was performed as previously described (Donovan and Foster-Frey, *supra*). Purified proteins for each construct were diluted in HydH5 activity buffer. Ten microliters of a 4 μ M solution and 1:2 dilutions of each construct were spotted onto a freshly spread lawn of *S. aureus* Sa9, *S. aureus* MRSA N315 and the strains of Table 1 that had air dried for 30 min on tryptic soy agar plates. The spotted plates were air dried for 10 min in a laminar flow hood, and incubated overnight in a 37°C environment. A cleared spot in the lawn indicates lysis of the pathogen. Scoring of the cleared spots occurred within 20 hr of plating the cells.

[0079] In the plate lysis assay (Fig. 2B) the full-length HydH5 showed clearing of *S. aureus* at ≥ 2 μ M concentration while the HydH5CHAP domain required higher concentrations (≥ 16 μ M). All the fusion proteins showed significantly greater activity than the parental protein HydH5, even against a methicillin-resistant *S. aureus* MRSA N315 strain. HydH5 has clear staphylolytic activity at 4 μ M in contrast with fusion proteins which shown lysis activity at much lower concentrations (1 μ M). Fusion of SH3b to the CHAP domain (HydH5CHAP-SH3b) also increased CHAP activity in plate lysis by a factor of 64.

[0080] In order to determine the specificity of the HydH5 and its derivative fusions, the plate lysis assay was performed using cells from different species and genera grown at OD_{600nm} = 0.5. The different bacterial cells suspensions were exposed to 2 μ M protein solutions in the plate lysis assay. We found that 10 μ l of 2 μ M HydH5-derived fusions, HydH5-SH3b and HydH5CHAP-SH3b, were able to lyse all *S. aureus* and *S. epidermidis* strains (14 tested; Table 1). In contrast, 10 μ l of 2 μ M HydH5-Lysostaphin was able to lyse all the *S. aureus* strains and only 2 out of 4 *S. epidermidis* strains. *S. epidermidis* lysis could be achieved at 4 μ M (data not shown). Other bacteria belonging genera *Bacillus*, *Streptococcus*, *Clostridium*, *Lactococcus*, *Leuconostoc*, *Lactobacillus*, *Listeria* and *Enterococcus* were not affected by any of the staphylolytic proteins. The lytic activity of HydH5 and its derivative fusions is specific for staphylococci. It was

observed that the degree of lytic activity on *S. epidermidis* strains was generally lower than that observed on *S. aureus* group. Moreover, a different spectrum of lytic activity was noted for each protein. HydH5 and HydH5-Lysostaphin seem to be the less active proteins in this assay. HydH5CHAP-SH3b showed the higher lytic activity even against *S. epidermidis* strains (Table 1). In addition to the differences between species, strain origin also seems to play a role in determining the lytic spectrum since HydH5-SH3b and HydH5-Lysostaphin were more active against bovine strains than clinical strains. This difference is more pronounced in the case of HydH5-Lysostaphin (Table 1).

Table 1. Lytic spectrum of HydH5 and its derivative fusions. Bacterial strains were exposed to 2 μ M of each protein.

STRAIN	PROTEIN				
	HydH5	HydH5Lyso	HydH5SH3b	CHAPSH3b	Lysostaphin
<u><i>S. aureus</i>:</u>					
<u>Bovine strains</u>					
Sa9	+	++	+++	+++	+++
Sa6	-	+++	+++	+++	+++
Sa11	+	+++	+++	+++	+++
AFG1	+	++	+++	+++	+++
AC9	+	+	+++	+++	+++
<u><i>S. aureus</i>:</u>					
<u>Human strains</u>					
N315	-	++	+++	+++	+++
96	-	+	++	+++	+++
143	-	+	+	++	+++
445	-	++	+++	+++	+++
c4	+	++	+++	+++	+++
<u><i>S. epidermidis</i></u>					
B1CD2	+	-	+++	+++	+
C213	-	++	+++	+++	++
Z2LDC17	+	+	++	+++	-
SILDC3	-	-	++	+++	++

* Strong lytic halo (+++), medium (++) , weak (+) and no halo (-) was indicated.

