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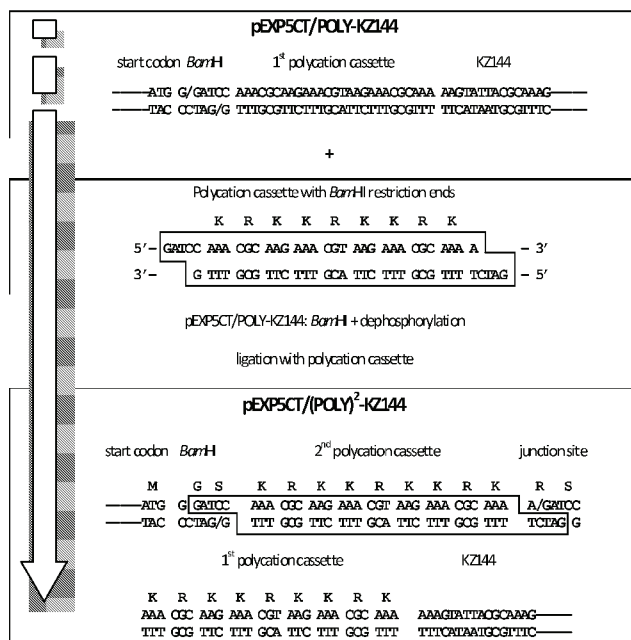
(54) Title: METHOD OF REDUCING BIOFILMS

Figure 1

(57) Abstract: The present invention relates to methods of eliminating, reducing or preventing bacterial biofilms by means of a fusion protein comprising an endolysin, an autolysin or a bacteriocin to which a peptide with membrane or LPS disrupting activity is fused. Further, the present invention relates to fusion proteins for use as a medicament, in particular for the treatment or prevention of Gram-negative and/or Gram-positive bacterial infections associated with bacterial biofilm, as diagnostic means, disinfectant or as cosmetic substance. The present invention also relates to the removal or reduction or prevention of Gram-negative and/or Gram-positive bacterial contamination associated with bacterial biofilm 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. Furthermore, the present invention relates to the use of said fusion protein as a diagnostic means in medicinal, food or feed or environmental diagnostic associated with bacterial biofilm.

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Method of Reducing Biofilms

The present invention relates to methods of eliminating, reducing or preventing bacterial biofilms by means of a fusion protein comprising an endolysin, an autolysin or a bacteriocin to which a peptide with membrane or LPS disrupting activity is fused. Further, the present invention relates to fusion proteins for use as a medicament, in particular for the treatment or prevention of Gram-negative and/or Gram-positive bacterial infections associated with bacterial biofilm, as diagnostic means, disinfectant or as cosmetic substance. The present invention also relates to the removal or reduction or prevention of Gram-negative and/or Gram-positive bacterial contamination associated with bacterial biofilm 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. Furthermore, the present invention relates to the use of said fusion protein as a diagnostic means in medicinal, food or feed or environmental diagnostic associated with bacterial biofilm.

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 β -(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 C1 endolysin towards group A streptococci (Nelson et al., 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. Nowadays, the most important challenge of endolysin therapy
5 lies in the insensitivity of Gram-negative bacteria towards the exogenous action of endolysins, since the outer membrane shields the access of endolysins from the peptidoglycan. This currently prevents the expansion of the range of effective endolysins to important Gram-negative pathogens.

