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(57) Abstract: The present invention relates to antimicrobial agents against Gram-positive bacteria, in particular to fusion proteins composed of an enzyme having the activity of degrading the cell wall of Gram-positive bacteria and an additional peptide stretch fused to the enzyme at the N- or C-terminus. Moreover, the present invention relates to nucleic acid molecules encoding said fusion protein, vectors comprising said nucleic acid molecules and host cells comprising either said nucleic acid molecules or said vectors. In addition, the present invention relates to said fusion protein for use as a medicament, in particular for the treatment or prevention of Gram-positive bacterial infections, as diagnostic means or as cosmetic substance. The present invention also relates to the treatment or prevention of Gram-positive bacterial contamination of foodstuff, of food processing equipment, of food processing plants, of surfaces coming into contact with foodstuff, of medical devices, of surfaces in hospitals and surgeries. Further, the present invention relates to a pharmaceutical composition comprising said fusion protein.

ANTIMICROBIAL AGENTS

The present invention relates to antimicrobial agents against Gram-positive bacteria, in particular to fusion proteins composed of an enzyme having the activity of degrading the cell wall of Gram-positive bacteria and an additional peptide stretch fused to the enzyme at the N- or C-terminus. Moreover, the present invention relates to nucleic acid molecules encoding said fusion protein, vectors comprising said nucleic acid molecules and host cells comprising either said nucleic acid molecules or said vectors. In addition, the present invention relates to said fusion protein for use as a medicament, in particular for the treatment or prevention of Gram-positive bacterial infections, as diagnostic means or as cosmetic substance. The present invention also relates to the treatment or prevention of Gram-positive bacterial contamination of foodstuff, of food processing equipment, of food processing plants, of surfaces coming into contact with foodstuff, of medical devices, of surfaces in hospitals and surgeries. Further, the present invention relates to a pharmaceutical composition comprising said fusion protein.

In contrast to Gram-negative bacteria, Gram-positive bacteria do not possess an outer membrane. The cytoplasmic membrane is surrounded by an up to 25 nm thick layer of peptidoglycan (which is only up to 5 nm for Gram-negative bacteria) which forms the cell wall. Main purpose of the cell wall of Gram-positives is to maintain bacterial shape and to counteract the internal bacterial cell pressure. Peptidoglycan, or murein, is a polymer consisting of sugars and amino acids. The sugar component consists of alternating residues of β-(1,4) linked N-acetylglucosamine and N-acetylmuramic acid residues compose the sugar components. A peptide chain of three to five amino acids is attached to the N-acetylmuramic acid. The peptide chain can be cross-linked to the peptide chain of another strand forming a 3D mesh-like layer. The peptide chain may contain D- and L- amino acid residues and the composition may vary for different bacteria.

A special situation is found in case of Mycobacteria which are usually considered Gram-positive. Mycobacteria were recently shown to posses an outer cell membrane. All Mycobacteria share a characteristic thick cell wall, which is hydrophobic, waxy, and rich in mycolic acids/mycolates. The cell wall consists of the hydrophobic mycolate layer and a

peptidoglycan layer held together by arabinogalactan, a polysaccharide. To overcome the thick cell wall of Mycobacteria a combined action of the new antimicrobial agents with a chitinase or a similar protein to disrupt the polysaccharide layer may be necessary.

Various types of agents having bactericidal or bacteriostatic activity are known, e.g. antibiotics, endolysins, antimicrobial peptides and defensins. Increasing microbial resistance to antibiotics, however, is creating difficulties in treating more and more infections caused by bacteria. Particular difficulties arise with infections caused by Gram-positive bacteria like *Staphylococcus aureus, Enterococci, Streptococci, Listeria monocytogenes* and *Clostridium difficile*, especially with e.g. Methicillin-resistant *Staphylococcus aureus* and Vancomycin-resistant *Enterococci.*

Endolysins are peptidoglycan hydrolases encoded by bacteriophages (or bacterial viruses). They are synthesized during late gene expression in the lytic cycle of phage multiplication and mediate the release of progeny virions from infected cells through degradation of the bacterial peptidoglycan. They are either ß(1,4)-glycosylases (lysozymes), transglycosylases, amidases or endopeptidases. Antimicrobial application of endolysins was already suggested in 1991 by Gasson (GB2243611). Although the killing capacity of endolysins has been known for a long time, the use of these enzymes as antibacterials was ignored due to the success and dominance of antibiotics. Only after the appearance of multiple antibiotic resistant bacteria this simple concept of combating human pathogens with endolysins received interest. A compelling need to develop totally new classes of antibacterial agents emerged and endolysins used as 'enzybiotics' - a hybrid term of 'enzymes' and 'antibiotics' - perfectly met this need. In 2001, Fischetti and coworkers demonstrated for the first time the therapeutic potential of bacteriophage Cl endolysin towards group A streptococci (Nelson et a1., 2001). Since then many publications have established endolysins as an attractive and complementary alternative to control bacterial infections, particularly by Gram-positive bacteria. Subsequently different endolysins against other Gram-positive pathogens such as *Streptococcus pneumoniae* (Loeffler et al., 2001), *Bacillus anthracis* (Schuch et al., 2002), *S. agalactiae* (Cheng et al., 2005) and *Staphylococcus aureus* (Rashel et al, 2007) have proven their efficacy as enzybiotics. However, it is also known that endolysins can, under some conditions (e.g. high ionic strength), create stable protoplast, where the internal bacterial cell pressure is not sufficient to lead to a cell burst. Under these conditions the bacterial cell wall can regenerate and the bacteria will survive.

Antimicrobial peptides (AMPs) represent a wide range of short, cationic or amphipatic gene-encoded peptide antibiotics that can be found in virtually every organism. Different AMPs display different properties, and many peptides in this class are being intensively researched not only as antibiotics, but also as templates for cell-penetrating peptides. Despite sharing a few common features (e.g., cationicity, amphipathicity and short size), AMP sequences vary greatly, and at least four structural groups (α-helical, β-sheet, extended and looped) have been proposed to accommodate the diversity of the observed AMP conformations. Likewise, several modes of action as antibiotics have been proposed, and it was shown e.g. that the primary target of many of these peptides is the cell membrane whereas for other peptides the primary target is cytoplasmic invasion and disruption of core metabolic functions. AMPs may become concentrated enough to exhibit cooperative activity despite the absence of specific target binding; for example, by forming a pore in the membrane, as is the case for most AMPs. However, this phenomenon has only been observed in model phospholipid bilayers, and in some cases, AMP concentrations in the membrane that were as high as one peptide molecule per six phospholipid molecules were required for these events to occur. These concentrations are close to, if not at, full membrane saturation. As the minimum inhibitory concentration (MIC) for AMPs is typically in the low micromolar range, scepticism has understandably arisen regarding the relevance of these thresholds and their importance *in vivo* (Melo et al., Nature reviews, Microbiology, 2009, 245).

Defensins are a large family of small, cationic, cysteine- and arginine-rich antimicrobial peptides, found in both vertebrates and invertebrates. Defensins are divided into five groups according to the spacing pattern of cysteines: plant, invertebrate, α-, β-, and θ-defensins. The latter three are mostly found in mammals. α -defensins are proteins found in neutrophils and intestinal epithelia. β-defensins are the most widely distributed and are secreted by leukocytes and epithelial cells of many kinds. θ-defensins have been rarely found so far e.g. in leukocytes of rhesus macaques. Defensins are active against bacteria, fungi and many enveloped and nonenveloped viruses. However, the concentrations needed for efficient killing of bacteria are mostly high, i.e. in the μ-molar range. Activity of many peptides may be limited in presence of physiological salt conditions, divalent cations and serum. Depending on the content of hydrophobic amino acid residues Defensins also show haemolytic activity.

Thus, there is a need for new antimicrobial agents against Gram-positive bacteria.

This object is solved by the subject matter defined in the claims.

The term "protein" as used herein refers synonymously to the term "polypeptide". The term “protein” as used herein refers to a linear polymer of amino acid residues linked by peptide bonds in a specific sequence. The amino-acid residues of a protein may be modified by e.g. covalent attachments of various groups such as carbohydrates and phosphate. Other substances may be more loosely associated with the polypeptide chains, such as heme or lipid, giving rise to the conjugated proteins which are also comprised by the term “protein” as used herein. The various ways in which the polypeptide chains fold have been elucidated, in particular with regard to the presence of alpha helices and beta-pleated sheets. The term “protein” as used herein refers to all four classes of proteins being all-alpha, all-beta, alpha/beta and alpha plus beta. Moreover, the term “protein” refers to a complex, wherein the complex refers to a homomer.

The term "fusion protein" as used herein refers to an expression product resulting from the fusion of two nucleic acid sequences. Such a protein may be produced, e.g., in recombinant DNA expression systems. Moreover, the term “fusion protein” as used herein refers to a fusion of a first amino acid sequence as e.g. an enzyme, with a second or further amino acid sequence. The second or further amino acid sequence may define a domain or any kind of peptide stretch. Preferably, said second and/or further amino acid sequence is foreign to and not substantially homologous with any domain of the first amino acid sequence.

The term “peptide stretch” as used herein refers to any kind of peptide linked to a protein such as an enzyme.

The term “peptide” as used herein refers to short polypeptides consisting of from about 2 to about 100 amino acid residues, more preferably from about 4 to about 50 amino acid residues, more preferably to about 5 to 30 amino acid residues, wherein the amino group of one amino acid residue is linked to the carboxyl group of another amino acid residue by a peptide bond. A peptide may have a specific function. A peptide can be a naturally occurring peptide or a synthetically designed and produced peptide. The peptide can be, for example, derived or removed from a native protein by enzymatic or chemical cleavage, or can be prepared using

conventional peptide synthesis techniques (e.g., solid phase synthesis) or molecular biology techniques (see Sambrook, J. et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Press, Cold Spring Harbor, N.Y. (1989)). Examples of naturally occurring peptides are antimicrobial peptides, defensins, sushi peptides. Examples of synthetically produced peptides are polycationic, amphiphatic or hydrophobic peptides. A peptide in the meaning of the present invention does not refer to His-tags, Strep-tags, thioredoxin or maltose binding proteins (MBP) or the like, which are used to purify or locate proteins.

The term "endolysin" as used herein refers to an enzyme which is suitable to hydrolyse bacterial cell walls. "Endolysins" comprise of at least one "enzymatically active domain" (EAD) having at least one of the following activities: endopeptidase, chitinase, T4 like muraminidase, lambda like muraminidase, N-acetyl-muramoyl-L-alanine-amidase (amidase), muramoyl-L-alanine-amidase, muramidase, lytic transglycosylase (C), lytic transglycosylase (M), N-acetyl-muramidase, N-acetyl-glucosaminidase (lysozyme) or transglycosylases as e.g. KZ144 and EL188. In addition, the endolysins may contain also regions which are enzymatically inactive, and bind to the cell wall of the host bacteria, the so-called CBDs (cell wall binding domains).

The term "CBD" as used herein refers to the cell wall binding domain of an endolysin. In endolysins being specific for Gram-positive bacteria the CBD is often found at the C-terminus, but may also be found N-terminal or somewhere else within the protein. Often CBD domains mediate binding of the endolysin to the bacterial cell wall but have no enzymatic activity in terms of hydrolyzing the cell wall.