[0081] Proteins were also tested by turbidity reduction assay (Fig. 2C) performed against live *S. aureus* Sa9 cells prepared as previously described (Donovan and Foster-Frey, *supra*; Becker *et al.* 2009, *supra*). The turbidity assay measures the drop in optical density (OD) resulting from lysis of the target bacteria with the HydH5-derived protein. A standardized turbidity assay modified from (Donovan *et al.* 2006a. *Appl. Environ. Microbiol.* 72:2988-2996) with *S. aureus* grown to logarithmic phase ($A_{600nm} = 0.4 - 0.6$) at 37°C in TSB (Tryptic Soy Broth, DIFCO, Franklin Lakes, NJ) were performed in a 96 well dish and analyzed in a plate reader as described previously (Becker *et al.* 2009b., *supra*). Log phase cultures were harvested at 4°C by centrifugation and stored on ice less than 4 hours until just before the assay when they were suspended in 150 mM NaCl, 10mM Tris-Cl, pH 7.5 or 50mM Phosphate buffer, pH 7.5 to an $A_{600nm} \sim 1.0$. Enzyme samples are added to three wells of a 96 well dish in 100 μ l of buffer (Ni-NTA elution buffer or storage buffer were shown to be equivalent). All samples are performed in triplicate. The assay is started by the addition of 100 μ l of cells in buffer at $A_{600nm} \sim 1.0$ via multichannel pipettor. A 'no enzyme control' of buffer and cells is included. A_{600nm} readings are taken every 20 seconds for 5 minutes. The readings for each well are transferred electronically to an Excel spreadsheet where they are analyzed in a sliding window over each group of 3 consecutive time points during the five minute period, to identify the highest instantaneous change in A_{600nm} for each well. The absolute values of ΔA_{600nm} for each group of 3 time points are ranked and the optimum chosen based on highest absolute value and reproducibility in the triplicate wells. A similarly calculated buffer plus cells alone control value from triplicate wells is then subtracted from the highest ranked value for each experimental well, and the values for the triplicate wells averaged to give a ΔOD_{600nm} /minute. This value is then divided by the concentration (μ M) of enzyme protein in the sample tested to give a specific activity ΔOD_{600nm} / μ M/min. The turbidity reduction assays are repeated with multiple independent protein isolations to verify the results, but only representative assays are presented, due to the high day-to-day variability in the results, presumably due to variations in the cell culture preparations.

[0082] This assay was performed using 1 μM of purified proteins. Specific activity of the proteins was expressed as $\Delta\text{OD}_{600\text{ nm}} \text{ min}^{-1} \mu\text{M}^{-1}$. The full-length recombinant HydH5, synthesized from the *E. coli* codon optimized version of *orf58opt*, showed a lytic activity in this assay in contrast to that previously observed in HydH5 from the standard *orf58* (Rodriguez *et al.* 2011, *supra*). The recombinant HydH5 was able to lyse live *S. aureus* Sa9 cells and had a specific activity of $0.033 \pm 0.009 \Delta\text{OD}_{600\text{ nm}} \text{ min}^{-1} \mu\text{M}^{-1}$ (Fig. 2C). Similar results were obtained for HydH5CHAP domain but about 1.4-fold reduction in specific activity ($0.023 \pm 0.001 \Delta\text{OD}_{600\text{ nm}} \text{ min}^{-1} \mu\text{M}^{-1}$) was observed when compared to full-length HydH5 activity. Fusion proteins, HydH5-Lysostaphin and HydH5-SH3b, showed a 2.5- and 1.7-fold higher specific activity than the parental protein HydH5, respectively. HydH5CHAP-SH3b showed an activity 4.8-fold greater than the CHAP domain. The specific activity of HydH5CHAP-SH3b was calculated to be $0.109 \pm 0.023 \Delta\text{OD}_{600\text{ nm}} \text{ min}^{-1} \mu\text{M}^{-1}$ the highest lytic activity obtained from HydH5 fusions. Therefore, C-terminal lysostaphin fusions conferred an enhanced staphylolytic activity to HydH5 and its catalytic domain CHAP.

