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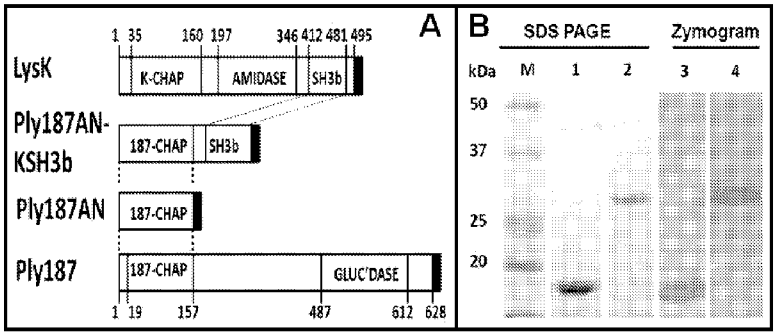


Fig. 1

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

WO 2013/149010 A1

Enhanced Antimicrobial Lytic Activity of a Chimeric Ply187 Endolysin

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 fusion polypeptide comprising a truncated ply187 endolysin from the *Staphylococcus aureus* phage 187. The invention further relates to compositions and a method of making the polypeptides and method of treating staphylococcal-associated diseases, including methicillin-resistant *Staphylococcus aureus* (MRSA).

Description of the Relevant Art

[0002] *Staphylococcus aureus* is a pathogen that causes a broad spectrum of human and animal diseases and has adapted to antibiotic selective pressures resulting in a high prevalence of multi-drug resistant strains (de Lencastre *et al*. 2007. *Curr. Opin. Microbiol.* 10:428-435). The spread of these antibiotic-resistant strains is a threat to public health and a critical concern to health care providers worldwide.

[0003] Phage endolysins are cell wall hydrolases that are produced near the end of the phage lytic cycle to help the nascent phage escape the infected host. Endolysins are ideally suited as antimicrobials for several reasons as described previously (Loessner, M. J. 2005. *Curr. Opin. Microbiol.* 8:480-487; Donovan *et al.* 2009. *Biotech. International* 21:6-10). Most importantly, phage endolysins are believed to have co-evolved with their host such that they target cell wall bonds that are believed essential and difficult for the host cell to alter. Thus bacterial resistance is unlikely. Due to the absence of an outer membrane in Gram-positive bacteria, endolysins are able to kill these bacteria when added from without.

[0004] The *S. aureus* bacteriophage 187 endolysin (Ply187) gene was initially reported by Loessner *et al.* (1999. *J. Bacteriol.* 181:4452-4460). Ply187 consists of 628 amino acids and has a calculated molecular mass of 71.6 kDa. Typically, endolysins from a Gram-positive background have a modular structure with an N-terminal catalytic domain for peptidoglycan hydrolysis and a C-terminal cell wall binding domain (Loessner, *supra*). However, the Pfam domain database indicates that the amino terminus of Ply187 harbors a Cysteine, Histidine-dependent Amidohydrolase/ Peptidase (CHAP) domain (Bateman and Rawlings. 2003. *Trends Biochem. Sci.* 28:234-237; Rigden *et al.* 2003. *Trends Biochem. Sci.* 28:230-234) and the C-terminus contains a glucosaminidase domain with no known C-terminal cell wall binding domain (Loessner *et al.*, *supra*; Fig. 1A). Cell wall binding domains are essential for the lytic activity of some endolysins and often determine specificity (Baba and Schneewind. 1996. *EMBO J.* 15:4789-4797; Grundling and Schneewind. 2006. *J. Bacteriol.* 188:2463-2472; Loessner *et al.* 2002. *Mol. Microbiol.* 44:225-349; Lu *et al.* 2006. *J. Biol. Chem.* 281:549-558; Sass and Bierbaum. 2007. *Appl. Environ. Microbiol.* 73:347-352).

[0005] The Phage K endolysin, LysK, has been shown to kill a wide range of staphylococci including multiple MRSA in plate lysis assays (O'Flaherty *et al.* 2005. *J. Bacteriol.* 187:7161-7164). Blast analysis of the LysK protein sequence reveals two

lytic domains, a CHAP endopeptidase domain, an amidase (N-acetyl-muramyl-L-alanine amidase) domain, and a C-terminal SH3b cell wall binding domain (O'Flaherty *et al.*, *supra*). It is common for phage endolysins to have an N-terminal lytic domain (or two) with a C-terminal cell wall binding domain (Loessner, M. J. 2005, *supra*), although recently an endolysin with two lytic domains flanking two mid-protein cell wall binding domains (Cpl-7) was reported for the LambdaSa2 prophage (Pritchard *et al.* 2007. *Appl. Environ. Microbiol.* 73: 7150-7154).

[0006] Novel antimicrobials that are specific for staphylococcal species, including methicillin-resistant *Staphylococcus aureus* (MRSA) and that are also refractory to resistance development are needed to contend with the rise of drug-resistant pathogenic bacteria.

SUMMARY OF THE INVENTION

[0007] We have discovered that a polypeptide comprising the complete truncated Ply187AN peptidoglycan hydrolase polypeptide and the SH3b cell wall binding domain(s) of native LysK are highly suitable for creating fusion polypeptides. Thus, both domains are versatile tools for creating new fusion polypeptides, i.e. Ply187AN can successfully be combined with a non-Ply187 derived cell wall binding domain and the SH3 domain of LysK can successfully be fused to other lytic domains such as Ply187AN. In particular we have discovered a nucleic acid encoding an antimicrobial fusion peptidoglycan hydrolase polypeptide comprising the complete truncated Ply187AN peptidoglycan hydrolase polypeptide and one or more of the SH3b cell wall binding domain(s) of native LysK where the encoded fusion polypeptide is capable of "lysis from without" lytic activity and can be used as an antimicrobial treatment for *Staphylococcal*-induced infections and diseases, including those caused by multidrug-

resistant strains. Particularly surprising was the high efficiency of the fusion polypeptide in milk, making the polypeptides according to the present invention particularly useful for the treatment of diseases such as mastitis.

[0008] In accordance with this discovery, it is an object of the invention to provide an isolated recombinant nucleic acid encoding an antimicrobial fusion peptidoglycan hydrolase polypeptide comprising a truncated Ply187 polypeptide, said truncated Ply187 polypeptide being Ply187AN comprising the amino terminal Ply187CHAP domain having exolytic function for the peptidoglycan cell wall of staphylococcal bacteria.