The term "EAD" as used herein refers to the enzymatically active domain of an endolysin. The EAD is responsible for hydrolysing bacterial peptidoglycans. It exhibits at least one enzymatic activitiy of an endolysin. The EAD can also be composed of more than one enzymatically active module.

The term "autolysins" refers to enzymes related to endolysins but encoded by bacteria and involved in e.g. cell division. An overview of autolysins is can be found in "Bacterial peptidoglycan (murein) hydrolases. Vollmer W, Joris B, Charlier P, Foster S. FEMS Microbiol Rev. 2008 Mar;32(2):259-86".

The term "bacteriocin" as used herein refers to protein-like, polypeptide-like or peptide-like substances which are able to inhibit the growth of other bacteria. Some bacteriocins are capable of degrading bacterial cell walls like Lysostaphin (degrading *Staphylococcus* cell walls), Mutanolysin (degrading *Streptococcus* cell walls) and Enterolysin (degrading *Enterococcus* cell walls). Preferably said inhibition is specifically by means of absorption of said other bacteria to specific receptors of the bacteriocin. In general, bacteriocins are produced by microorganisms. However, the term “bacteriocin” as used herein refers both to an isolated form procuded by a microorganism or to a synthetically produced form, and refers also to variants which substantially retain the activities of their parent bacteriocins, but whose sequences have been altered by insertion or deletion of one or more amino acid residues.

The term, "antimicrobial peptide" (AMP) as used herein refers to any peptide that has microbicidal and/or microbistatic activity. Thus, the term “antimicrobial peptide” as used herein refers in particular to any peptide having anti-bacterial, anti-fungal, anti-mycotic, anti-parasitic, anti-protozoal, anti-viral, anti-infectious, anti-infective and/or germicidal, algicidal, amoebicidal, microbicidal, bactericidal, fungicidal, parasiticidal, protozoacidal, protozoicidal properties.

The term "defensin" as used herein refers to a peptide present within animals, preferably mammals, more preferably humans, wherein the defensin plays a role in the innate host defense system as the destruction of foreign substances such as infectious bacteria and/or infectious viruses and/or fungi. A defensin is non-antibody microbicidal and/or tumoricidal protein, peptide or polypeptide. Examples for "defensins" are "mammalian defensins," alpha-defensins, beta-defensins, indolicidin and magainins. The term "defensins" as used herein refers both to an isolated form from animal cells or to a synthetically produced form, and refers also to variants which substantially retain the cytotoxic activities of their parent proteins, but whose sequences have been altered by insertion or deletion of one or more amino acid residues.

The term “sushi peptide” as used herein refers to complement control proteins (CCP) having short consensus repeats. The sushi module of sushi peptides functions as a protein-protein interaction domain in many different proteins. Peptides containing a Sushi domain have been shown to have antimicrobial activities.

As used herein, the term "cationic peptide" refers to a peptide having positively charged amino acid residues. Preferably a cationic peptide has a pKa-value of 9.0 or greater. Typically, at least four of the amino acid residues of the cationic peptide can be positively charged, for example, lysine or arginine. "Positively charged" refers to the side chains of the amino acid residues which have a net positive charge at about physiological conditions. Examples of naturally occurring cationic peptides which can be recombinantly produced are defensins, magainins, melittin, and cecropins.

The term “polycationic peptide” as used herein refers to a synthetically produced peptide composed of mostly lysine and/or arginine residues.

The term "amphipathic peptide" as used herein refers to peptides having both hydrophilic and hydrophobic functional groups. Preferably, the term “amphipathic peptide” as used herein refers to a peptide having a defined arrangement of hydrophilic and hydrophobic groups e.g. amphipatic peptides may be e.g. alpha helical, having predominantly non polar side chains along one side of the helix and polar residues along the remainder of its surface.

The term "hydrophobic group" as used herein refers to chemical groups such as amino acid side chains which are substantially water insoluble, but soluble in an oil phase, with the solubility in the oil phase being higher than that in water or in an aqueous phase. In water, amino acids having a hydrophobic side chain interact with one another to generate a nonaqueous environment. Examples of amino acids with hydrophobic side chains are alanine, valine, leucine, isoleucine, phenylalanine, histidine, tryptophane and tyrosine.

The term “deletion” as used herein refers to the removal of 1, 2, 3, 4, 5 or more amino acid residues from the respective starting sequence.

The term “insertion” or “addition” as used herein refers to the insertion or addition of 1, 2, 3, 4, 5 or more amino acid residues to the respective starting sequence.

The term “substitution” as used herein refers to the exchange of an amino acid residue located at a certain position for a different one.

The present invention relates to new antibacterial agents against Gram-positive bacteria, in particular to fusion proteins composed of an enzyme having the activity of degrading the cell wall of Gram-positive bacteria and an additional peptide stretch fused to the enzyme at the N- or C-terminus or at both termini.

The fusion proteins according to the present invention have the advantage that they may prevent the regeneration of stable protoplasts and thus, preventing the survival of the bacteria which should be eliminated. The regeneration of the protoplast by the bacteria occurs under some conditions (e.g. high ionic strength), where the internal bacterial cell pressure is not sufficient to lead to a cell burst and leads to the survival of the bacteria.

In one aspect of the present invention the enzyme having the activity of degrading the cell wall of Gram-positive bacteria is an endolysine, autolysine and/or bacteriocin.

In another aspect of the present invention the enzyme may contain also regions which are enzymatically inactive, and bind to the cell wall of the host bacteria, the so-called CBDs (cell wall binding domains).

Preferred fusion proteins according to the present invention are depicted in SEQ ID NO:63 to 90. The fusion proteins according to SEQ ID NO:63 to 90 may comprise one or more additional amio acid residues on the N-terminus. Preferably the additional amino acid residue is methionine.

Preferably, the endolysin is encoded by bacteriophages specific for Gram-positive bacteria such as Gram-positive bacteria of bacterial groups, families, genera or species comprising strains pathogenic for humans or animals as listed in the following table.

Table 1:

I. Phylum Actinobacteria
Class: Actinobacteridae
Order Actinomycetales
Families:
Actinomycineae: Actinomycetaceae (Actinomyces, Mobiluncus)
Corynebacterineae: Mycobacteriaceae (Mycobacterium), Nocardiaceae, Corynebacteriaceae
Frankineae: Frankiaceae

Micrococcineae: Brevibacteriaceae
Propionibacteriaceae (Propionibacterium)
Order: Bifidobacteriales
Families:
Bifidobacteriaceae (Bifidobacterium, Falcivibrio, Gardnerella)
Other subclasses:Acidimicrobidae, Coriobacteridae, Rubrobacteridae, Sphaerobacteridae

II. Phylum Firmicutes
Class: Bacilli
Order: Bacillales:
Families:
Bacillaceae (Bacillus), Listeriaceae (Listeria), Staphylococcaceae (Staphylococcus, Gemella, Jeotgalicoccus)
Order: Lactobacillales:
Families: Enterococcaceae (Enterococcus), Lactobacillaceae (Lactobacillus, Pediococcus), Leuconostocaceae (Leuconostoc), Streptococcaceae (Lactococcus, Streptococcus)

Class: Clostridia
Order: Clostridiales (Clostridium, Peptostreptococcus, Selenomonas)
Order: Halanaerobiales
Order: Thermoanaerobacterales

Class: Tenericutes/Mollicutes
Order: Mycoplasmatales (Mycoplasma, Ureaplasma)
Order: Entomoplasmatales (Spiroplasma)
Order: Anaeroplasmatales (Erysipelothrix)
Order: Acholeplasmatales (Acholeplasma)
Order: Haloplasmatales (Haloplasma)

Preferably, the autolysin is encoded by Gram-positive bacteria such as Gram-positive bacteria of bacterial groups, families, genera or species comprising strains pathogenic for humans or animals as listed in table 1.

Preferably, the bacteriocin is encoded by Gram-positive bacteria such as Gram-positive bacteria of bacterial groups, families, genera or species comprising strains pathogenic for humans or animals as listed in table 1.

The enzyme according to the present invention has cell wall degrading activity against Gram-positive bacteria of bacterial groups, families, genera or species comprising strains pathogenic for humans or animals like *Listeria monocytogenes, Staphylococcus aureus, Enterococcus faecalis, Enterococcus faecium, Streptococcus pneumoniae, Streptococcus pyogenes, Streptococcus mutans, Streptococcus equi, Clostridium difficile, Clostridium botulinum,*

Clostridium tetani, Clostridium perfringens, Bacillus anthracis, Bacillus cereus, Propionibacterium acnes, Mycobacterium avium, Mycobacterium tuberculosis, Corynebacterium diphteriae, Mycoplasma pneumoniae, Actinomyces.

Preferred endolysins are Listeria phage endolysins PlyA118, PlyA500, PlyPSA, PlyA511, PlyP35, PlyP40, Staph phage Phi 11, Phi MR11, LysK, *Clostridium perfringens* PlyS6, Ply3626, *Clostridium difficile*: CD27L endolysin, Streptococcus: B30 endolysin, phage Dp-1 Pal amidase, C1 endolysin, Cpl-1 endolysin, PlyGBS, Enterococccus: PlyV12, Bacillus anthracis: Phage gamma endolysin PlyG.

Preferred autolysins are described in: Bacterial peptidoglycan (murein) hydrolases. Vollmer W, Joris B, Charlier P, Foster S. FEMS Microbiol Rev. 2008 Mar;32(2):259-86. Epub 2008 Feb 11. Review. An example of a preferred autolysin is the AtlA Autolysine.

Preferred bacteriocines are Lysostaphin (degrading *Staphylococcus* cell walls), Mutanolysin (degrading *Streptococcus* cell walls) and Enterolysin (degrading *Enterococcus* cell walls).

More preferably, the endolysin part is selected from the group consisting of Cpl-1 according to SEQ ID NO:57, Ply511 according to SEQ ID NO:58, LysK according to SEQ ID NO:59, Lysostaphin according to SEQ ID NO:60 and PA6-gp20 according to SEQ ID NO:61.

In another preferred embodiment of the present invention the endolysins, autolysins and bacteriocins of the fusion protein according to the present invention comprise modifications and/or alterations of the amino acid sequences. Such alterations and/or modifications may comprise mutations such as deletions, insertions and additions, substitutions or combinations thereof and/or chemical changes of the amino acid residues, e.g. biotinylation, acetylation, pegylation, chemical changes of the amino-, SH- or carboxyl- groups. Said endolysins, autolysins and bacteriocins of the fusion protein according to the present invention exhibit the lytic activity of the respective wild-type endolysin. However, said activity can be the same, higher or lower as the activity of the respective wild-type endolysin. Said activity can be about 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190 or about 200 % of the activity of the respective wild-type endolysin or even more. The activity can be measured by assays well known in the art by a person skilled in the art as e.g. the plate lysis assay or the liquid lysis assay which are e.g. described *in Briers et al., J. Biochem.*

Biophys Methods 70: 531-533, (2007) or *Donovan DM, Lardeo M, Foster-Frey J. FEMS Microbiol Lett. 2006 Dec;265(1)* or similar publications.