EXAMPLE 4

Synergy

[0083] To determine the interaction of HydH5 and its derivative fusions with the endolysin LysH5 a standard checkerboard dilution assay was performed using live *S. aureus* Sa9 cells in HydH5 activity buffer to a final $\text{OD}_{600\text{ nm}}$ of ~ 0.8 . Checkerboard tests were performed between LysH5 (Obeso *et al.*, *supra*) and HydH5 and between LysH5 (Acc. Number EU573240) and HydH5-derived constructs as described in Garcia *et al.* (2010, *supra*). Initially, we determined the minimum lytic concentration (MLC) of each protein: HydH5 (56.7 $\mu\text{g/ml}$), HydH5-Lysostaphin (12.5 $\mu\text{g/ml}$), HydH5-SH3b (13.5 $\mu\text{g/ml}$), HydH5CHAP-SH3b (8.7 $\mu\text{g/ml}$) and LysH5 (2.5 $\mu\text{g/ml}$). MLCs were defined as

the lowest concentration at which an OD_{600nm} less than 0.1 after 15 minutes of incubation at 37°C was obtained. Ranges of enzyme concentrations were thus: LysH5 (2.5 µg/ml to 0.04 µg/ml) ; HydH5 (56.7 µg/ml to 0.06 µg/ml), HydH5-Lysostaphin (25.1 µg/ml to 0.02 µg/ml), HydH5-SH3b (13.5 µg/ml to 0.01 µg/ml) and HydH5CHAP-SH3b (17.34 µg/ml to 0.03 µg/ml). The fractional inhibitory concentration (FIC) was calculated as the minimum lytic concentration (MLC) of the antimicrobial in combination divided by the MLC of the antimicrobial acting alone. Strong synergy exists if the sum of the two FICs [$\Sigma FIC = FICA + FICB$] is <0.5 (Hall *et al.* 1983. *J. Antimicrob. Chemother.* 11(5):427-433). All the experiments were performed in duplicate.

[0084] When HydH5 or its derived fusions were combined with LysH5, a synergistic effect was observed in all combinations. In the presence of subinhibitory concentrations of the HydH5-derived proteins, a lower endolysin LysH5 concentration was needed to fully decrease the *S. aureus* Sa9 OD_{600nm}. Representative graphs of the synergistic interaction between LysH5 and HydH5 and between LysH5 and each HydH5-lysostaphin construct are shown in Fig. 3. HydH5 and its fusions act synergistically with LysH5 against *S. aureus*. From the MLC checkerboard test, the average ΣFIC values for the four protein combinations were calculated (Fig. 3). All these values are indicative of strong synergistic interaction and it could be concluded that the increased lysis observed with these mixtures would also be more effective to inhibit *S. aureus* bacterial growth.

[0085] 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.

[0086] 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. An isolated or recombinant nucleic acid encoding an antimicrobial fusion peptidoglycan hydrolase polypeptide comprising the complete HydH5 peptidoglycan hydrolase polypeptide or a truncated HydH5 polypeptide (HydH5 156 or HydH5 166), said truncated HydH5 protein being the HydH5CHAP domain having exolytic function for the peptidoglycan cell wall of staphylococcal bacteria.
2. The nucleic acid of Claim 1, wherein said nucleic acid encodes an antimicrobial fusion peptidoglycan hydrolase polypeptide comprising the complete, non-truncated HydH5 peptidoglycan hydrolase polypeptide and an SH3 cell wall-binding domain of native lysostaphin.
3. The nucleic acid of Claim 1, wherein said nucleic acid encodes an antimicrobial fusion peptidoglycan hydrolase polypeptide comprising the HydH5CHAP domain of truncated HydH5 peptidoglycan hydrolase and the SH3 cell wall-binding domain of native lysostaphin.
4. The nucleic acid of Claim 1, wherein said nucleic acid encodes an antimicrobial fusion peptidoglycan hydrolase polypeptide comprising the complete, non-truncated HydH5 peptidoglycan hydrolase polypeptide and lysostaphin.
5. The nucleic acid of Claim 2, wherein said nucleic acid encodes an antimicrobial fusion peptidoglycan hydrolase polypeptide HydH5-SH3b having the sequence of SEQ ID NO: 10