[0009] It is a further object of the invention to provide an isolated recombinant polynucleotide formed from a nucleic acid encoding a complete truncated Ply187AN peptidoglycan hydrolase polypeptide in combination with a nucleic acid encoding one or more of the SH3b cell wall binding domain(s) of native LysK.

[0010] It is also an object of the invention to provide an antimicrobial fusion peptidoglycan hydrolase polypeptide comprising a truncated Ply187 polypeptide, said truncated Ply187 polypeptide is Ply187AN comprising the amino terminal Ply187CHAP domain having exolytic function for the peptidoglycan cell wall of staphylococcal bacteria, and wherein said polypeptide comprises a cell wall binding domain, in particular one or more of the SH3b cell wall binding domain(s) of native LysK.

[0011] It is also an object of the invention to provide a recombinant antimicrobial fusion protein comprising Ply187AN, a truncated Ply187 peptidoglycan hydrolase comprising the amino terminal CHAP domain of Ply187 endolysin in combination with one or more of the SH3b cell wall binding domain(s) of native LysK.

[0012] It is also an object of the invention to provide a polypeptide comprising one or more of the SH3b cell wall binding domain(s) of native LysK, wherein the polypeptide is not LysK, and wherein the polypeptide exhibits preferably exolytic function for the peptidoglycan cell wall of staphylococcal bacteria.

[0013] It is a further object of the invention to provide the fusion protein of Ply187AN and the Sh3b cell wall binding domain of LysK, KSh3b, such as Ply187AN-KSh3b.

[0014] An added object of the invention is to provide a nucleic acid sequence encoding a complete truncated Ply187AN peptidoglycan hydrolase polypeptide in combination with a nucleic acid encoding one or more of the SH3b cell wall binding domain(s) of native LysK 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.

[0015] An additional object of the invention is to provide nucleic acid constructs comprising the isolated recombinant polynucleotide formed from a nucleic acid encoding a complete truncated Ply187AN peptidoglycan hydrolase polypeptide in combination with a nucleic acid encoding one or more of the SH3b cell wall binding domain(s) of native LysK, wherein said nucleic acid is in operable linkage to a promoter that drives expression in a host cell.

[0016] Another 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.

[0017] Another object of the invention is to provide compositions comprising the poylpeptides, nucleic acids, nucleic acid constructs, vectors, and/or host cells of the

invention. Preferably said compositions comprise milk and the polypeptides of the present invention are active therein,

[0018] An added object of the invention is to provide compositions useful for the treatment of disease caused by the *Staphylococcus* strains and the multidrug-resistant staphylococcal strains including methicillin-resistant *S. aureus* (MRSA), for which the fusion proteins of the invention are specific and effective, wherein said composition comprises the recombinant antimicrobial fusion protein comprising Ply187AN in combination with one or more of the SH3b cell wall binding domain(s) of native LysK and a pharmaceutically acceptable carrier. Such compositions may thus be useful in preparing a medicament for the treatment of such diseases. Any use thereof is specifically contemplated herein.

[0019] An additional object of the invention is to provide compositions useful for the treatment of disease comprising the composition above in combination with another sequence, or sequences, encoding one or more disease-resistance properties.

[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 Ply187AN peptidoglycan hydrolase and fusions comprising the truncated Ply187AN 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] Figures 1A and 1B depict the constructs utilized and the SDS-PAGE and zymogram analysis of endolysin constructs. Fig. 1A is a schematic of the constructs. Black box = pET21a derived 6 x His tag; GLUC'DASE = glucosaminidase domain. Fig. 1B shows the SDS-PAGE and zymogram analysis of the Ply187 constructs. The proteins migrate as expected for their predicted molecular weights: Ply187AN (Lanes 1 and 3): 18.9kDa, Ply187AN-KSH3b (Lanes 2 and 4): 30.6 kDa. 4µg of each Ni-NTA purified protein was loaded per lane M = excess prestained Kaleidoscope protein standards (Bio-Rad).

[0023] Figures 2A-2C show that the fusion of LysK SH3b domain to the Ply187AN domain enhances Ply187AN antimicrobial activity in plate lysis, turbidity reduction and Minimal Inhibitor Concentration (MIC) assays. Fig. 2A depicts a representative plate lysis assay with *S. aureus* strain Newman. Zones of clearing represent lysis of the lawn. Fig. 2B depicts the Specific Activities (ΔOD_{600nm}/µmol/min) observed in the Turbidity Reduction Assays. The assay volume was 200 µl. Error bars represent SEM for three or more independent experiments. Fig. 2C shows results for LysK and purified Ply187 derivatives in the MIC Assay.

[0024] Figure 3. Fusion of LysK SH3b domain enhances Ply187AN antimicrobial activity in pasteurized cow milk. At five time points over a time period of 3 hours, CFU counts increased dramatically in inoculated milk samples following the addition of enzyme storage buffer [■], Ply187AN [◆], and LysK [▲]. However, following the addition of Ply187AN-KSH3b [X], the CFU counts were reduced to, and stayed, at 0% of the enzyme storage buffer control at time point zero, indicating effective killing. Error bars = SEM.

DETAILED DESCRIPTION OF THE INVENTION

[0025] Previous experimental data indicate that native *S. aureus* bacteriophage 187 endolysin (Ply187 endolysin) is nearly inactive while a truncated derivative, the C-terminal-truncated Ply187, *i.e.*, Ply187AN, comprising amino acids 1-157 of Ply187, is much more active than the full-length protein. The C-terminus of the native Ply187 endolysin is known to contain a glucosaminidase domain and no known C-terminal cell wall binding domain. That fact that truncation of the C-terminus resulted in increased activity suggested an inhibitory domain at the C-terminus (Loessner *et al.*1999, *supra*).

[0026] SEQ ID NO: 1 as disclosed herein is Ply187AN. SEQ ID No: 2 is the very same sequence, however without N-terminal methionine residue. Utilization of such sequence may prove particularly useful if in the inventive polypepeptide further amino acid sequences are present N-terminal of the Ply187AN sequence. SEQ ID No: 3 represents the LysK SH3b cell wall binding domain.

[0027] In preferred embodiments of the invention an inventive polypeptide is a fusion polypeptide comprising the sequence of Ply187An and at least one LysK SH3b cell wall binding domain, e.g. comprises the sequence of SEQ ID NO: 1 or 2, and the sequence of SEQ ID NO: 3. Examples of such polypeptides are shown in SEQ ID NOs: 4, 5, 6 and 7.

[0028] An inventive polypeptide may further comprise for example a tag to facilitate for instance purification of the inventive polypeptide. Examples of such polypeptides are shown in SEQ ID NOs: 8 and 9, respectively. A tag may for example be a His Tag. A particular preferred tag is the His_6 tag (see SEQ ID NO: 10).