Preferably, the peptide stretch of the fusion protein according to the invention is fused to the N-terminus and/or to the C-terminus of the endolysin. In a particular preferred embodiment said peptide stretch is only fused to the N-terminus of the enzyme. In another preferred embodiment the peptide stretch is only fused to the C-Terminus of the enzyme. However, also preferred are modified fusion proteins having a peptide stretch both on the N-terminus and on the C-terminus. Said peptide stretches on the N-terminus and on the C-terminus can be the same or distinct peptide stretches. The peptide stretch can be linked to the enzyme by additional amino acid residues e.g. due to cloning reasons. Preferably, said peptide stretch can be linked to the fusion protein by at least 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 additional amino acid residues. In a preferred embodiment the peptide stretch is linked to the enzyme by the additional amino acid residues glycine and/or serine (Gly-Ser). Moreover, the peptide stretch of the fusion protein according to the invention further comprises additional amino acids on its N-terminus. Preferably, the peptide stretch comprises the amino acid methionine (Met) or alanine, methionine and glycine (Ala-Met-Gly).

The peptide stretch of the fusion protein according to the present invention is preferably covalently bound to the enzyme. Preferably, said peptide stretch consists of at least 5, more preferably at least of 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or at least 100 amino acid residues. Especially preferred is a peptide stretch comprising about 5 to about 100 amino acid residues, about 5 to about 50 or about 5 to about 30 amino acid residues. More preferred is a peptide stretch comprising about 6 to about 42 amino acid residues, about 6 to about 39 amino acid residues, about 6 to about 38 amino acid residues, about 6 to about 31 amino acid residues, about 6 to about 25 amino acid residues, about 6 to about 24 amino acid residues, about 6 to about 22 amino acid residues, about 6 to about 21 amino acid residues, about 6 to about 20 amino acid residues, about 6 to about 19 amino acid residues, about 6 to about 16 amino acid residues, about 6 to about 14 amino acid residues, about 6 to about 12 amino acid residues, about 6 to about 10 amino acid residues or about 6 to about 9 amino acid residues.

Preferably, the peptide stretch is no tag such as a His_6-tag, Strep-tag, Avi-tag, Myc-tag, Gst-tag, JS-tag, cystein-tag, FLAG-tag or other tags known in the art and no thioredoxin or maltose binding proteins (MBP). However, the peptide stretch and/or the endolysin, autolysin or bacteriocin according to the present invention may comprise in addition such tag or tags.

More preferably the peptide stretch has the function to facilitate the burst of the bacterial cell via interaction of the fusion protein with: first the peptidoglycan layer, degrading the peptidoglycan and second the cytoplasmic membrane, destabilizing the cytoplasmic membrane.

In one aspect of the present invention the fused peptide stretch is a cationic peptide, more preferably a polycationic peptide. Preferably the cationic peptide comprises one or more of the positively charged amino acid residues of lysine, arginine and/or histidine. Preferably, more than about 60, 65, 70, 75, 80, 85, 90, 95 or about 100 %, of the amino acid residues in said peptide are positively charged amino acid residues. Advantageously, the cationic peptide is fused at the N-terminal and/or the C-terminal end of the enzyme having cell wall degrading activity, thus enhancing the cationicity of the fusion proteins and/or antimicrobial agents of the present invention. In another embodiment of the invention, the cationic peptide fused to the enzyme consists of at least 5, more preferably of at least 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49 or 50 amino acid residues. Preferably at least about 60, 65, 70, 75, 80, 85, 90, 95 or about 100 %, of the amino acid residues of the cationic peptide are either arginine or lysine. In another embodiment of the present invention the cationic peptide comprises about 3 to about 50, more preferably about 5 to about 20, for instance about 5 to about 15 amino acid residues and the said amino acid residues are either arginine or lysine residues. Preferred cationic peptides are depicted in SEQ ID NOs:13 and 14.

Especially preferred are cationic and/or polycationic peptide stretches comprising at least one motive according to SEQ ID NO: 62 (KRKKRK). In particular cationic peptide stretches comprising at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16 or 17 motives according to SEQ ID NO: 62 (KRKKRK) are preferred. More preferred are cationic peptide stretches comprising at least one KRK motive (lys-arg-lys), preferable at least 2, 3, 4, 5, 6, 7, 8, 9, 10,

11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32 or 33 KRK motives.

In another preferred embodiment of the present invention the cationic peptide stretch comprises beside the positively charged amino acid residues, in particular lysine and/or arginine residues, neutrally charged amino acid residues, in particular glycine and/or serine residues. Preferred are cationic peptide stretches consisting of about 70 % to about 100 %, or about 80 % to about 95 %, or about 85 % to about 90 % positively charged amino acid residues, in particular lysine, arginine and/or histidine residues, more preferably lysine and/or arginine residues and of about 0 % to about 30 %, or about 5 % to about 20 %, or about 10 % to about 20 % neutrally charged amino acid residues, in particular glycine and/or serine residues. Preferred are polypeptide stretches consisting of about 4 % to about 8 % serine residues, of about 33 % to about 36 % arginine residues and of about 56 % to about 63 % lysine residues. Especially preferred are polypeptide stretches comprising at least one motive according to SEQ ID NO: 45 (KRXKR), wherein X is any other amino acid than lysine, arginine and histidine. Especially preferred are polypeptide stretches comprising at least one motive according to SEQ ID NO: 46 (KRSKR). More preferred are cationic stretches comprising at least about 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or about 20 motives according to SEQ ID NO: 45 (KRXKR) or SEQ ID NO: 46 (KRSKR).

Also preferred are polypeptide stretches consisting of about 9 to about 16 % glycine residues, of about 4 to about 11 % serine residues, of about 26 to about 32 % arginine residues and of about 47 to about 55 % lysine residues. Especially preferred are polypeptide stretches comprising at least one motive according to SEQ ID NO: 47 (KRGSG). More preferred are cationic stretches comprising at least about 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or about 20 motives according to SEQ ID NO: 47 (KRGSG).

In another preferred embodiment of the present invention the cationic peptide stretch comprises beside the positively charged amino acid residues, in particular lysine and/or arginine residues, hydrophobic amino acid residues, in particular valine, isoleucine, leucine, methionine, phenylalanine, tryptophan, cysteine, alanine, tyrosine, histidine, threonin, serine, proline and glycine residues, more preferably alanine, valine, leucine, isoleucine, phenylalanine, and/or tryptophan residues. Preferred are cationic peptide stretches consisting of about 70 % to about 100 %, or about 80 % to about 95 %, or about 85 % to about 90 %

positively charged amino acid residues, in particular lysine and/or arginine residues and of about 0 % to about 30 %, or about 5 % to about 20 %, or about 10 % to about 20 % hydrophobic amino acid residues, valine, isoleucine, leucine, methionine, phenylalanine, tryptophan, cysteine, alanine, tyrosine, histidine, threonin, serine, proline and glycine residues, more preferably alanine, valine, leucine, isoleucine, phenylalanine, and/or tryptophan residues.

Especially preferred are peptide stretches selected from the group consisting of the following sequences:

Table 2:

peptide stretch	length	SEQ ID NO:
KRKKRK	6	SEQ ID NO:24
KRKKRKKRK	9	SEQ ID NO:13
RRRRRRRRR	9	SEQ ID NO:25
KKKKKKKK	8	SEQ ID NO:26
KRKKRKKRKK	10	SEQ ID NO:27
KRKKRKKRKKRK	12	SEQ ID NO:28
KRKKRKKRKKRKKR	14	SEQ ID NO:29
KKKKKKKKKKKKKKKK	16	SEQ ID NO:30
KRKKRKKRKKRKKRKKRK	18	SEQ ID NO:31
KRKKRKKRKKRKKRKKRKK	19	SEQ ID NO:32
RRRRRRRRRRRRRRRRRRR	19	SEQ ID NO:33
KKKKKKKKKKKKKKKKKKK	19	SEQ ID NO:34
KRKKRKKRKRSKRKKRKKRK	20	SEQ ID NO:35
KRKKRKKRKRSKRKKRKKRKK	21	SEQ ID NO:36
KRKKRKKRKKRKKRKKRKKRK	21	SEQ ID NO:37
KRKKRKKRKRGSGKRKKRKKRK	22	SEQ ID NO:38
KRKKRKKRKRGSGSGKRKKRKKRK	24	SEQ ID NO:39
KRKKRKKRKKRKKRKKRKKRKKRKK	25	SEQ ID NO:40
KRKKRKKRKRSKRKKRKKRKRSKRKKRKKRK	31	SEQ ID NO:41
KRKKRKKRKRGSGSGKRKKRKKRKGSGSGKRKKRKKRK	38	SEQ ID NO:42
KRKKRKKRKKRKKRKKRKKRKKRKKRKKRKKRKKRKKRK	39	SEQ ID NO:43
KRKKRKKRKRSKRKKRKKRKRSKRKKRKKRKRSKRKKRKKRK	42	SEQ ID NO:44

In a further aspect of the present invention the fused peptide stretch is an amphipatic peptide, which comprises one or more of the positively charged amino acid residues of lysine, arginine and/or histidine, combined to one or more of the hydrophobic amino acid residues of valine,

isoleucine, leucine, methionine, phenylalanine, tryptophan, cysteine, alanine, tyrosine, histidine, threonin, serine, proline and/or glycine. Side chains of the amino acid residues are oriented in order that cationic and hydrophobic surfaces are clustered at opposite sides of the peptide. Preferably, more than about 30, 40, 50, 60 or 70% of the amino acids in said peptide are positively charged amino acids. Preferably, more than about 30, 40, 50, 60 or 70%, of the amino acid residues in said peptide are hydrophobic amino acid residues. Advantageously, the amphipathic peptide is fused at the N-terminal and/or the C-terminal end of the enzyme having cell wall degrading activity, thus enhancing the amphipathicity of the latter proteins.

In another embodiment of the invention, the amphipathic peptide fused to the enzyme consists of at least 5, more preferably at least of 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49 or 50 amino acid residues. In a preferred embodiment at least about 30, 40, 50, 60 or 70% of the said amino acid residues of the amphipatic peptide are either arginine or lysine residues and/or at least about 30, 40, 50, 60 or 70% of the said amino acid residues of the amphipathic peptide are of the hydrophobic amino acids valine, isoleucine, leucine, methionine, phenylalanine, tryptophan, cysteine, alanine, tyrosine, histidine, threonin, serine, proline and/or glycine.

Preferred amphipatic peptides are Pleurocidin according to SEQ ID NO:1, Cecropin P1 according to SEQ ID NO:2, Buforin II according to SEQ ID NO:3, Buforin I according to SEQ ID NO:23 and Magainin according to SEQ ID NO:4. Further preferred amphipatic peptides are Cathelidicine e.g. LL-37 according to SEQ ID NO:5, Nigrocine 2 according to SEQ ID NO: 48 and Ascaphine 5 according to SEQ ID NO:49.

In a further aspect of the present invention the fused peptide stretch is an antimicrobial peptide, which comprises a positive net charge and around 50% hydrophobic amino acids. The antimicrobial peptides are amphipathic, with a length of about 12 to about 50 amino acid residues.

Examples for antimicrobial peptides are listed in the following table.