6. The nucleic acid of Claim 3, wherein said nucleic acid encodes an antimicrobial fusion peptidoglycan hydrolase polypeptide HydH5CHAP- SH3b having the sequence of SEQ ID NO: 12
7. The nucleic acid of Claim 4, wherein said nucleic acid encodes an antimicrobial fusion peptidoglycan hydrolase polypeptide HydH5-Lysostaphin having the sequence of SEQ ID NO: 14
8. The nucleic acid of Claim 5 having the sequence of SEQ ID NO: 9.
9. The nucleic acid of Claim 6 having the sequence of SEQ ID NO: 11.
10. The nucleic acid of Claim 7 having the sequence of SEQ ID NO: 13.
11. 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.
12. A cloning vector comprising the construct of Claim 11.
13. An expression vector comprising the construct of Claim 11.
14. A process for transforming a host cell, comprising stably integrating the nucleic acid of Claim 1 or the construct of Claim 11 into the host cell.
15. An isolated host cell transformed with the nucleic acid according to Claim 1.
16. An isolated host cell transformed with the construct according to Claim 11.

17. The host cell of Claim 15 or 16, 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 peptidoglycan hydrolase.

18. A method of making a recombinant peptidoglycan hydrolase fusion protein, said method comprising steps:

- a. introducing into a host cell a nucleic acid or construct encoding a peptidoglycan hydrolase fusion protein;
- b. culturing said cell under conditions suitable for expression of said protein;
- c. recovering the protein so expressed.

19. An isolated antimicrobial fusion peptidoglycan hydrolase protein comprising either a non-truncated HydH5 peptidoglycan hydrolase or a functioning HydH5 CHAP domain from truncated HydH5 in combination with either lysostaphin or the SH3b cell wall-binding (CWB) domain of native lysostaphin.

20. The protein of Claim 19 wherein said fusion protein is HydH5 peptidoglycan hydrolase- lysostaphin, HydH5 peptidoglycan hydrolase-SH3b, or HydH5CHAP-SH3b.

21. The protein of Claim 20 wherein said protein is identified by SEQ ID NO: 10, SEQ ID NO: 12, and SEQ ID NO: 14, respectively.

22. A composition useful for the treatment of disease caused by the *Staphylococcus* strains for which the fusion proteins of the invention are specific and effective, wherein said composition comprises any one of the proteins of Claim 20 and a pharmaceutically acceptable carrier.
23. A composition useful for the treatment of a disease caused by multidrug-resistant staphylococcal strains including methicillin-resistant *S. aureus* (MRSA), wherein said composition comprises any one of the fusion proteins of Claim 20 and a pharmaceutically acceptable carrier.
24. A antimicrobial staphylococcal-specific composition comprising an isolated or recombinant LysH5 endolysin together with HydH5 peptidoglycan hydrolase or with an HydH5-derived fusion protein selected from HydH5 peptidoglycan hydrolase-lysostaphin, HydH5 peptidoglycan hydrolase-SH3b, or HydH5CHAP-SH3b wherein said composition is functional for treatment of diseases and infections caused by *Staphylococcus* strains where the composition comprises LysH5 and a particular HydH5 fusion protein each in amounts that are ineffective alone but act synergistically together to effectively treat said diseases and infections.
25. A composition useful for the treatment of a disease caused by multidrug-resistant staphylococcal strains including methicillin-resistant *S. aureus* (MRSA), wherein said composition comprises any one of the fusion proteins of Claim 24 and a pharmaceutically acceptable carrier.
26. A composition useful for the treatment of disease comprising the composition of Claim 20 in combination with another sequence, or sequences, encoding one or more disease-resistance properties.