The polypeptides of the invention may thus comprise the sequence of any one of SEQ ID NOs. 1 to 9 and sequences substantially similar thereto, in particular substantially similar to SEQ ID NO: 1 (or 2, respectively) and/or to SEQ ID NO: 3.

[0029] In an effort to improve the Ply187 lytic activity, we have fused the nucleotide sequence (SEQ ID NO:11) encoding Ply187AN (SEQ ID NO:1; comprising the CHAP domain of the amino terminus of the native Ply187) to the nucleotide sequence (SEQ ID NO:12) encoding the LysK SH3b cell wall binding domain (KSH3b; SEQ ID NO:3) in order to generate a chimeric nucleotide sequence (SEQ ID NO:13) encoding the Ply187AN-KSH3b fusion protein (SEQ ID NO:8; Fig. 1A), similar to work reported with the streptococcal LambdaSa2 (LSA2) endolysin N-terminal lytic domain (Becker *et al.* 2009a. *Gene* 443:32-41). The Ply187 CHAP domain shows only weak homology (40% identity) with the CHAP domain of LysK (O'Flaherty *et al.*, *supra*).

[0030] Although the bacterial SH3b domain is readily identified in multiple domain databases, it is still poorly understood at the level of the site of binding. SH3b domains often determine an endolysin's specificity (Baba and Schneewind, *supra*; (Becker *et al.* 2009a, *supra*; Grundling and Schneewind, *supra*; Lu *et al.*, *supra*; Sass and Bierbaum, *supra*). Low *et al.* have proposed a model to explain the role of the SH3b domain in lysin lytic activity (Low *et al.* 2005. *J. Biol. Chem.* 280: 35433-35439). Their model suggests that the SH3b domain folds back, binds to and inhibits the lytic domain until the lytic domain recognizes and binds to peptidoglycan at which point the SH3b domain releases its grip on the lytic domain, thereby allowing digestion of the peptidoglycan to occur. Although believed to play a role in substrate recognition and binding specificity, its role must be empirically determined. In deletion experiments, Horgan *et al.* (2009. *Appl. Environ. Microbiol.* 75:872-874) suggested that deletion of the LysK SH3b domain enhanced LysK CHAP domain enzymatic activity while Becker *et al.* (2009b. *FEMS Microbiol. Lett.* 294:52-60) showed that fusion of the LysK SH3b domain to the LysK CHAP domain was necessary for CHAP domain activity. Other labs have demonstrated

that the SH3b domain of the bacteriocin, lysostaphin, binds to the *S. aureus* pentaglycine bridge (Baba and Schneewind, *supra*; Grundling and Schneewind, *supra*; Lu *et al.*, *supra*). Recently, the Fischetti group used a non-SH3b cell wall binding domain to generate a chimeric staphylolytic lysin using the Twort phage endolysin CHAP domain (Daniel *et al.* 2010. *Antimicrob. Agents Chemother.* 54:1603-1612). Our lab has used staphylococcal SH3b domains in fusions with the streptococcal phage LambdaSA2 endolysin endopeptidase domain in order to shift the activity from *Streptococcus*-specificity to an enzyme that recognized both streptococcal and staphylococcal cell walls (Becker *et al.* 2009a, *supra*).

[0031] We have generated a potent chimeric Ply187AN-KSH3b protein (SEQ ID NO:8) by fusing the nucleotide sequences encoding the CHAP endopeptidase domain of endolysin Ply187 from phage 187 and the SH3b cell wall- binding domain of LysK from phage K. In a series of functional assays, we have demonstrated that this chimeric Ply187AN-KSH3b is a more effective antimicrobial than the full length Ply187 and the Ply187 truncation (Ply187AN) and also outperforms the known high activity lysin, LysK in two out of three functional assays. Despite the numerous cell wall binding domain fusion constructs previously reported, the Ply187-KSH3b fusion in this study represents the first time that the activity of a lytic domain from an endolysin that naturally lacks a cell wall binding domain was enhanced by adding a known cell wall binding domain.

[0032] In the Ply187AN-SH3b construct, the LysK SH3b domain was fused to truncated Ply187AN increasing the lytic activity, suggesting that this cell wall binding domain helps the lytic domain to recognize and 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.* 2009b, *supra*). This result indicates the importance of the SH3b cell wall binding 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 when compared to the native (dual domain) Ply187 endolysin and truncated Ply187AN.

[0033] In summary, we report the development of a novel chimeric peptidoglycan hydrolases with improved lytic activity against *S. aureus* strain Newman. The effectiveness of the Ply187AN-KSH3b fusion polypeptide indicates that it can serve as an additional new weapon to combat multidrug-resistant *S. aureus* infections in agricultural and clinical environments.

[0034] 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 recombinant truncated Ply187AN peptidoglycan hydrolase fusion protein 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 Ply187AN and KSH3b genes according to the invention can be introduced. The regulatory elements required for expressing the nucleic acid sequence encoding the Ply187AN-KSh3b fusion polypeptide 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.

[0035] The present invention also relates to a cloning and/or expression vector for transforming a host organism containing at least the Ply187AN-KSh3b fusion peptidoglycan hydrolase gene as defined herein above. This vector comprises, in addition, to the above Ply187AN-KSh3b chimeric 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.

[0036] 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.

[0037] 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 Ply187AN-KSh3b chimeric 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.

[0038] 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 exists 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.

[0039] 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.

[0040] 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.
[0041] 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).

[0042] "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.

[0043] "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.

[0044] 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.

[0045] 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.

[0046] 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.

[0047] 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.

[0048] 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).

[0049] 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.

[0050] The invention includes functional fragments of the Ply187AN-KSh3b fusion peptidoglycan hydrolase polypeptide and functional fusion polypeptides encompassing a functional Ply187AN-KSh3b fusion 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 Ply187AN-KSh3b fusion peptidoglycan hydrolase" refers to all fragments of Ply187AN-KSh3b fusion peptidoglycan hydrolase that retain Ply187AN-KSh3b fusion peptidoglycan hydrolase activity and function to lyse staphylococcal bacteria.

[0051] Modifications of the Ply187AN-KSh3b fusion peptidoglycan hydrolase primary amino acid sequence may result in further mutant or variant proteins having substantially equivalent activity to the Ply187AN-KSh3b fusion 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 Ply187AN-KSh3b fusion peptidoglycan hydrolase polypeptide. Any polypeptides produced by minor modifications of the Ply187AN-KSh3b fusion peptidoglycan hydrolase primary amino acid sequence are included herein as long as the biological activity of Ply187AN-KSh3b fusion peptidoglycan hydrolase is present; *e.g.*, having a role in pathways leading to lysis of staphylococcal bacteria.