Table 3:

Peptid	**Sequenz**	
LL-37	LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES	SEQ ID NO:5

SMAP-29	RGLRRLGRKIAHGVKKYGPTVLRIIRIAG	SEQ ID NO:6
Indolicidin	ILPWKWPWWPWRR	SEQ ID NO:7
Protegrin	RGGRLCYCRRRFCVCVGR	SEQ ID NO:8
Cecropin P1	SWLSKTAKKLENSAKKRISEGIAIAIQGGPR	SEQ ID NO:2
Magainin	GIGKFLHSAKKFGKAFVGEIMNS	SEQ ID NO:4
Pleurocidin	GWGSFFKKAAHVGKHVGKAALTHYL	SEQ ID NO:1
Cecropin A (A.aegypti)	GGLKKLGKKLEGAGKRVFNAAEKALPVVAGAKALRK	SEQ ID NO:9
Cecropin A (D. melanogaster)	GWLKKIGKKIERVGQHTRDATIQGLGIPQQAANVAATARG	SEQ ID NO:10
Buforin II	TRSSRAGLQFPVGRVHRLLRK	SEQ ID NO:3
Sarcotoxin IA	GWLKKIGKKIERVGQHTRDATIQGLGIAQQAANVAATAR	SEQ ID NO:11
Apidaecine	ANRPVYIPPPRPPHPRL	SEQ ID NO:50
Ascaphine 5	GIKDWIKGAAKKLIKTVASHIANQ	SEQ ID NO:49
Nigrocine 2	GLLSKVLGVGKKVLCGVSGLVC	SEQ ID NO:48
Pseudin 1	GLNTLKKVFQGLHEAIKLINNHVQ	SEQ ID NO:51
Ranalexin	FLGGLIVPAMICAVTKKC	SEQ ID NO:52
Melittin	GIGAVLKVLTTGLPALISWIKRKRQQ	SEQ ID NO:53

In a further aspect of the present invention the fused peptide stretch is a sushi peptide which is described by Ding JL, Li P, Ho B Cell Mol Life Sci. 2008 Apr;65(7-8):1202-19. The Sushi peptides: structural characterization and mode of action against Gram-negative bacteria. Especially preferred is the sushi 1 peptide according to SEQ ID NO:54.

Preferred sushi peptides are sushi peptides S1 and S3 and multiples thereof; FASEB J. 2000 Sep;14(12):1801-13.

In a further aspect of the present invention the fused peptide stretch is a defensin, preferably Cathelicidine, Cecropin P1, Cecropin A or Magainin II.

In a further aspect of the present invention the fused peptide stretch is a hydrophobic peptide e.g. Apidaecine having the amino acid sequence according to SEQ ID NO: 50, WLBU2-Variant having the amino acid sequence according to SEQ ID NO: 55 and Walmagh1 having the amino acid sequence according to SEQ ID NO: 56. The hydrophobic peptide having the amino acid sequence Phe-Phe-Val-Ala-Pro (SEQ ID NO: 12) is not part of the present invention.

In another preferred embodiment of the present invention the peptide stretches of the fusion protein according to the present invention comprise modifications and/or alterations of the amino acid sequences. Such alterations and/or modifications may comprise mutations such as deletions, insertions and additions, substitutions or combinations thereof and/or chemical

changes of the amino acid residues, e.g. biotinylation, acetylation, peglyation, chemical changes of the amino-, SH- or carboxyl- groups.

Especially preferred are fusion proteins according to the SEQ ID NOs: 63 to 90 and the fusion proteins selected from the group consisting of the following fusion proteins:

Table 4:

Fusion protein	Fusion protein	Enzyme part	Peptide stretch (N-terminal unless otherwise indicated)
P1-E1	SEQ ID NO: 63	Cpl-1 (SEQ ID NO: 57)	Ascaphine 5 (SEQ ID NO:49)
P2-E1	SEQ ID NO: 64	Cpl-1 (SEQ ID NO: 57)	Apiadaecine (SEQ ID NO:50)
P3-E1	SEQ ID NO: 65	Cpl-1 (SEQ ID NO: 57)	Nigrocine 2 (SEQ ID NO:48)
P4-E1	SEQ ID NO: 66	Cpl-1 (SEQ ID NO: 57)	Pseudin 1 (SEQ ID NO:51)
P7-E1	SEQ ID NO: 67	Cpl-1 (SEQ ID NO: 57)	Ranalexin (SEQ ID NO:52)
P8-E1	SEQ ID NO: 68	Cpl-1 (SEQ ID NO: 57)	WLBU2-Variant (SEQ ID NO:55)
P9-E1	SEQ ID NO: 69	Cpl-1 (SEQ ID NO: 57)	Sushi 1 (SEQ ID NO:54)
P10-E1	SEQ ID NO: 70	Cpl-1 (SEQ ID NO: 57)	Melittin (SEQ ID NO:53)
P11-E1	SEQ ID NO: 71	Cpl-1 (SEQ ID NO: 57)	LL-37 (SEQ ID NO:5)
P12-E1	SEQ ID NO: 72	Cpl-1 (SEQ ID NO: 57)	Indolicidin (SEQ ID NO:7)
P13-E1	SEQ ID NO: 73	Cpl-1 (SEQ ID NO: 57)	SMAP-29 (SEQ ID NO:6)
P14-E1	SEQ ID NO: 74	Cpl-1 (SEQ ID NO: 57)	Protegrin (SEQ ID NO:8)
P15-E1	SEQ ID NO: 75	Cpl-1 (SEQ ID NO: 57)	Cecropin P1 (SEQ ID NO:2)
P16-E1	SEQ ID NO: 76	Cpl-1 (SEQ ID NO: 57)	Magainin (SEQ ID NO:4)
P17-E1	SEQ ID NO: 77	Cpl-1 (SEQ ID NO: 57)	Pleurocidin (SEQ ID NO:1)
P18-E1	SEQ ID NO: 78	Cpl-1 (SEQ ID NO:57)	Cecropin A (A. aegypti) (SEQ ID NO:9)
P19-E1	SEQ ID NO: 79	Cpl-1 (SEQ ID NO:57)	Cecropin A (D. melanogaster) (SEQ ID NO:10)
P20-E1	SEQ ID NO: 80	Cpl-1	Buforin II

		(SEQ ID NO:57)	(SEQ ID NO:3)
P21-E1	SEQ ID NO: 81	Cpl-1 (SEQ ID NO:57)	Sarcotoxin IA (SEQ ID NO:11)
P5-E1	SEQ ID NO: 82	Cpl-1 (SEQ ID NO:57)	PK (SEQ ID NO:13)
P22-E2	SEQ ID NO: 83	Ply511 (SEQ ID NO:58)	Pentapeptid (SEQ ID NO:12)
P5-E7	SEQ ID NO: 84	LysK (SEQ ID NO:59)	PK (N-terminal) (SEQ ID NO:13)
P6-E7	SEQ ID NO: 85	LysK (SEQ ID NO:59)	PK2 (SEQ ID NO:31)
P5-E8	SEQ ID NO: 86	Lysostaphin (SEQ ID NO:60)	PK (C-terminal) (SEQ ID NO:13)
P6-E8	SEQ ID NO: 87	Lysostaphin (SEQ ID NO:60)	PK2 (SEQ ID NO:31)
P23-E9	SEQ ID NO: 88	PA6-gp20 (SEQ ID NO:61)	Walmagh1 (SEQ ID NO:56)
P5-E7	SEQ ID NO: 89	LysK (SEQ ID NO:59)	PK (C-terminal) (SEQ ID NO:13)
P5-E8	SEQ ID NO: 90	Lysostaphin (SEQ ID NO:60)	PK (N-terminal) (SEQ ID NO:13)

The fusion protein according to the present invention, and thus in particular the especially preferred fusion proteins according to SEQ ID NO:63 to 90, may additional comprise a methionine on the N-terminus.

The fusion protein according to the present invention, and thus in particular the especially preferred fusion proteins according to SEQ ID NO:63 to 90, may additional comprise a tag e.g. for purification. Preferred is a His_6-tag, preferably at the C-terminus of the fusion protein. Said tag can be linked to the fusion protein by additional amino acid residues e.g. due to cloning reasons. Preferably said tag can be linked to the fusion protein by at least 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 additional amino acid residues. In a preferred embodiment the fusion protein comprises a His_6-tag at its C-terminus linked to the fusion protein by the additional amino acid residues lysine and glycine (Lys-Gly) or leucine and glutamic acid (Leu-Glu). In another preferred embodiment the fusion protein comprises a His_6-tag at its N-terminus linked to the fusion protein by the additional amino acid residues lysine and glycine (Lys-Gly) or leucine and glutamic acid (Leu-Glu). In a more preferred embodiment the fusion protein comprises a His_6-tag at its N-terminus linked to the fusion protein by the additional amino acid residues leucine and glutamic acid (Leu-Glu). In another preferred embodiment the fusion protein

comprises a His_6-tag at its C-terminus linked to the fusion protein by the additional amino acid residues leucine and glutamic acid (Leu-Glu).

In a more preferred embodiment the fusion protein comprises a His_6-tag at its C-terminus linked to the fusion protein by the additional amino acid residues leucine and glutamic acid (Leu-Glu) and the peptide stretch of the fusion protein according to the invention is linked to the N-terminus of the enzyme by the additional amino acid residues glycine and serine (Gly-Ser). In another preferred embodiment the fusion protein comprises a His_6-tag at its C-terminus linked to the fusion protein by the additional amino acid residues leucine and glutamic acid (Leu-Glu) and the peptide stretch of the fusion protein according to the invention is linked to the N-terminus of the enzyme by the additional amino acid residues glycine and serine (Gly-Ser) and the fusion protein comprises on the N-terminus the additional amino acid residues methionine (Met) or methionine and glycine (Met-Gly) or alanine, methionine and glycine (Ala-Met-Gly). Preferably the fusion proteins are according to SEQ ID NO: 108 to 123.

In another preferred embodiment the fusion protein comprises a His_6-tag at its N-terminus, wherein the His_6-tag further comprises on its N-terminus the additional amino acids serine and serine (Ser-Ser) or methionine and glycine (Met-Gly) or methionine, glycine, serine and serine (Met-Gly-Ser-Ser).