27. A method of treating infection and disease caused by staphylococci in an individual comprising:

administering to said individual an effective dosage of a composition of Claim 20, wherein said composition comprises an isolated recombinant peptidoglycan hydrolase fusion protein having specificity and exolytic activity for the peptidoglycan cell wall of untreated staphylococci and wherein said administration is effective for the treatment of diseases and infections caused by *Staphylococcus* strains including multidrug-resistant staphylococcal strains and methicillin-resistant *S. aureus* (MRSA).

28. A method of treating infection and disease caused by staphylococci in an individual comprising:

administering to said individual an effective dosage of a composition of Claim 24, wherein said composition comprises LysH5 and an isolated recombinant peptidoglycan hydrolase fusion protein having specificity and exolytic activity for the peptidoglycan cell wall of untreated staphylococci, each in amounts that are ineffective alone but act synergistically together to effectively treat said disease and infection caused by *Staphylococcus* strains including multidrug-resistant staphylococcal strains and methicillin-resistant *S. aureus* (MRSA).

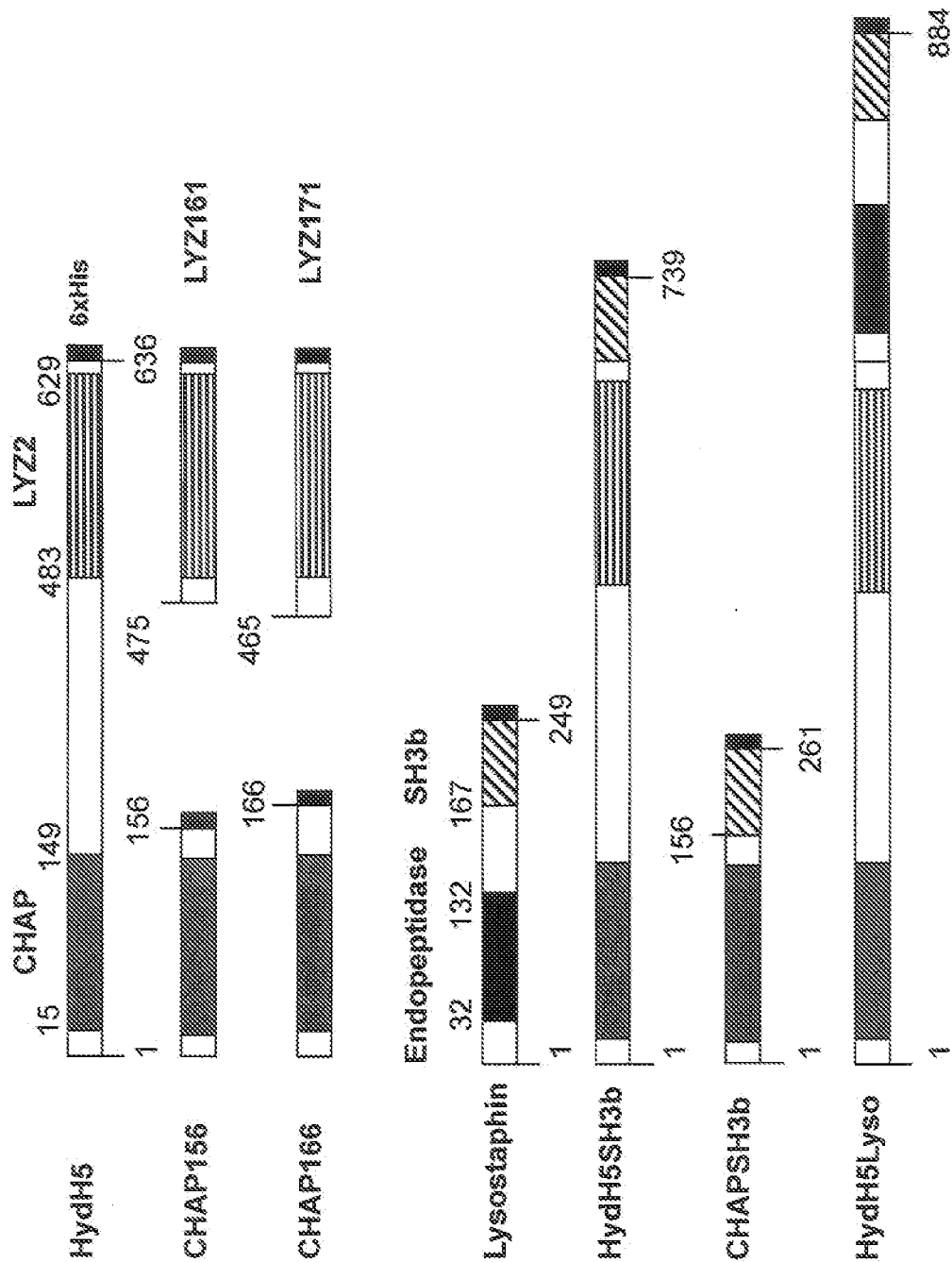
29. A method of treating mastitis in an animal comprising:

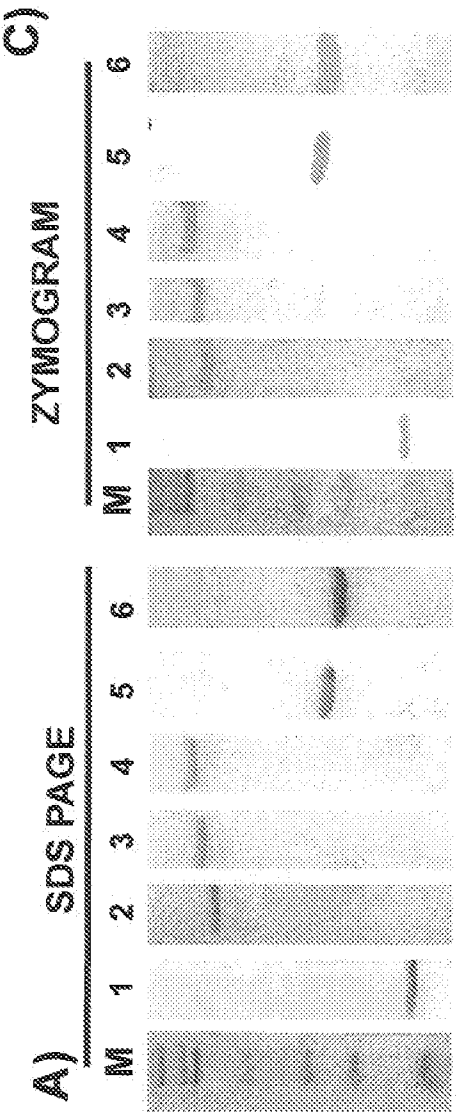
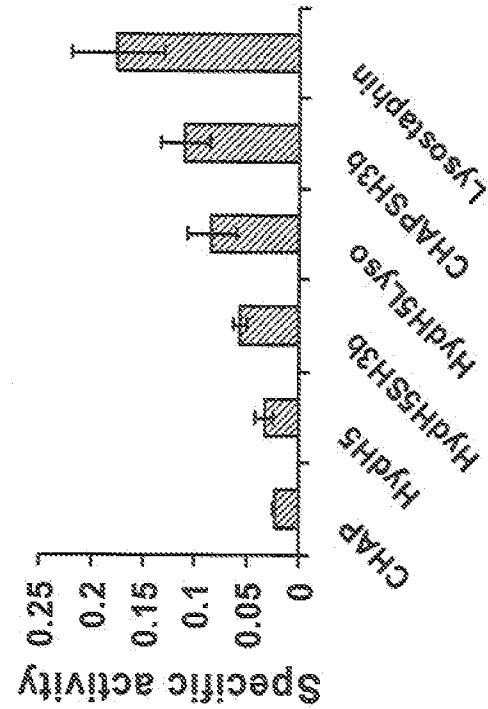
administering to said animal in need of treatment for mastitis an effective dosage of a composition of Claim 20, wherein said composition comprises an isolated recombinant peptidoglycan hydrolase fusion protein having specificity and exolytic activity for the peptidoglycan cell wall of untreated staphylococci and wherein said administration is effective for the treatment of and reduction of severity of mastitis caused by *Staphylococcus* strains

including multidrug-resistant staphylococcal strains and methicillin-resistant *S. aureus* (MRSA).