[0052] 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.

[0053] 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 (Tm) 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 Ply187AN-KSh3b peptidoglycan hydrolase polypeptide and which hybridize under stringent conditions to the Ply187AN-KSh3b fusion peptidoglycan hydrolase sequences disclosed herein, or to fragments thereof, are encompassed by the present invention.

[0054] 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).

[0055] 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.

[0056] 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.

[0057] 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.

[0058] 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.

[0059] 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).

[0060] 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 use 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.

[0061] 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 Ply187AN-KSh3b fusion peptidoglycan hydrolase-like activity. Alternatively, fragments of a nucleotide sequence that are useful as hybridization probes may not encode fragment proteins retaining biological activity.

[0062] 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 Ply187AN-KSh3b fusion 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.

[0063] 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, Ply187AN-KSh3b fusion peptidoglycan hydrolase activity as described herein. Such variants may result from, for example, genetic polymorphism or from human manipulation. Biologically active variants of the Ply187AN-KSh3b fusion 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.

[0064] 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 Ply187AN-KSh3b fusion 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.

[0065] 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 Ply187AN-KSh3b fusion peptidoglycan hydrolase protein can be observed.

[0066] "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.

[0067] 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.

[0068] Using highly specific antimicrobials which target specific sites of the specific organisms involved rather than relying on the generalized use of broad range antimicrobials can enhance our effectiveness in treating disease and also enable us to reduce the occurrence of antibiotic resistance.

EXAMPLES

[0069] 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

[0070] Overexpression of proteins was performed in *E. coli* BL21 (DE3) (Invitrogen, Carlsbad, CA) cultured at 37°C in modified Luria-Bertani (mLB) medium (15 g/l tryptone, 8 g/l yeast extract, 5 g/l NaCl) (Schmelcher *et al.* 2010. *Appl. Environ. Microbiol.* 76:5745-5756) supplemented with 150 µg/ml ampicillin for plasmid selection. Staphylococcal strains used are described in Table 1. All strains were grown in Tryptic Soy Broth (TSB) at 37°C.

EXAMPLE 2

Plasmid Constructs; DNA Manipulation

[0071] To enhance the heterologous expression of Ply187 endolysin in *E. coli*, the sequences encoding the truncated Ply187 N-terminal domain (Ply187AN; 1-157aa) were converted to an *E. coli* codon bias, commercially synthesized, and subcloned into pUC57 with engineered 5' NdeI (CATATG; ATG = start of translation) and 3' XhoI (CTCGAG; codes for aa's LE) restriction enzyme sites (Genscript; Piscataway, NJ). Subcloning of the Ply187AN construct into the pET21a expression vector was via conventional means for protein expression. Similarly, the Ply187AN was fused to the LysK SH3b by subcloning the Ply187AN NdeI-XhoI DNA fragment harboring all CHAP lytic domain coding sequences into a similarly digested pre-constructed pET21a-KSH3b vector described previously (Becker *et al.* 2009b. *FEMS Microbiol. Lett.* 294:52-60). Recombinant LysK was used in this work as a positive control (Becker *et al.* 2009b, *supra*).

EXAMPLE 3

Protein Purification and Analysis

[0072] Protein induction, purification and storage followed the protocols as described previously (Becker *et al.* 2009b, *supra*). Briefly, *Escherichia coli* cultures harboring vectors were harvested, then sonicated for 5 min using an automatic pulsing sonication (Bronson Sonifier; Bronson Sonic Power Co., Danbury, CT, USA). His-tagged proteins were isolated using Ni-NTA nickel column chromatography (Qiagen). Wash and elution profiles were empirically determined to be 10 ml of 10 mM imidazole, 20 ml of 20 mM imidazole and elution with 1.2 ml of 250 mM imidazole in phosphate buffered saline (50 mM NaH_2PO_4, 300 mM NaCl, pH 8.0) with 1% glycerol to prevent precipitation of the purified protein. All samples were then desalted with Zeba desalting column (Pierce, Rockford, IL) equilibrated in 2X PBS buffer and filter sterilized. Sterilized protein preparation was stored at 4ºC in 2X PBS buffer 30% glycerol until the time of assay.

[0073] Nickel-chromatography purified proteins were analyzed using 15% SDS-PAGE and Kaleidoscope protein standards (Bio-Rad, Hercules, CA) (Fig. 1B), with or without 300 ml culture equivalents of mid log phase *S. aureus* cells (OD_{600nm} = 0.4-0.6) embedded in the gel as described previously (Becker *et al.* 2009b, *supra*), to verify the absence of co-purifying lytic contaminants. Coomassie-stained SDS-PAGE of each purified protein C-His-Ply187AN and C-His-Ply187AN-KSH3b indicated that the two constructs were able to be expressed in *E. coli* and purified at greater than 95% purity. Zones of lysis on the zymogram gel run in parallel with the SDS-PAGE indicate that the predicted protein in each preparation is the only protein with staphylolytic activity (Fig. 1B).

EXAMPLE 4

Plate Lysis Assay

[0074] To verify and quantify the lytic activity against live *S. aureus, we* have tested Ply187AN-KSH3b, the parental truncation (Ply187AN), and a strong antimicrobial endolysin, LysK (Becker *et al.* 2009b, *supra*; Baba and Schneewind, *supra*) in three different antimicrobial assays. Purified proteins for each construct were diluted in sterile nickel column elution buffer and six microliters containing (10, 1.0 and 0.1 µg) was spotted onto a freshly spread lawn of growing cells 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. Scoring of the cleared spots occurred within 20 hr of plating the cells.

[0075] The plate lysis assay results in Fig. 2A demonstrate that both 10 µg and 1 µg LysK (0.2 and 0.02 µmol, respectively; molecular weight (MW: 55.8 kD) produce a zone of clearing, indicating that 1 µg LysK in 10 µl of buffer is effective at eliminating the *S. aureus* lawn, consistent with previous reports (Becker *et al.* 2009b., *supra*; Baba and Schneewind, *supra*). In contrast, only 10 µg of Ply187AN (0.5 µmol; MW:18.9 kD) produced a zone of clearing in the plate lysis assay, indicating that Ply187AN is much less effective than LysK in this assay. Surprisingly, Ply187AN-KSH3b produces a zone of clearing at 10, 1 and 0.1 µg (0.3, 0.03 and 0.003 µmol, respectively; MW: 30.6 kD), indicating that Ply187AN-KSH3b is more active than Ply187AN and LysK.