In another preferred embodiment the fusion protein comprises a His_6-tag at its N-terminus linked to the fusion protein by the additional amino acid residues serine, serine, glycine, leucine, valine, proline, arginine, glycine, serine and histidine (Ser-Ser-Gly-Leu-Val-Pro-Arg-Gly-Ser-His). In another preferred embodiment the fusion protein comprises a His_6-tag at its N-terminus linked to the fusion protein by the additional amino acid residues serine, serine, glycine, leucine, valine, proline, arginine, glycine, serine, histidine and methionine (Ser-Ser-Gly-Leu-Val-Pro-Arg-Gly-Ser-His-Met). In another preferred embodiment the fusion protein comprises a His_6-tag at its N-terminus linked to the fusion protein by the additional amino acid residues serine, serine, glycine, leucine, valine, proline, arginine, glycine, serine and histidine (Ser-Ser-Gly-Leu-Val-Pro-Arg-Gly-Ser-His) or serine, serine, glycine, leucine, valine, proline, arginine, glycine, serine, histidine and methionine (Ser-Ser-Gly-Leu-Val-Pro-Arg-Gly-Ser-His-Met) and the peptide stretch of the fusion protein according to the invention is linked to the C-terminus of the enzyme by the additional amino acid residue serine. In

another preferred embodiment the fusion protein comprises a His_6-tag at its N-terminus linked to the fusion protein by the additional amino acid residues serine, serine, glycine, leucine, valine, proline, arginine, glycine, serine and histidine (Ser-Ser-Gly-Leu-Val-Pro-Arg-Gly-Ser-His) or serine, serine, glycine, leucine, valine, proline, arginine, glycine, serine, histidine and methionine (Ser-Ser-Gly-Leu-Val-Pro-Arg-Gly-Ser-His-Met) and the peptide stretch of the fusion protein according to the invention is linked to the C-terminus of the enzyme by the additional amino acid residue serine and the His6-tag comprises on the N-terminus the additional amino acid residues serine and serine (Ser-Ser) or methionine, glycine, serine and serine (Met-Gly-Ser-Ser) or methionine and serine (Met-Ser). Preferably the fusion proteins are according to SEQ ID NO: 122 and 123.

Fusion proteins are constructed by linking at least two nucleic acid sequences using standard cloning techniques as described e.g. by Sambrook et al. 2001, Molecular Cloning: A Laboratory Manual. Such a protein may be produced, e.g., in recombinant DNA expression systems. Such fusion proteins according to the present invention can be obtained by fusing the nucleic acids for endolysin and the respective peptide stretch.

The fusion proteins according to the present invention may be fused or linked to other additional proteins. Example for this other additional protein is thioredoxin.

The present invention further relates to an isolated nucleic acid molecule encoding the fusion protein according to the present invention. The present invention further relates to a vector comprising the nucleic acid molecule according to the present invention. Said vector may provide for the constitutive or inducible expression of said fusion protein according to the present invention.

The invention also relates to a method for obtaining said fusion proteins from a micro-organism, such as a genetically modified suitable host cell which expresses said fusion proteins. Said host cell may be a micro-organism such as bacteria or yeast or an animal cell as e.g. a mammalian cell, in particular a human cell. In one embodiment of the present invention the host cell is a *Pichia pastoris* cell. The host may be selected due to mere biotechnological reasons, e.g. yield, solubility, costs, etc. but may be also selected from a medical point of view, e.g. a non-pathological bacteria or yeast, human cells. Another aspect of the present invention is related to a method for genetically transforming a suitable host cell in order to

obtain the expression of the fusion proteins according to the invention wherein the host cell is genetically modified by the introduction of a genetic material encoding said fusion proteins into the host cell and obtain their translation and expression by genetic engineering methods well known by the man skilled in the art.

In a further aspect the present invention relates to a composition, preferably a pharmaceutical composition, comprising a fusion protein according to the present invention and/or a host transformed with a nucleic acid molecule or a vector comprising a nucleotide sequence encoding a fusion protein according to the present invention.

The present invention also relates to a fusion protein according to the present invention and/or a host transformed with a nucleic acid comprising a nucleotide sequence encoding a fusion protein according to the present invention for use as a medicament. In a further aspect the present invention relates to the use of a fusion protein according to the present invention and/or a host transformed with a vector comprising a nucleic acid molecule comprising a nucleotide sequence encoding a modified, fusion protein according to the present invention in the manufacture of a medicament for the treatment and/or prevention of a disorder, disease or condition associated with Gram-positive bacteria. In particular the treatment and/or prevention of the disorder, disease or condition may be caused by Gram-positive bacteria of bacterial groups, families, genera or species comprising strains pathogenic for humans or animals like *Listeria monocytogenes, Staphylococcus aureus, Enterococcus faecalis, Enterococcus faecium, Streptococcus pneumoniae, Streptococcus pyogenes, Streptococcus mutans, Streptococcus equi, Clostridium difficile, Clostridium botulinum, Clostridium tetani, Clostridium perfringens, Bacillus anthracis, Bacillus cereus, Propionibacterium acnes, Mycobacterium avium, Mycobacterium tuberculosis, Corynebacterium diphteriae, Mycoplasma pneumoniae, Actinomyces.*

The present invention further relates to a medicament comprising a fusion protein according to the present invention and/or a host transformed with a nucleic acid comprising a nucleotide sequence encoding a fusion protein according to the present invention.

In a further aspect the present invention relates to a method of treating a disorder, disease or condition in a subject in need of treatment and/or prevention, which method comprises administering to said subject an effective amount of a fusion protein according to the present

invention and/or an effective amount of a host transformed with a nucleic acid comprising a nucleotide sequence encoding a fusion protein according to the present invention or a composition according to the present invention. The subject may be a human or an animal.

In particular said method of treatment may be for the treatment and/or prevention of infections of the skin, of soft tissues, the respiratory system, the lung, the digestive tract, the eye, the ear, the teeth, the nasopharynx, the mouth, the bones, the vagina, of wounds of bacteraemia and/or endocarditis caused by Gram-positive bacteria, in particular by the Gram-positive bacteria as listed above.

The dosage and route of administration used in a method of treatment (or prophylaxis) according to the present invention depends on the specific disease/site of infection to be treated. The route of administration may be for example oral, topical, nasopharyngeal, parenteral, intravenous, rectal or any other route of administration.

For application of a fusion protein according to the present invention and/or an effective amount of a host transformed with a nucleic acid comprising a nucleotide sequence encoding a fusion protein according to the present invention or a composition according to the present invention to a site of infection (or site endangered to be infected) a formulation may be used that protects the active compounds from environmental influences such as proteases, oxidation, immune response etc., until it reaches the site of infection. Therefore, the formulation may be capsule, dragee, pill, powder, suppository, emulsion, suspension, gel, lotion, cream, salve, injectable solution, syrup, spray, inhalant or any other medical reasonable galenic formulation. Preferably, the galenic formulation may comprise suitable carriers, stabilizers, flavourings, buffers or other suitable reagents. . For example, for topical application the formulation may be a lotion, cream, gel, salve or plaster, for nasopharyngeal application the formulation may be saline solution to be applied via a spray to the nose. For oral administration in case of the treatment and/or prevention of a specific infection site e.g. in the intestine, it can be necessary to protect a fusion protein according to the present invention from the harsh digestive environment of the gastrointestinal tract until the site of infection is reached. Thus, bacteria as carrier, which survive the initial steps of digestion in the stomach and which secret later on a fusion protein according to the present invention into the intestinal environment can be used.

In a specific embodiment of the present invention the use of a fusion protein according to the present invention and/or a host transformed with a vector comprising a nucleic acid molecule comprising a nucleotide sequence encoding a fusion protein according to the present invention in the manufacture of a medicament for the treatment and/or prevention of a disorder, disease or condition caused by *Listeria monocytogenes,* in particular Granulomatosis infantiseptica (listeriosis of newborns), mononucleosis, conjunctivitis, meningitis, granulomatosis septica and the listeriosis of pregnant women.

In another specific embodiment of the present invention the disorder, disease or condition is caused by *Staphylococcus aureus*, in particular infections of the skin like pyoderma, particularly folliculitis, furuncle, carbuncle, abscesses of the sweat glands and pemphigus, and like scaled skin syndrome. The scaled skin syndrome can appear in three clinical pictures: dermatitis exfoliativa, impetigo bullosa and scarlatiniform erythroderma. Moreover the disorder, disease or condition caused by *Staphylococcus aureus* is *Staphylococcus* pneumonia, hospitalism, in particular surgical wound infections, mastitis puerperalis and enterokolitis, and food poisonings.

In another specific embodiment of the present invention the disorder, disease or condition is caused by *Streptococcus pyogenes*, in particular tonsillitis, pharyngitis, scarlet, erysipelas, rheumatic fever and acute glomerulonephritis.

In another specific embodiment of the present invention the disorder, disease or condition is caused by Streptococcus pneumoniae, in particular pneumonia, ulcus serpens corneae, otitis media, meningitis, peritonitis, mastoiditis and osteomyelitis.

In another specific embodiment of the present invention the disorder, disease or condition is caused by *Clostridium perfringens*, in particular gas gangrene, enteritis necroticans ulcerosa and food poisonings.

In another specific embodiment of the present invention the disorder, disease or condition is caused by *Clostridium botulinum*, in particular botulism.

In another specific embodiment of the present invention the disorder, disease or condition is caused by *Clostridium difficile*, in particular pseudomembranoes enterokolitis.

In another specific embodiment of the present invention the disorder, disease or condition is caused by *Bacillus anthracis*, in particular cutaneous anthrax, inhalation anthrax, and gastrointestinal anthrax.

In another specific embodiment of the present invention the disorder, disease or condition is caused by *Enterococcus faecalis* or *E. faecium*, like nosokomial infections, and endokarditis.

In another specific embodiment of the present invention the disorder, disease or condition is caused by *Bacillus cereus*, in particular food poisonings, bronchial pneumonia, septicaemia and meningitis.

In another specific embodiment of the present invention the disorder, disease or condition is caused by *Mycobacterium avium, Mycobacterium paratuberculosis* and *Mycobacterium tuberculosis*, in particular tuberculosis.

In another specific embodiment of the present invention the disorder, disease or condition is caused by *Mycoplasma pneumoniae*, in particular pneumonia, diseases of the upper respiratory tract and inflammations of the ear drum.

In another specific embodiment of the present invention the disorder, disease or condition is caused by *Actinomyces*, in particular actinomycosis in human, cattle, cat and dog.

In another specific embodiment of the present invention the disorder, disease or condition is caused by *Corynebacterium diphteriae*, in particular localized diphtheria of the tonsils, the nose, the nasopharynx or the middle ear, progressive diphtheria of the larynx, the trachea and the bronchi, toxic or maligne diphtheria, skin and wound diphtheria.

Preferably, a fusion protein according to the present invention is used for medical treatment, if the infection to be treated (or prevented) is caused by multiresistant bacterial strains, in particular by strains resistant against one or more of the following antibiotics: streptomycin, tetracycline, cephalothin, penicillin, gentamicin, cefotaxime, cephalosporin, ceftazidime or imipenem. Furthermore, a fusion protein according to the present invention can be used in

methods of treatment by administering it in combination with conventional antibacterial agents, such as antibiotics, lantibiotics, bacteriocins or endolysins, etc.

The present invention also relates to a pharmaceutical pack comprising one or more compartments, wherein at least one compartment comprises one or more fusion protein according to the present invention and/or one or more hosts transformed with a nucleic acid comprising a nucleotide sequence encoding a fusion protein according to the present invention or a composition according to the present invention,

In another aspect the present invention relates to a process of preparation of a pharmaceutical composition, said process comprising admixing one or more fusion protein according to the present invention and/or one or more hosts transformed with a nucleic acid comprising a nucleotide sequence encoding a fusion protein according to the present invention with a pharmaceutically acceptable diluent, excipient or carrier.

In an even further aspect the composition according to the present invention is a cosmetic composition. Several bacterial species can cause irritations on environmentally exposed surfaces of the patient's body such as the skin. In order to prevent such irritations or in order to eliminate minor manifestations of said bacterial pathogens, special cosmetic preparations may be employed, which comprise sufficient amounts of the fusion protein according to the present invention in order to degrade already existing or freshly settling pathogenic Gram-positive bacteria.