30. A method of treating mastitis in an animal comprising:
administering to said animal in need of treatment for mastitis an effective dosage of a composition of Claim 24, wherein said composition comprises LysH5 and an isolated recombinant peptidoglycan hydrolase fusion protein having specificity and exolytic activity for the peptidoglycan cell wall of untreated staphylococci, each in amounts that are ineffective alone but act synergistically together to effectively treat or reduce the severity of said mastitis caused by *Staphylococcus* strains including multidrug-resistant staphylococcal strains and methicillin-resistant *S. aureus* (MRSA).
31. A kit, comprising a composition of any one of Claims 22-26.

Fig. 1





B)

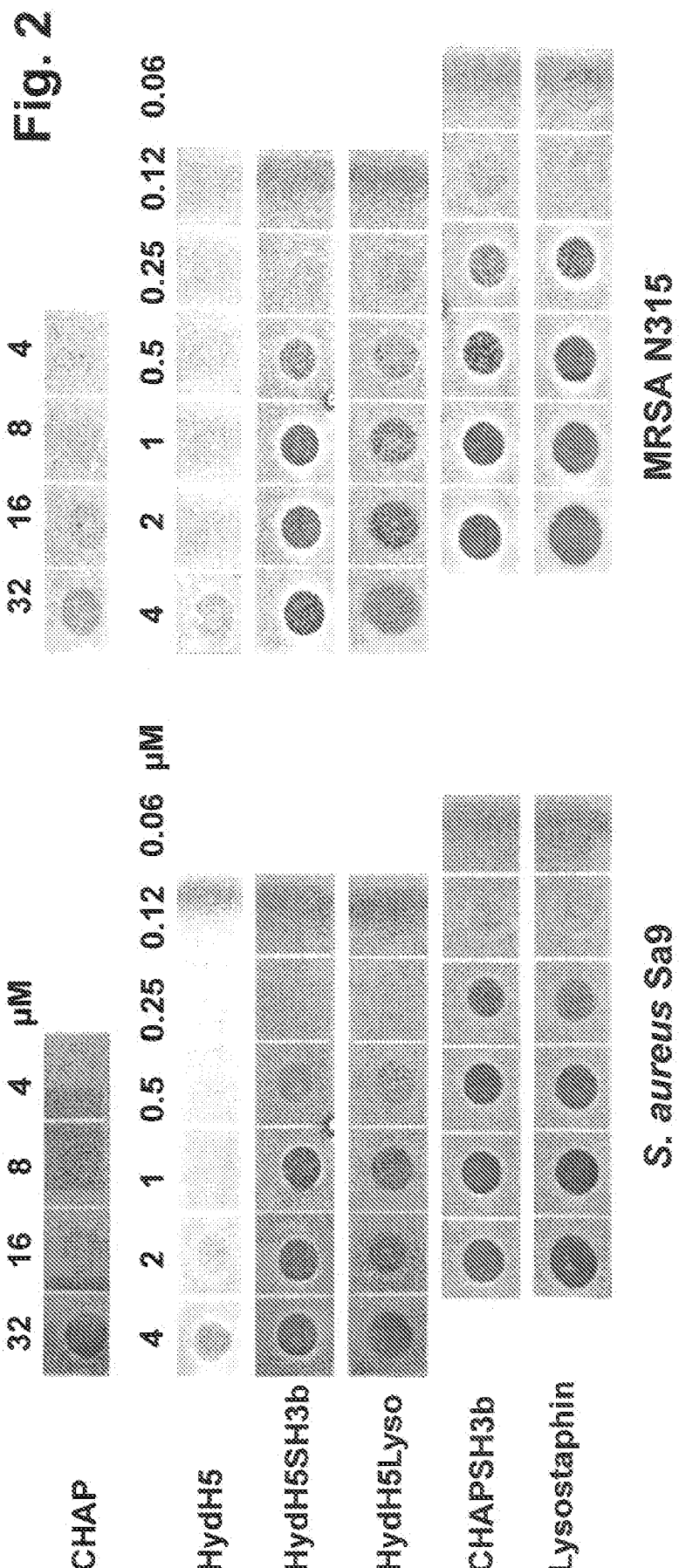


Fig. 2

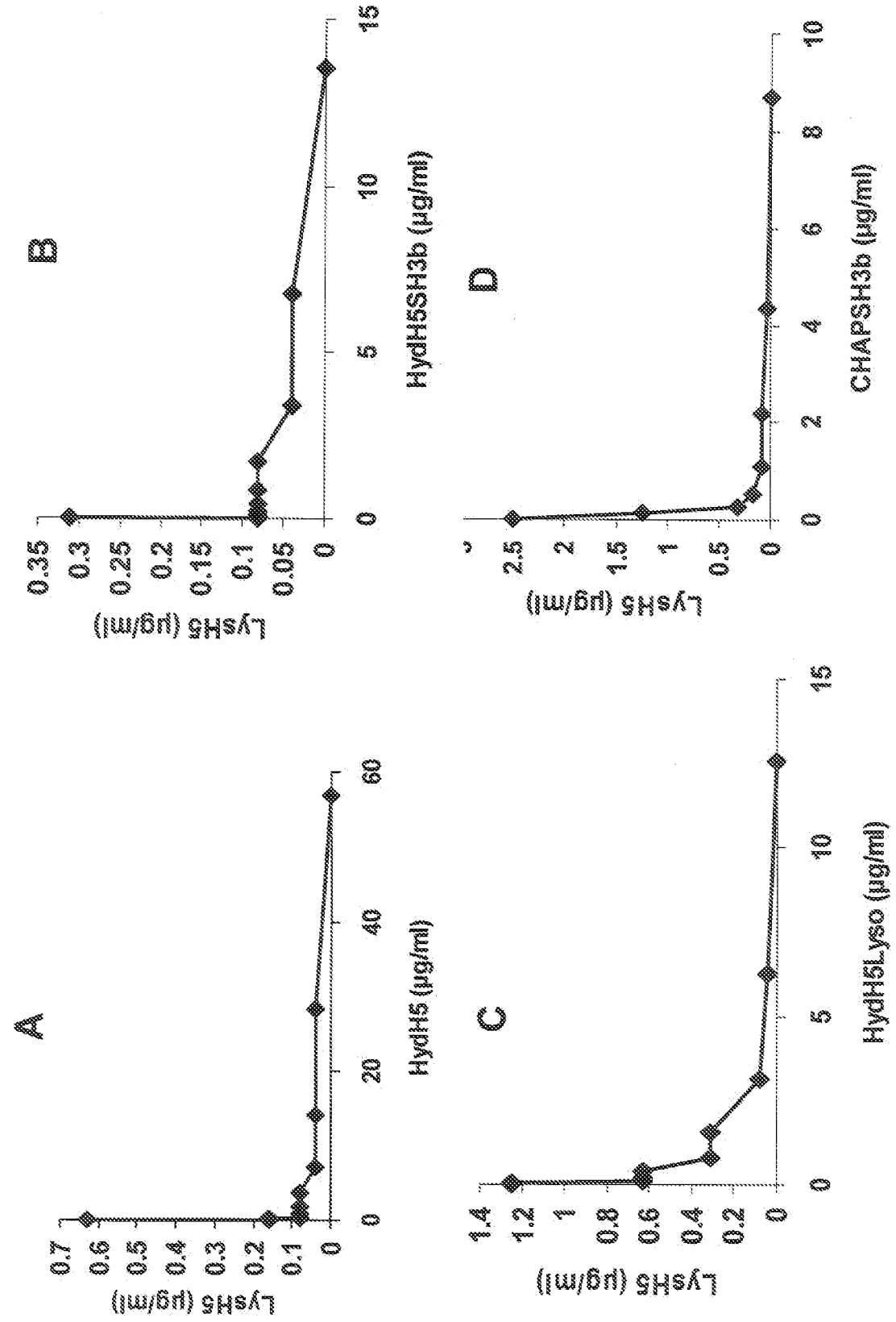


Fig. 3