[0076] To determine the specificity of Ply187AN-KSH3b for staphylococcal cells, we tested the enzyme in plate lysis assays against a collection of staphylococcal and non-staphylococcal strains in comparison with LysK (Table 1). The staphylococcal strains

included bovine mastitis isolates, MRSA strains, and coagulase negative staphylococci. Both the fusion protein and LysK were able to lyse all staphylococcal strains tested, with Ply187AN-KSH3b exhibiting higher activity than LysK against many strains when compared on a molar basis. In contrast, both enzymes were inactive against non-staphylococcal strains with the exception of Ply187AN-KSH3b showing weak activity against *Streptococcus dysgalactiae*.

Table 1: Susceptibility of multiple bacterial strains to lysis by the parental enzyme LysK and the fusion protein Ply187AN-KSH3b

Strain	Source	Susceptibility[1]	
		LysK	Ply187AN-KSH3b
Staphylococcus aureus strains			
Newman	[2]	++	++(+)
MN8	[2]	++(+)	+++
SA113	[2]	++	+++
Reynolds CP5	[2]	++(+)	+++
Newbould (305)	ATCC 29740	++(+)	+++
SA019	[3]	++(+)	+++
SA020	[3]	++(+)	++(+)
SA021	[3]	++	++
SA026	[3]	++	++(+)
NRS382 (MRSA)	NRS 382	+(+)	++(+)
NRS383 (MRSA)	NRS 383	++	++(+)
NRS384 (MRSA)	NRS 384	++	+++
NRS385 (MRSA)	NRS 385	++	++(+)
Staphylococcus chromogenes	[4]	++(+)	++(+)
Staphylococcus epidermidis	[4]	++	+++
Staphylococcus hyicus	[4]	+++	+++
Staphylococcus simulans	[4]	++(+)	+++

Staphylococcus warneri	[4]	++(+)	+++
Staphylococcus xylosus	[4]	+++(+)	+++(+)
Streptococcus agalactiae	ATCC 27541	-	-
Streptococcus dysgalactiae	[5]	-	+
Streptococcus uberis	[6]	-	-
Listeria monocytogenes Petite ScottA	ATCC 49594	-	-
Rhodococcus equi	[7]	-	-
Lactobacillus amylovorus 4540	[8]	-	-
Lactobacillus reuteri 14171	[8]	-	-
E. coli H5	[9]	-	-
E. coli DH5α	Invitrogen	-	-
Salmonella Enteritidis	ATCC13076	-	-
Klebsiella pneumoniae	[10]	-	-

[1] Smallest amount of protein in a volume of 10 µl causing a lysis zone after overnight incubation: ++++, 0.1 pmol; +++, 1 pmol; ++, 10 pmol; +, 100 pmol. "(+)" represents a faint lysis zone; "-", no lysis zone at the highest amount tested (100 pmol). Scores represent averaged results from two separate experiments.

[2] Jean C. Lee, Channing Laboratory, Brigham and Women's Hospital, Boston, MA, USA

[3] Yasunori Tanji, Tokyo Institute of Technology, Yokohama, Japan; bovine mastitis isolates

[4] Max Paape, ABBL, ANRI, ARS, USDA, Beltsville, MD, USA; bovine mastitis isolates

[5] W.D. Schultze, BARC Dairy, Beltsville, MD, USA; isolated from a clinical case

[6] Strain 0140; Dr. A.J. Bramley, Compton Laboratory, Newbury, United Kingdom; isolated from a clinical case

[7] Strain 33701; Steeve Giguere, College of Veterinary Medicine, University of Georgia, Athens, GA, USA

[8] Ken Bischoff, ARS, Peoria, Ill, USA.

[9] Manan Sharma, EMFSL, ANRI, ARS, USDA, Beltsville, MD, USA

[10] Strain K-6; E.J. Carroll, Dept. Vet Med, University of California, Davis, CA, USA; cow 2612 clinical case

NRS strains are obtained from the "Network on Antimicrobial Resistance in *Staphylococcus aureus*" repository (retrieved from the Internet: <URL: narsa.net).

EXAMPLE 5

Turbidity Reduction Assays;

[0077] To further quantify the degree of lytic enhancement obtained from the fusion of the Ply187 CHAP domain to the KSH3b domain we compared the staphylolytic activities in both turbidity reduction (Fig. 2B) and minimal inhibitory concentration (MIC) assays (Fig. 2C). The turbidity assay measures the drop in optical density (OD) resulting from lysis of the target bacteria with the phage endolysin-derived protein. A standardized turbidity assay modified from Donovan *et al.* (2006a. *Appl. Environ. Microbiol.* 72:2988-2996) with staphylococcal strains grown to logarithmic phase (OD_{600nm} = 0.4 - 0.6) at 37°C in Brain Heart Infusion broth (DIFCO, Franklin Lakes, NJ) was performed in a 96 well dish and analyzed in a plate reader as described previously (Becker *et al.* 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 resuspended to OD_{600nm} = 1.0 in 400 mM NaCl, 20 mM Tris-HCl, 1% glycerol, pH 7.5 unless otherwise stated. Enzyme samples are added to three wells of a 96 well dish in 100 µl of buffer. All samples are performed in triplicate. The assay is started by the addition of 100 µl of cells in buffer at OD_{600nm} ~1.0 via multi channel pipettor. A 'no enzyme control' of buffer and cells is included. OD_{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 40 second window over each group of 3 consecutive time points during the five minute period, to identify the highest instantaneous change in OD_{600nm} for each well. The absolute values of ΔOD_{600nm} for each group of 3 time points are ranked for the entire 5 min period. A plot of these values vs. time is examined for consistency and the highest consistent value is chosen. A similarly calculated buffer plus cells alone control value from triplicate wells is then subtracted from the highest ranked ΔOD_{600nm} value for each experimental well, and the 40 sec values for the triplicate wells averaged and multiplied by 1.5 to give a ΔOD_{600nm}

/minute. This value is then divided by the ug of enzyme protein in the sample tested to give a specific activity ΔOD_{600nm} /ug/min.

[0078] The specific activity for LysK, Ply187AN and Ply187AN-KSH3b is 1.2±0.4, 0.08±0.03, and 1.2±0.2 ΔOD_{600nm}/ µmol/min, respectively. Consistent with the plate lysis assay, Ply187AN was only about 10% as active as LysK. However, the addition of the KSH3b domain to the Ply187 CHAP domain yields a ten-fold increase in specific activity (Fig. 2B). The resulting activity of Ply187AN-KSH3b is similar to that of the recombinant LysK protein.