In a further aspect the present invention relates to the fusion protein according to the present invention for use as diagnostic means in medicinal, food or feed or environmental diagnostics, in particular as a diagnostic means for the diagnostic of bacteria infection caused in particular by Gram-positive bacteria. In this respect the fusion protein according to the present invention may be used as a tool to specifically degrade pathogenic bacteria, in particular Gram-positive pathogenic bacteria. The degradation of the bacterial cells by the fusion protein according to the present invention can be supported by the addition of detergents like Triton X-100 or other additives which weaken the bacterial cell envelope like polymyxin B. Specific cell degradation is needed as an initial step for subsequent specific detection of bacteria using nucleic acid based methods like PCR, nucleic acid hybridization or NASBA (Nucleic Acid Sequence Based Amplification), immunological methods like IMS, immunofluorescence or

ELISA techniques, or other methods relying on the cellular content of the bacterial cells like enzymatic assays using proteins specific for distinct bacterial groups or species (e.g. β-galactosidase for enterobacteria, coagulase for coagulase positive strains).

In a further aspect the present invention relates to the use of the fusion protein according to the present invention for the treatment or prevention of Gram-positive bacterial contamination of foodstuff, of food processing equipment, of food processing plants, of surfaces coming into contact with foodstuff such as shelves and food deposit areas and in all other situations, where pathogenic, facultative pathogenic or other undesirable bacteria can potentially infest food material, of medical devices and of all kind of surfaces in hospitals and surgeries.

In particular, a fusion protein of the present invention may be used prophylactically as sanitizing agent. Said sanitizing agent may be used before or after surgery, or for example during hemodialysis. Moreover, premature infants and immunocompromised persons, or those subjects with need for prosthetic devices may be treated with a fusion protein according to the present invention. Said treatment may be either prophylactically or during acute infection. In the same context, nosocomial infections, especially by antibiotic resistant strains like *Methicillin-resistant Staphylococcus aureus, Vancomycin-resistant Enterococcus faecalis, Vancomycin-resistant Enterococcus faecium, Streptococcus pneumoniae, Propionibacterium acnes, multidrug-resistant Mycobacterium tuberculosis,* may be treated prophylactically or during acute phase with a fusion protein of the present invention. Therefore, a fusion protein according to the present invention may be used as a disinfectant also in combination with other ingredients useful in a disinfecting solution like detergents, tensids, solvents, antibiotics, lanthibiotics, or bacteriocins.

For the use of the fusion protein according to the present invention as a disinfectant e.g. in hospital, dental surgery, veterinary, kitchen or bathroom, the fusion protein can be prepared in a composition in form of e.g. a fluid, a powder, a gel, or an ingredient of a wet wipe or a disinfection sheet product. Said composition may additionally comprise suitable carrier, additives, diluting agents and/or excipients for its respective use and form, respectively, - but also agents that support the antimicrobial activity like EDTA or agents enhance the antimicrobial activity of the fusion proteins. The fusion protein may also be used with common disinfectant agents like, Alcohols, Aldehydes, Oxidizing agents, Phenolics, Quaternary ammonium compounds or UV-light. For disinfecting for example surfaces,

objects and/or devices the fusion protein can be applied on said surfaces, objects and/or devices. The application may occur for instance by wetting the disinfecting composition with any means such as a cloth or rag, by spraying, pouring. The fusion proteins may be used in varying concentration depending on the respective application and the „reaction time“ intended to obtain full antimicrobial activity.

Further scope of applicability of the present invention will become apparent from the detailed description given hereinafter, however, it should be understood that the detailed description and specific examples, while indicating preferred embodiments of the invention, are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description. It is to be understood that both the foregoing general description and the following detailed description are exemplary and explanatory only and are not restrictive of the invention, as claimed.

The following examples explain the present invention but are not considered to be limiting. Unless indicated differently, molecular biological standard methods were used, as e.g., described by Sambrock et al., 1989, Molecular Cloning: A Laboratory Manual, 2nd edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

EXAMPLE 1: Cloning, expression and purification of Cpl-1, Ply511, LysK, Lysostaphin (Lss) and PA6-gp20 enzymes modified with various peptide stretches on the N-terminus or the C-terminus.

Enzymes

Cpl-1 according to SEQ ID NO: 57 is an endolysin originating from *Streptococcus pneumoniae* phage Cpl-1. The endolysin Cpl-1 is encoded by the nucleic acid molecule according to SEQ ID NO: 91. The nucleic acid molecule according to SEQ ID NO: 91 was synthetically produced with a BamH I (5´-GGA TCC-3´) restriction site at the 5´-end of the nucleic acid molecule and an Xho I (5´-CTC GAG-3´) restriction site at the 3´-end of the nucleic acid molecule.

Ply511 according to SEQ ID NO: 58 is an endolysin originating from *Listeria monocytogenes* phage A511. The endolysin Ply511 is encoded by the nucleic acid molecule according to SEQ

ID NO: 92. The nucleic acid molecule according to SEQ ID NO: 92 was synthetically produced with a BamH I (5´-GGA TCC-3´) restriction site at the 5´-end of the nucleic acid molecule and an Xho I (5´-CTC GAG-3´) restriction site at the 3´-end of the nucleic acid molecule.

LysK according to SEQ ID NO: 59 is an endolysin originating from *Staphylococcus aureus* phage K. The endolysin LysK is encoded by the nucleic acid molecule according to SEQ ID NO: 93. The nucleic acid molecule according to SEQ ID NO: 93 was synthetically produced with a BamH I (5´-GGA TCC-3´) restriction site at the 5´-end of the nucleic acid molecule and an Xho I (5´-CTC GAG-3´) restriction site at the 3´-end of the nucleic acid molecule.

Lysostaphin (Lss) according to SEQ ID NO: 60 is a bacteriocin originating from *Staphylococcus simulans*. The bacteriocin Lss is encoded by the nucleic acid molecule according to SEQ ID NO: 94. The nucleic acid molecule according to SEQ ID NO: 94 was synthetically produced with a BamH I (5´-GGA TCC-3´) restriction site at the 5´-end of the nucleic acid molecule and an Xho I (5´-CTC GAG-3´) restriction site at the 3´-end of the nucleic acid molecule.

PA6-gp20_according to SEQ ID NO: 61 is an endolysin originating from *Propionibacterium acnes* phage. The endolysin PA6-gp20 is encoded by the nucleic acid molecule according to SEQ ID NO: 123. The nucleic acid molecule according to SEQ ID NO: 123 was synthetically produced with a BamH I (5´-GGA TCC-3´) restriction site at the 5´-end of the nucleic acid molecule and an Xho I (5´-CTC GAG-3´) restriction site at the 3´-end of the nucleic acid molecule.

The following peptide stretches in table 5 were used for production of fusion proteins with the enzymes Cpl-1, Ply511, LysK, Lysostaphin (Lss) and PA6-gp20:

Table 5 :

Peptide stretch	Nucleic acid molecule encoding the peptide stretch
Pseudin 1 (SEQ ID NO:51)	SEQ ID NO: 95
WLBU2-Variant	SEQ ID NO: 96

(SEQ ID NO:55)	
LL-37 (SEQ ID NO: 5)	SEQ ID NO: 97
Indolicidin (SEQ ID NO: 7)	SEQ ID NO: 98
Magainin (SEQ ID NO:4)	SEQ ID NO: 99
Pleurocidin (SEQ ID NO:1)	SEQ ID NO: 100
Cecropin A (A. aegypti) (SEQ ID NO:9)	SEQ ID NO:101
Buforin II (SEQ ID NO:3)	SEQ ID NO: 102
Sarcotoxin IA (SEQ ID NO:11)	SEQ ID NO: 103
PK (SEQ ID NO:13)	SEQ ID NO:104
Pentapeptide (SEQ ID NO:12)	SEQ ID NO:105
PK2 (SEQ ID NO: 31)	SEQ ID NO:106

The nucleic acid molecules encoding the respective peptide stretches were synthetically produced with a Nde I (5´-CAT ATG-3´) restriction site at the 5´-end of the nucleic acid molecule and a BamH I (5´-GGA TCC-3´) restriction site at the 3´-end of the nucleic acid molecule, except the nucleic acid molecule encoding the PK and PK2 for ligation with the bacteriocin Lss, which was produced with a Nco I restriction site plus two additional nucleotides (5´-CCA TGG GC-3´) at the 5´-end of the nucleic acid molecule.

Fusion proteins are constructed by linking at least two nucleic acid sequences using standard cloning techniques as described e.g. by Sambrook et al. 2001, Molecular Cloning: A Laboratory Manual. Therefore the nucleic acid molecules encoding the peptide stretches were cleaved in a digest with the respective restriction enzymes Nde I and BamH I and in case of the nucleic acid molecule encoding the peptide stretch PK and PK2 for ligation with the Lss the digest was performed with the restriction enzymes Nco I and BamH I. Subsequently the cleaved nucleic acids encoding the peptide stretches were ligated into the pET21 b expression vector (Novagen, Darmstadt, Germany), which was also cleaved in a digest with the respective restriction enzymes Nde I and BamH I before. The cleaved nucleic acid molecule

encoding the peptide stretch PK and PK2 for ligation with Lss was ligated into a modified pET32 b expression vector (unmodified vector obtainable from Novagen, Darmstadt, Germany), which was also cleaved in a digest with the respective restriction enzymes Nco I and BamH I before. The modification of the pET32b expression vector refers to the deletion of the sequence encoding a S-tag and the central His_6-tag.

Afterwards, the nucleic acid molecules encoding the enzymes Cpl-1, Ply511, PA6-gp20, LysK and Lss were cleaved in a digest with the restriction enzyme BamH I and Xho I, so that the endolysin could be ligated into the pET21b expression vector (Novagen, Darmstadt, Germany) and the modified pET32 b expression vector, respectively, which were also cleaved in a digest with the respective restriction enzymes BamH I and Xho I before.

In the case of the peptide stretch PK, which was ligated to the C-terminus of the Lysostaphin and the LysK, the resulting fusion protein has a His_6-tag on the N-terminus, wherein the His_6-tag is linked to the N-terminus by a linker. For the cloning of the respective nucleic acid molecules the pET32 b expression vector (Novagen, Darmstadt, Germany) was used.

Thus, the nucleic acid molecule encoding the peptide stretch is ligated into the respective vector at the 5´-end of the nucleic acid molecule encoding the respective enzyme. Moreover, the nucleic acid molecule encoding the respective enzyme is ligated into the respective plasmid, so that a nucleic acid molecule encoding a His_6-tag consisting of six histidine residues is associated at the 3´-end of the nucleic acid molecule encoding the endolysin.