EXAMPLE 6

Minimal Inhibitory Concentration (MIC) Assay

[0079] A classical microdilution broth method for determination of the Minimal Inhibitory Concentration (MIC) was used (Jones *et al.* 1985. In *Manual of Clinical Microbiology*, Balows *et al.* (Eds.), American Society for Microbiology, Washington D.C., pages 972-977) with modifications as described previously (Becker *et al.* 2009b, *supra*) to determine the MIC for each construct. Briefly, logarithmic-phase bacteria were cultivated in tryptic soy broth and adjusted in broth to a concentration of 1 x 10^6 CFU/ml. Sterile microtiter plates containing 100µl of either LysK or lysostaphin diluted in TSB are inoculated with 100 µl of the bacterial suspension to yield 5 x 10^5 CFU/ml. The plate is incubated at 37°C for 20 h, at which point the plate is analyzed for clear vs. turbid wells. Each MIC assay represents at least two identical determinations per experiment.

[0080] In the MIC assay, LysK inhibits growth of *S. aureus* Newman at concentrations of 35.6 ± 10.5 µg/ml, corresponding to 0.6 ± 0.2 µmol/ml, which is comparable to

previous results (Becker *et al.* 2009b, *supra*). The MIC for Ply187AN is 53.5 ± 12.0 µg/ ml (2.8 ± 0.6 µmol/ ml). Similar to the prior two antimicrobial assays, Ply187AN-KSH3b is much more active than Ply187AN, with an MIC of only 7.6 ± 2.9 µg/ml (0.3 ± 0.02 µmol/ml) which is five-fold lower than that of LysK's (two-fold, when compared on a molar basis). We know that LysK is a very potent antibacterial, showing higher activities in turbidity reduction assays than lysostaphin (Becker *et al.* 2009b, *supra*). These data indicate that Ply187AN-KSH3b is a potent staphylolytic agent.

EXAMPLE 7

Chimeric Ply187AN-KSH3b lysin is active in pasteurized cows milk

[0081] Milk Assays: Fresh milk samples obtained from the USDA BARC, Beltsville, MD dairy herd were pasteurized for 30 minutes at 63°C. The milk was brought to 37°C and was spiked with exponentially growing (OD_{600nm} = 0.4 - 0.6) cells of the mastitis causing *S. aureus* strain Newbould 305 at a concentration of ~1 x 10^3 cfu/ml. Immediately after inoculation, purified enzyme was added at a concentration of 8 µM, and the milk was incubated at 37°C without shaking for 3 hours. Samples were taken immediately before and immediately after addition of enzyme, as well as after 30, 60, 120, and 180 minutes, and the CFUs determined by direct plating on duplicate TSA plates. A sample spiked with lysin buffer alone, no enzyme served as a control. The absence of CFUs in pasteurized milk was verified by direct plating on TSA plates.

[0082] The lytic activity of Ply187-AN-KSH3b, Ply187-AN, and LysK were determined via serial diluting plating of timed samples of milk inoculated with *S. aureus*, and either a lytic enzyme or enzyme buffer alone (Fig. 3). Immediately after addition of enzyme, Ply187-AN-KSH3b samples showed immediate eradication of all CFUs at time zero and

remained undetectable throughout the 3-hour period. Ply187-AN and LysK showed no significant difference from the buffer alone control at any time point.

[0083] 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.

[0084] 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 antimicrobial fusion peptidoglycan hydrolase polypeptide comprising a truncated Ply187 polypeptide, said truncated Ply187 polypeptide is Ply187AN comprising the amino terminal Ply187CHAP domain having exolytic function for the peptidoglycan cell wall of staphylococcal bacteria, and wherein said polypeptide comprises a cell wall binding domain.

2. The polypeptide of Claim 1, wherein said antimicrobial fusion peptidoglycan hydrolase polypeptide comprises the complete truncated Ply187AN peptidoglycan hydrolase polypeptide and one or more of the SH3b cell wall binding domain(s) of native LysK.

3. The polypeptide of Claim 1 or 2, wherein the polypeptide comprises the sequence of SEQ ID NO: 1 or 2.

4. The polypeptide of Claim 3, wherein the polypeptide comprises the sequence of SEQ ID NO: 3.

5. The polypeptide of Claim 4, wherein the polypeptide comprises the sequence of SEQ ID NO: 4 or 5, respectively.

6. The polypeptide of Claim 5, wherein the polypeptide comprises the sequence of SEQ ID NO: 6 or 7, respectively.

7. A polypeptide comprising one or more of the SH3b cell wall binding domain(s) of native LysK, wherein the polypeptide is not LysK.

8. The polypeptide of Claim 7, wherein the polypeptide comprises the sequence of SEQ ID NO: 3.

9. The polypeptide of Claim 7 or 8, wherein the polypeptide exhibits exolytic function for the peptidoglycan cell wall of staphylococcal bacteria.

10. The polypeptide of Claim 9, wherein said polypeptide comprises a truncated Ply187AN peptidoglycan hydrolase polypeptide.

11. The polypeptide Claim 10, wherein the polypeptide comprises the sequence of SEQ ID NO: 1 or 2.

12. The polypeptide of Claim 11, wherein the polypeptide comprises the sequence of SEQ ID NO: 4 or 5, respectively.

13. The polypeptide of Claim 12, wherein the polypeptide comprises the sequence of SEQ ID NO: 6 or 7, respectively.

14. A nucleic acid encoding a polypeptide of any one of Claims 1 to 13.

15. The nucleic acid of Claim 14, wherein the nucleic acid comprises the sequence of SEQ ID NO: 11, 12 or 13.

16. A construct comprising the nucleic acid of Claim 14, wherein said nucleic acid is in operable linkage to a promoter that drives expression in a host cell.

17. A cloning vector comprising the nucleic acid of claim 14 or the construct of Claim 16.

18. An expression vector comprising the nucleic acid of claim 14 or the construct of Claim 16.

19. A process for transforming a host cell, comprising stably integrating the nucleic acid of Claim 14 or the construct of Claim 16 into the host cell.

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

21. An isolated host cell transformed with the construct according to Claim 16.

22. The host cell of Claim 20 or 21, 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, in particular so as to produce an antimicrobial peptidoglycan hydrolase.