As some fusion proteins may either be toxic upon expression in bacteria, or not homogenous due to protein degradation, the strategy might be to express these fusion proteins fused or linked to other additional proteins. Example for these other additional protein is thioredoxin, which was shown to mediate expression of toxic antimicrobial peptides in *E.coli* (TrxA mediating fusion expression of antimicrobial peptide CM4 from multiple joined genes in Escherichia coli. Zhou L, Zhao Z, Li B, Cai Y, Zhang S. Protein Expr Purif. 2009 Apr;64(2):225-230). In the case of the fusion protein consisting of the N-terminal PK or PK2 peptide and the bacteriocin Lss, the peptide was ligated into the modified pET32 b expression vector, so that an additional thioredoxin is associated at the 5´-end of the peptide. The thioredoxin could be removed from the expressed fusion protein by the use of enterokinase,

therefore between the nucleic acid molecule encoding the peptide and the one encoding the thioredoxin is an enterokinase restriction site introduced.

The sequence of the endolysin-peptide-fusions was controlled via DNA-sequencing and correct clones were transformed into E.coli BL21(DE3) and in *E. coli* BL21 (DE3) pLysS cells (Novagen, Darmstadt, Germany) for protein expression.

Recombinant expression of the fusion proteins according to SEQ ID NO: 107 to 122 and 124 is performed in *E. coli* BL21 (DE3) and *E. coli* BL21 (DE3) pLysS cells (Novagen, Darmstadt, Germany). The cells were growing until an optical density of OD600nm of 0.5-0.8 was reached. Then the expression of the fusion protein was induced with 1 mM IPTG (isopropylthiogalactoside) and the expression was performed at 37°C for a period of 4 hours.

E.coli BL21 cells were harvested by centrifugation for 20 min at 6000g and disrupted via sonication on ice. Soluble and insoluble fraction of the *E.coli* crude extract were separated by centrifugation (Sorvall, SS34, 30 min, 15 000 rpm). All proteins were purified by Ni^{2+} affinity chromatography (Akta FPLC, GE Healthcare) using the C-terminal His_6-tag, encoded by the pET21b or pET32b vectors.

Some proteins were expressed using a modified pET32b vector (S-tag and central His_6-tag deleted) as described above, which fuses thioredoxin on the N-terminus of the proteins of interest. The vector also contains an enterokinase cleavage site right before the protein of interest. This site allows the proteolytic cleavage between thioredoxin and the protein of interest, which can purified via the remaining C-terminal His_6-tag. For antimicrobial function of the fusion protein it may be necessary to remove the thioredoxin by proteolytic cleavage. Therefore the fusion protein was cleaved with 2-4 units/mg recombinant enterokinase (Novagen, Darmstadt, Germany) to remove the thioredoxin following the protocol provided by the manufacturer. After enterokinase cleavage the fusion protein was purified via His_6-tag purification as described below.

The Ni^{2+} affinity chromatography is performed in 4 subsequent steps, all at room temperature:

1. *Equilibration* of the *Histrap FF 5 ml* column (GE Healthcare) with up to 10 column volumes of Washing Buffer (20 mM imidazole, 1 M NaCl and 20 mM Hepes on pH 7.4) at a flow rate of 3-5 ml/min.
2. *Loading* of the total lysate (with wanted fusion protein) on the *Histrap FF 5 ml* column at a flow rate of 3-5 ml/min.
3. Washing of the column with up to 10 column volumes of Washing Buffer to remove unbound sample followed by a second washing step with 10% Elution buffer (500 mM imidazole, 0.5 M NaCl and 20 mM Hepes on pH 7.4)at a flow rate of 3-5 ml/min.
4. Elution of bounded fusion proteins from the column with a linear gradient of 4 column volumes of Elution Buffer (500 mM imidazole, 0.5 M NaCl and 20 mM Hepes on pH 7.4) to 100% at a flow rate of 3-5 ml/min.

Purified stock solutions of fusion proteins in Elution Buffer (20 mM Hepes pH 7.4; 0.5 M NaCl; 500 mM imidazole) were at least 90% pure as determined visually on SDS-PAGE gels (data not shown).

EXAMPLE 2: Antimicrobial activity of Cpl-1 enzymes modified with various peptide stretches on the N-terminus.

The fusion proteins Cpl 1 with the N-terminal peptide stretches Pseudin 1, WLBU2-Variant, LL-37, Indolicidin, Magainin, Pleurocidin, Cecropin A (A. aegypti), Buforin II, Sarcotoxin IA and PK were produced as described in example 1. The antimicrobial activity of said fusion protein against *Streptococcus pneumoniae* DSMZ 11967 and *Streptococcus pneumoniae* DSMZ 14378 were tested by using the plating test described below. The measured activity of the fusion protein is shown in Table 6.

The results presented in Table 6 show high antimicrobial activity of all fusion proteins against *Streptococcus pneumoniae* DSMZ 11967 and *Streptococcus pneumoniae* DSMZ 14378.

Plating assay:
Exponentially growing cells of e.g. Streptococci, Listeria, Propionibacteria or Staphylococci were taken (1ml) cooled on ice and washed with distilled water. The bacteria were

resuspended in 20mM Tris pH 7.0, 1 mM $MgCl_2$, 0.5 M Saccharose. Fusion proteins were diluted in resuspension buffer, adding sucrose to a final concentration of 0.5 M and incubated (final concentration of the fusion protein about 10µg/ml) with the respective bacteria for 60 minutes at room temperature. After that bacteria were plated on appropriated agar plates (e.g. Streptococci: Columbia blood agar) containing 0.5 M sucrose and the resulting colonies were counted after incubation.

The residual colonies were counted after an overnight incubation at 37°C. Based on the counted cell numbers the antibacterial activity as logarithmic units ($=\log_{10}N_0/N_i$ with N_0 = number of untreated cells and N_i = number of treated cells) was calculated. All samples were replicated at least in four fold.

Table 6:

Fusion protein	Enzyme part	Peptide stretch (N-terminal unless otherwise indicated)	Activity against *Streptococcus pneumoniae* DSMZ 11967	Activity against *Streptococcus pneumoniae* DSMZ 14378
SEQ ID NO:107	Cpl-1 (SEQ ID NO:57)	Pseudin 1 (SEQ ID NO:51)	+++	+++
SEQ ID NO:108	Cpl-1 (SEQ ID NO:57)	WLBU2-Variant (SEQ ID NO:55)	++	++
SEQ ID NO:109	Cpl-1 (SEQ ID NO:57)	LL-37 (SEQ ID NO:5)	+++	+++
SEQ ID NO:110	Cpl-1 (SEQ ID NO:57)	Indolicidin (SEQ ID NO:7)	+++	+++
SEQ ID NO:111	Cpl-1 (SEQ ID NO:57)	Magainin (SEQ ID NO:4)	+++	+++
SEQ ID NO:112	Cpl-1 (SEQ ID NO:57)	Pleurocidin (SEQ ID NO:1)	+++	+++
SEQ ID NO:113	Cpl-1 (SEQ ID NO:57)	Cecropin A (A. aegypti) (SEQ ID NO:9)	+++	+++
SEQ ID NO:114	Cpl-1 (SEQ ID NO:57)	Buforin II (SEQ ID NO:3)	+++	+++
SEQ ID NO:115	Cpl-1 (SEQ ID NO:57)	Sarcotoxin IA (SEQ ID NO:11)	+++	+++
SEQ ID NO:116	Cpl-1 (SEQ ID NO:57)	PK (SEQ ID NO:13)	+++	+++

Abbreviations: +: 1 log; ++: 2-3 log; +++: 4 or more logs.

EXAMPLE 3: Antimicrobial activity of Ply511 enzyme modified with the pentapeptide on the N-terminus.

The fusion protein Ply511 with the N-terminal peptide stretch pentapeptide according to SEQ ID NO: 12 was produced as described in example 1. The antimicrobial activity of said fusion protein against *Listeria monocytogenes* DSMZ 15675 and *Listeria monocytogenes* DSMZ 20600 was tested by using the plating test described in example 2. The measured activity of the fusion protein is shown in Table 7.

The results presented in Table 7 show high antimicrobial activity of the fusion protein pentapeptide: Ply511 against *Listeria monocytogenes* DSMZ 15675 and *Listeria monocytogenes* DSMZ 20600.

Table 7:

Fusion protein	Enzyme part	Peptide stretch (N-terminal unless otherwise indicated)	Activity against *Listeria monocytogenes* DSMZ 15675	Activity against *Listeria monocytogenes* DSMZ 20600
SEQ ID NO:117	Ply511 (SEQ ID NO:58)	Pentapeptid (SEQ ID NO:12)	+++	+++

Abbreviations: +: 1 log; ++: 2-3 log; +++: 4 or more logs.

EXAMPLE 4: Antimicrobial activity of Lss and LysK enzyme modified with polycationic peptides on the N-terminus or C-terminus.

The fusion proteins Lss and LysK, respectively, with the N-terminal peptide stretch PK according to SEQ ID NO: 13, the fusion protein Lss with the N-terminal peptide stretch PK2 according to SEQ ID NO:31, as well as the fusion proteins Lss and LysK, respectively, with the C-terminal peptide stretch PK were produced as described in example 1. The antimicrobial activity of said fusion proteins against *Staphylococcus aureus* DSMZ 346 and

Staphylococcus epidermidis DSMZ 20041 was tested by using the plating test described in example 2, as well as by using the lysis test as described in the following.

Lysis test

The Lysis test was used for the modified LysK and Lysostaphins to examine the antimicrobial effect of these fusion proteins.

Staphylococcal cells of were grown in BHI medium until and optical density at 600nm of 0.7-1 was reached indicating exponential growth. Cells were harvested by centrifugation and resuspended in lysis buffer (20 mM Tris-HCl (pH 7.4), 60 mM NaCl, 2 mM $CaCl_2$. Cells were resuspended at an optical density at 600nm of 1.0 and incubated with fusion proteins. Activity was measured spectrophotometrically at 600nm.

The measured activity of the fusion protein is shown in Table 8.

The results presented in Table 8 show high antimicrobial activity of the fusion proteins Lss with the N-terminal peptide PK or PK2 against *Staphylococcus aureus* DSMZ 346 and*Staphylococcus epidermidis* DSMZ 20041. But also the other fusion proteins show antimicrobial activity against the two tested bacterial strains.

Table 8

Fusion protein	Enzyme part	Peptide stretch (N-terminal unless otherwise indicated)	Activity against *Staphylococcus aureus* DSMZ 346	Activity against *Staphylococcus epidermidis* DSMZ 20041
SEQ ID NO:118	LysK (SEQ ID NO:59)	PK (SEQ ID NO:13)	+	+
SEQ ID NO:119	Lysostaphin (SEQ ID NO:60)	PK (SEQ ID NO:13)	+++	+++
SEQ ID NO:120	Lysostaphin (SEQ ID NO:60)	PK2 (SEQ ID NO:31)	+++	+++
SEQ ID NO:121	LysK (SEQ ID NO:59)	PK (C-terminal) (SEQ ID NO:13)	+	+
SEQ ID NO:122	Lysostaphin (SEQ ID NO:60)	PK (C-terminal) (SEQ ID NO:13)	+	+

Abbreviations: +: 1 log; ++: 2-3 log; +++: 4 or more logs.

EXAMPLE 5: Antimicrobial activity of PA6-gp20 enzyme modified with the hydrophobic peptide stretch Walmagh 1

The fusion protein PA6-gp20 with the N-terminal peptide stretch Walmagh 1 according to SEQ ID NO: 56 was produced as described in example 1. The antimicrobial activity of said fusion protein against *Propionibacterium acnes* DSMZ 1897 and *Propionibacterium acnes* DSMZ 16379 was tested by using the plating test described in example 2. The measured activity of the fusion protein is shown in Table 9.

The results presented in Table 9 show antimicrobial activity of the fusion protein against both bacterial strains of *Propionibacterium acnes*.

Table 9:

Fusion protein	Enzyme part	Peptide stretch (N-terminal unless otherwise indicated)	Activity against *Propionibacterium acnes* DSMZ 1897	Activity against *Propionibacterium acnes* DSMZ 16379
SEQ ID NO: 124	PA6-gp20 (SEQ ID NO:61)	Walmagh 1 (SEQ ID NO:56)	++	++

Abbreviations: ++: 2-3 log;

The fusion proteins in Table 6 to 9 without any tag and linker were also tested with the activity assays described above. They all showed antimicrobial activity against the used bacterial strains in Table 6 to 9.

CLAIMS

1. A fusion protein composed of an enzyme having the activity of degrading the cell wall of Gram-positive bacteria and a peptide stretch fused to the enzyme at the N- or C-terminus or at both termini.

2. The fusion protein according to claim 1, wherein said fusion protein comprises an additional amino acid residue on the N-terminus.

3. The fusion protein according to claim 1 or 2, wherein said fusion protein exhibits an amino acid sequence according to SEQ ID NO: 63 to 90.

4. The fusion protein according to any of the preceding claims, wherein said fusion protein comprises a tag or additional protein on the C- and/or N-terminus.

5. The fusion protein according to claim 4, wherein said tag or additional protein is linked to the fusion protein by one or more additional amino acid residues.

6. The fusion protein according to any of the preceding claims, wherein the peptide stetch is linked to the fusion protein by one or more additional amino acid residues.

7. The fusion protein according to claim 1, wherein the enzyme is an endolysin, autolysin or a bacteriocin.

8. The fusion protein according to claim 7, wherein the enzyme exhibits an amino acid sequence according to SEQ ID NO: 57 to 61.

9. The fusion protein according to any of the preceding claims, wherein the Gram-positive bacteria are selected from the group consisting of the bacteria listed in table 1.

10. The fusion protein according to any one of the preceding claims, wherein the peptide stretch is a cationic, more preferably a polycationic peptide, amphipatic peptide, sushi peptide, defensin, hydrophobic peptide or an antimicrobial peptide.

11. The fusion protein according to claim 10, wherein the sushi peptide exhibits an amino acid sequence according to SEQ ID NO: 54.

12. The fusion protein according to claim 10, wherein the cationic peptide comprises at least one amino acid residue selected out of the group consisting of arginine, histidine and lysine residues.

13. The fusion protein according to claim 10, wherein the antimicrobial peptide exhibits an amino acid sequence according to SEQ ID NO:1 to 11 or 48 to 53.

14. The fusion protein according to claim 10, wherein the hydrophobic peptide exhibits an amino acid sequence according to SEQ ID NO: 12, 50, 55 or 56.

15. The fusion protein according to claim 10, wherein at least about 70% of the amino acid residues comprised in said cationic peptide are either arginine, histidine or lysine residues, or wherein at least about 70% of the amino acid residues comprised in said peptide are either arginine or lysine, or wherein the amino acid residues comprised in said peptide are either arginine or lysine residues.

16. The fusion protein according to claim 15, wherein the cationic peptide exhibits an amino acid sequence according to SEQ ID NO: 13 or 24 to 44.

17. The fusion protein according to claim 10, wherein the amphipatic peptide comprises at least one positively charged amino acid residues selected out of the group consisting of lysine, arginine and histidine residues, combined to at least one hydrophobic amino acid residue selected out of the group consisting of valine, isoleucine, leucine, methionine, phenylalanine, tryptophan, cysteine, alanine, tyrosine, histidine, threonin, serine, proline and glycine residues.

18. The fusion protein according to claim 17, wherein at least about 70% of the said amino acid residues in said amphipatic peptide are either arginine or lysine residues and at least about 30% of the said amino acid residues in said amphipatic peptide are valine, isoleucine, leucine, methionine, phenylalanine, tryptophan, cysteine, alanine, tyrosine, histidine, threonin, serine, proline or glycine residues.

19. The fusion protein according to claim 18, wherein the amphipatic peptide exhibits an amino acid sequence according to SEQ ID NO: 1 to 5, 23, 48 or 49.

20. The fusion protein according to any one of claims 1 to 19, wherein the peptide stretch comprises about 5 to about 100 amino acid residues, in particular about 5 to 50 amino acid residues, in particular about 5 to 30 amino acid residues.

21. An isolated nucleic acid molecule encoding a fusion protein according to any of claims 1 to 19.

22. A vector comprising the nucleic acid molecule according to claim 21.

23. A host cell comprising the nucleic acid molecule according to claim 21 or the vector according to claim 122.

24. A host cell according to claim 23, wherein the cell is a bacterial cell or a yeast cell.

25. A host cell according to claim 24, wherein the yeast cell is *Pichia pastoris* cell.

26. The fusion protein according to any one of claims 1 to 20 for use as a medicament, diagnostic means or cosmetic substance.

27. The fusion protein according to any one of claims 1 to 20 for use as a medicament for treatment or prevention of Gram-positive bacterial infections.

28. The fusion protein according to any one of the claims 1 to 20 for use as a disinfectant.

29. The use of the fusion protein according to any one of claims 1 to 20 for the treatment or prevention of Gram-positive bacterial contamination of foodstuff, of food processing equipment, of food processing plants, of surfaces coming into contact with foodstuff, of medical devices, of surfaces in hospitals and surgeries.

30. The use of the fusion protein according to any one of claims 1 to 20 as a diagnostic means in medicinal, food or feed or environmental diagnostics.

31. Pharmaceutical composition comprising a fusion protein according to any one of claims 1 to 20.

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2010/059152

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N9/36 C12N9/52

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 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, BIOSIS, WPI Data, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	WO 2010/023207 A2 (UNIV LEUVEN KATH [BE]; BRIERS YVES [CH]; LAVIGNE ROB [BE]; VOLCKAERT G) 4 March 2010 (2010-03-04) the whole document	1-31
X	DONOVAN D M ET AL: "Peptidoglycan hydrolase fusions maintain their parental specificities" APPLIED AND ENVIRONMENTAL MICROBIOLOGY, AMERICAN SOCIETY FOR MICROBIOLOGY, US LNKD- DOI:10.1128/AEM.72.4.2988-2996.2006, vol. 72, no. 4, 1 April 2006 (2006-04-01), pages 2988-2996, XP002582772 ISSN: 0099-2240 abstract	1-10,12, 13,15, 17-31

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[X] Further documents are listed in the continuation of Box C.

[X] 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 document 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 another 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
19 August 2010	12/11/2010

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INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2010/059152

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ARIMA H ET AL: "Bactericidal action of lysozymes attached with various sizes of hydrophobic peptides to the C-terminal using genetic modification" FEBS LETTERS, ELSEVIER, AMSTERDAM, NL LNKD- DOI:10.1016/S0014-5793(97)01071-5, vol. 415, no. 1, 22 September 1997 (1997-09-22), pages 114-118, XP004261147 ISSN: 0014-5793 abstract	1-10,12, 13,15, 17-31
X	MANOHARADAS S ET AL: "Antimicrobial activity of a chimeric enzybiotic towards Staphylococcus aureus" JOURNAL OF BIOTECHNOLOGY, ELSEVIER SCIENCE PUBLISHERS, AMSTERDAM, NL LNKD- DOI:10.1016/J.JBIOTEC.2008.09.003, vol. 139, no. 1, 1 January 2009 (2009-01-01), pages 118-123, XP025796283 ISSN: 0168-1656 [retrieved on 2008-09-26] abstract	1-10,12, 13,15, 17-31
X	IBRAHIM H R ET AL: "Enhanced bactericidal action of lysozyme to Escherichia coli by inserting a hydrophobic pentapeptide into its C terminus" JOURNAL OF BIOLOGICAL CHEMISTRY, AMERICAN SOCIETY FOR BIOCHEMISTRY AND MOLECULAR BIOLOGY, INC, US, vol. 269, no. 7, 18 February 1994 (1994-02-18), pages 5059-5063, XP002579297 ISSN: 0021-9258 page 5059	1-31
X	DIAZ E ET AL: "CHIMERIC PHAGE-BACTERIAL ENZYMES A CLUE TO THE MODULAR EVOLUTION OF GENES" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES (PNAS), NATIONAL ACADEMY OF SCIENCE, US LNKD- DOI:10.1073/PNAS.87.20.8125, vol. 87, no. 20, 1 October 1990 (1990-10-01), pages 8125-8129, XP002477600 ISSN: 0027-8424 figures 1, 2 left-hand column, paragraph 1 - page 8127	1-10,12, 13,15, 17-31

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INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2010/059152

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	LOPEZ R ET AL: "Enzymes for anti-infective therapy: phage lysins" DRUG DISCOVERY TODAY: THERAPEUTIC STRATEGIES, ELSEVIER LNKD-DOI:10.1016/J.DDSTR.2004.09.002, vol. 1, no. 4, 1 December 2004 (2004-12-01), pages 469-474, XP004694307 ISSN: 1740-6773 figure 3 page 473, right-hand column	1-10,12, 13,15, 17-31
A	JADO ISABEL ET AL: "Phage lytic enzymes as therapy for antibiotic-resistant Streptococcus pneumoniae infection in a murine sepsis model." JOURNAL OF ANTIMICROBIAL CHEMOTHERAPY, vol. 52, no. 6, December 2003 (2003-12), pages 967-973, XP002597152 ISSN: 0305-7453 the whole document	1-10,12, 13,15, 17-31
A	CONLON ET AL: "Peptidomic analysis of skin secretions supports separate species status for the tailed frogs, Ascaphus truei and Ascaphus montanus" COMPARATIVE BIOCHEMISTRY AND PHYSIOLOGY. PART D: GENOMICS ANDPROTEOMICS, ELSEVIER, AMSTERDAM, NL LNKD-DOI:10.1016/J.CBD.2007.01.003, vol. 2, no. 2, 27 April 2007 (2007-04-27), pages 121-125, XP022052772 ISSN: 1744-117X the whole document	1-10,12, 13,15, 17-31

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2010/059152

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.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
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:

3. ☐ Claims Nos.:
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:

see additional sheet

1. ☐ As all required additional 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 fees, this Authority did not invite payment of additional fees.

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.:

1-10, 12, 13, 15, 17-31(all partially)

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.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

Invention: 1; Claims: 1-10, 12, 13, 15, 17-31(all partially)

Fusion protein P1-E1 according to SEQ ID NO: 63, comprising CPL-1 (SEQ ID NO:57) and ascaphin-5 (SEQ ID NO:49), see also page 17 of the description. Furthermore nucleic acids, vectors, host cells, uses and compositions relating thereto.

Inventions: 2-28; Claims: 1-31(partially)

As invention 1 but relating to SEQ ID NOs 64-90.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2010/059152

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2010023207 A2	04-03-2010	NONE	