23. A method of making a protein, in particular a recombinant peptidoglycan hydrolase fusion protein, said method comprising the steps:

a. introducing into a host cell a nucleic acid or construct encoding a polypeptide according to Claim 1 or 7;

b. culturing said cell under conditions suitable for expression of said protein;

c. recovering the protein so expressed.

24. A composition comprising a polypeptide of any one of Claims 1 to 13.

25. The composition of Claim 19, wherein the composition comprises milk.

26. A composition useful for the treatment of disease caused by the *Staphylococcus* strains for which the fusion protein of the invention is specific and effective, wherein said composition comprises the polypeptide of Claim 1 or 7 and a pharmaceutically acceptable carrier.

27. 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 the polypeptide of Claim 1 or 7 and a pharmaceutically acceptable carrier.

28. A composition useful for the treatment of disease comprising the composition of Claim 24 or 26 in combination with another sequence, or sequences, encoding one or more disease-resistance properties.

29. 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 or 26, 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).

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 or 26, 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).

31. A kit, comprising a composition of any one of Claims 24-28.

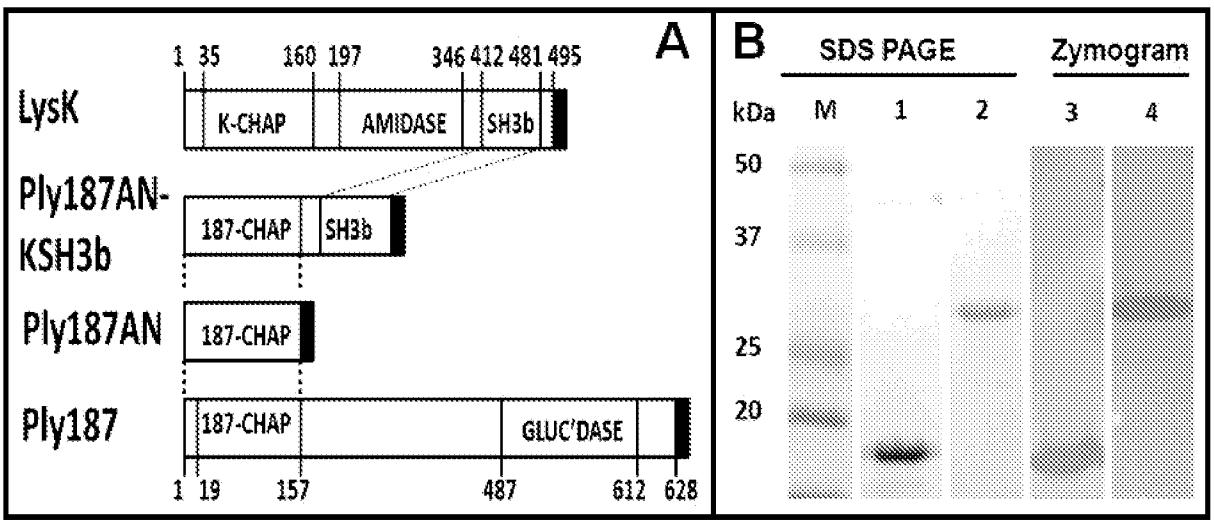

A
1 35 160 197 346 412 481 495
LysK
K-CHAP
AMIDASE
SH3b
Ply187AN-KSH3b
187-CHAP
SH3b
Ply187AN
187-CHAP
Ply187
187-CHAP
GLUC'DASE
1 19 157 487 612 628
B
SDS PAGE
Zymogram
kDa
M
1
2
3
4
50
37
25
20

Fig. 1

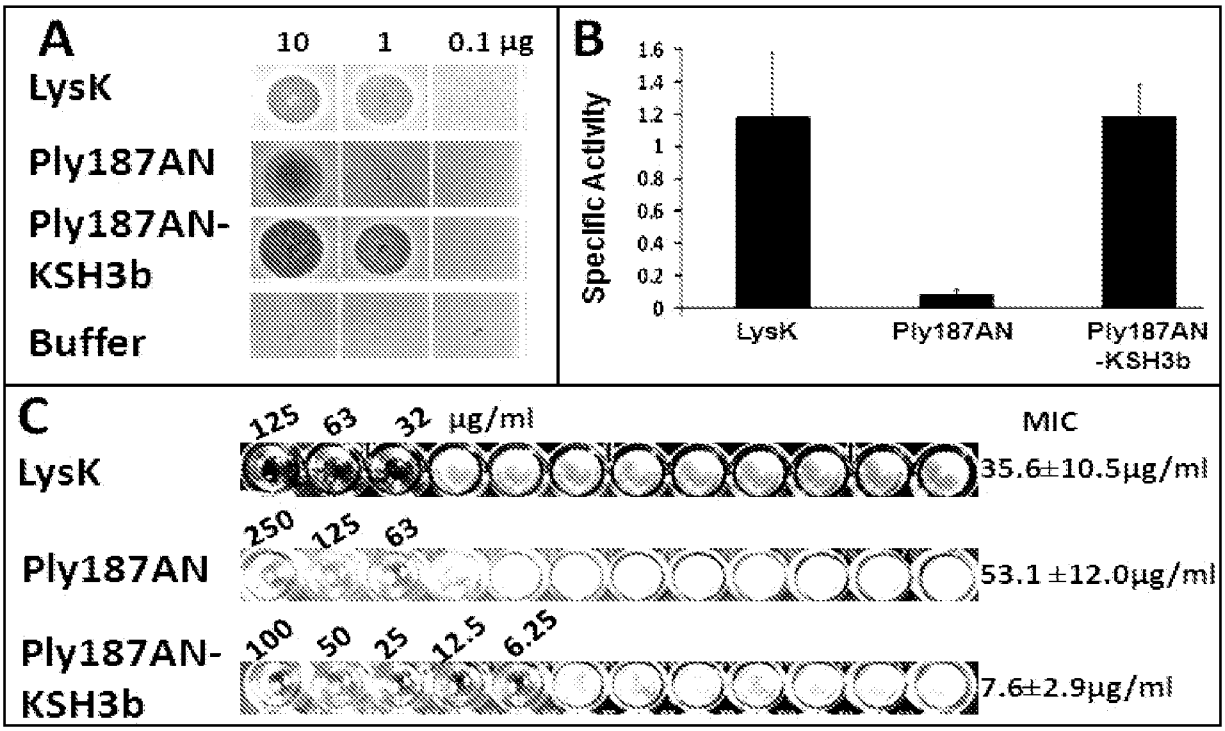

A
10
1
0.1 µg
LysK
Ply187AN
Ply187AN-KSH3b
Buffer
B
Specific Activity
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
LysK
Ply187AN
Ply187AN-KSH3b
C
125
63
32
µg/ml
MIC
LysK
35.6±10.5µg/ml
250
125
63
Ply187AN
53.1 ±12.0µg/ml
100
50
25
12.5
6.25
Ply187AN-KSH3b
7.6±2.9µg/ml

Fig. 2

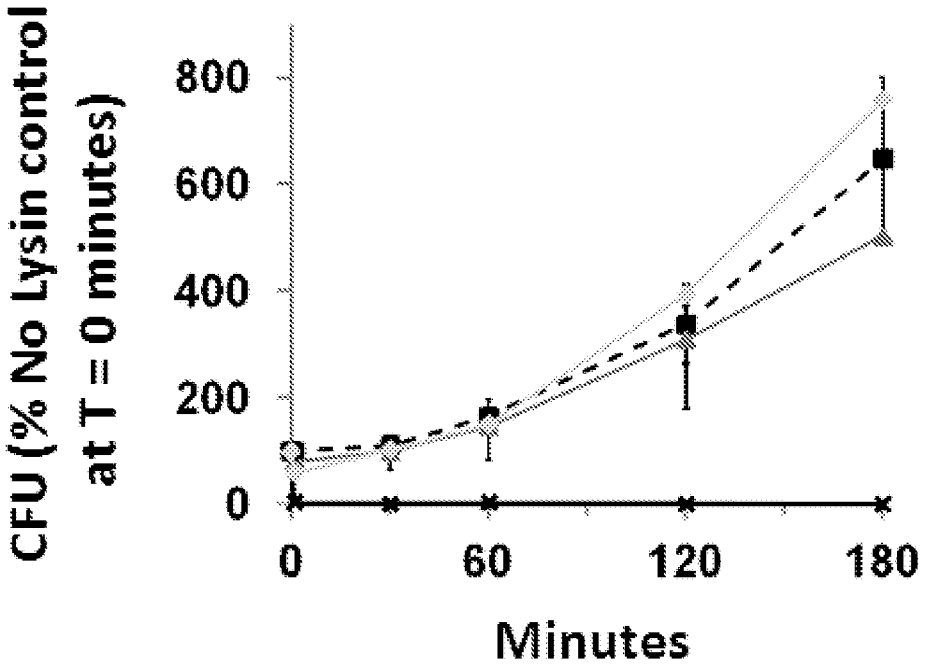

CFU (% No Lysin control at T = 0 minutes)
800
600
400
200
0
0
60
120
180
Minutes

Fig. 3

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2013/034358

A. CLASSIFICATION OF SUBJECT MATTER

C07K 19/00(2006.01)i, C07K 14/31(2006.01)i, C12N 15/62(2006.01)i, C12N 15/63(2006.01)i, A61K 38/16(2006.01)i, A61P 31/04(2006.01)i

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07K 19/00; C07H 21/04; A61K 38/54; C07K 14/435; C12N 9/14; C07K 14/31; C12N 15/62; C12N 15/63; A61K 38/16; A61P 31/04

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Korean utility models and applications for utility models
Japanese utility models and applications for utility models

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
eKOMPASS(KIPO internal) & Keywords: antimicrobial fusion peptididoglycan hydrolase, endolysin, Ply187, SH3b cell wall binding domain, staphylococcus

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2010-020657 A1 (PROFOS AG) 25 February 2010 See abstract; pages 1, 6, 19 and 21; claims 1, 5-6 and 8.	7,9
Y		1-3,10-11
A		4-6,8,12-13
Y	LOESSNER, MARTIN J., `Evidence for a holin-like protein gene fully embedded out of frame in the endolysin gene of Staphylococcus aureus bacteriophage 187` Journal of Bacteriology, August 1999, Vol. 181, No. 15, pp. 4452-4460, INSN 0021-9193. See the whole document, especially page 4455 and figures 2-3.	1-3,10-11
A		4-9,12-13
A	DONOVAN, DAVID M. et al., `Peptidoglycan hydrolase fusions maintain their parental specificities` Applied and Environmental Microbiology, April 2006, Vol. 72, No. 4, pp. 2988-2996, ISSN 0099-2240. See the whole document.	1-13
A	US 2011-0027249 A1 (DONOVAN, DAVID) 3 February 2011 See abstract; paragraphs [0008]-[0009]; claim 1.	1-13

☒ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents,such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
26 July 2013 (26.07.2013)	**26 July 2013 (26.07.2013)**

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2013/034358

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	LOESSNER, MARTIN J., `Bacteriophage endolysins--current state of research and applications` August 2005, Current Opinion in Microbiology, August 2005, Vol. 8, No. 4, pp. 480-487, ISSN 1369-5274. See the whole document.	1-13
A	BECKER, STEPHEN C. et al., `Differentially conserved staphylococcal SH3b_5 cell wall binding domains confer increased staphylolytic and streptolytic activity to a streptococcal prophage endolysin domain` Gene, 15 August 2009, Vol. 443, No. 1-2, pp. 32-41, ISSN 0378-1119. See the whole document.	1-13
PX	MAO, JINZHE et al., `Chimeric Ply187 endolysin kills Staphylococcus aureus more effectively than the parental enzyme` FEMS Microbiology Letters, Epub. 15 March 2013, Vol. 342, No. 1, pp. 30-36, ISSN 0378-1097. See the whole document.	1-3,7,9-11

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2013/034358

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 29
because they relate to subject matter not required to be searched by this Authority, namely:
Claim 29 pertains to methods for treatment of the human body by therapy and thus relatse to a subject matter which this International Searching Authority is not required, under Article 17(2)(a)(i) of the PCT and Rule 39.1(iv) of the Regulations under the PCT, to search.

2. ☒ Claims Nos.: 15,16,20,21,25
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
Claims 15, 16, 20, 21, and 25 are unclear since they are referring to the multiple dependent claims which do not comply with PCT Rule 6.4(a).

3. ☒ Claims Nos.: 14,17-19,22-24,26-31
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required addtional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/US2013/034358

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2010-020657 A1	25/02/2010	AU 2009-284175 A1	25/02/2010
		CA 2734829 A1	25/02/2010
		CN 102186878 A	14/09/2011
		EP 2157100 A1	24/02/2010
		JP 2012-500015 A	05/01/2012
		US 2012-0171188 A1	05/07/2012
		US 2012-0244595 A1	27/09/2012
US 2011-0027249 A1	03/02/2011	US 8383102 B2	26/02/2013