10 Gram-negative bacteria possess an outer membrane, with its characteristic asymmetric bilayer as a hallmark. The outer membrane bilayer consists of an inner monolayer containing phospholipids (primarily phosphatidyl ethanolamine) and an outer monolayer that is mainly composed of a single glycolipid, lipopolysaccharide (LPS). There is an immense diversity of LPS structures in the bacterial kingdom and the LPS structure may be modified in response to
15 prevailing environmental conditions. The stability of the LPS layer and interaction between different LPS molecules is mainly achieved by the electrostatic interaction of divalent ions (Mg^{2+} , Ca^{2+}) with the anionic components of the LPS molecule (phosphate groups in the lipid A and the inner core and carboxyl groups of KDO). Therefore, the cation-binding sites are essential for the integrity of the outer membrane (Vaara, 1992). Polycationic agents such as
20 poly-L-lysine polymers (of at least 20 residues) increase the outer membrane permeability by displacement of these stabilizing divalent cations. In addition, they exert a so-called 'self-promoted uptake' mechanism (Hancock and Wong, 1984). Due to their bulkiness, they disrupt the normal barrier function of the outer membrane and create transient cracks, promoting their own uptake (Vaara and Vaara, 1983). Furthermore, the dense and ordered packing of the
25 hydrophobic moiety of lipid A, favored by the absence of unsaturated fatty acids, forms a rigid structure with high viscosity. This makes it less permeable for lipophilic molecules and confers additional stability to the outer membrane (OM).

In contrast to Gram-negative bacteria, Gram-positive bacteria do not possess an outer
30 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.

Most Gram-negative bacteria, as well as many Gram-positive bacteria develop a bacterial biofilm. Biofilm is defined as an aggregate or association of microorganisms, which adhere to a surface. The adherent bacteria are often surrounded and protected by an extracellular polymer substance which is produced by the Gram-negative and Gram-positive bacteria. Due to the biofilm the bacteria are much more resistant to antimicrobial substances like antibiotics, disinfectants and cell wall degrading enzymes. In addition, the treatment of biofilms is currently not feasible because the extracellular polymer substance protects itself against degradation by antimicrobial substances, disinfectants or biofilm degrading substances.

Thus, there is a need for methods of eliminating, reducing or preventing bacterial biofilms.

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

The following figures illustrate the present invention.

Figure 1 is a schematic overview showing plasmid construction for recombinant production of (POLY)ⁿ-gp144 ((POLY)ⁿ-KZ144). Previously, pEXP5CT/POLY-gp144 (pEXP5CT/POLY-KZ144) was constructed by a tail PCR (with the BamHI restriction site and first polycation cassette in the 5' tail primer). The plasmid was linearized with BamHI, dephosphorylated and ligated with a cassette containing overhanging BamHI ends. This cassette originates from the hybridization of two complementary oligonucleotides and encodes 9 positively charged residues. One additional positive arginine residue is created at the junction site between the first and second cassette, together with a serine. Longer pEXP5CT/(POLY)ⁿ-gp144 (pEXP5CT/(POLY)ⁿ-KZ144) variants were constructed similarly by repeated cycles.

Figure 2 shows the expression and secretion of POLY-gp144 by *Pichia pastoris*. An amount of 30 μ l supernatant of a *P. pastoris* X33 expression culture [after 1 day (square), 3 days (triangle) and 4 days (circle)] is added to 270 μ l chloroform-permeabilized *P. aeruginosa* PAO1p cells. The buffer conditions were the optimal enzymatic conditions of POLY-gp144 (KH₂PO₄/K₂HP0₄) I = 120 mM pH 6.2). Subsequently, the optical density was spectrophotometrically recorded. A drop in optical density indicates the secretion of a muralytic enzyme by *P. pastoris*. As a negative control, *P. pastoris* X33 without expression plasmid is included (diamond).

Figure 3 shows in a graphical representation the antibacterial activity of the unmodified phiKZgp144 and ELgp188 endolysins, of the modified endolysin variants POLY-gp144 and POLY-gp188 comprising a peptide comprising 9 positively charged amino acid residues and of the modified variants (POLY)²-gp144 and (POLY)²-gp188 comprising a peptide comprising 18 positively charged amino acid residues on *Pseudomonas aeruginosa* PAO1p cells. The error bars render the standard deviations of the mean.

Figure 4 shows a picture of a Coomassie-stained SDS-PAGE showing the results of the expression and purification of the unmodified endolysin PSP3gp10 and its modified endolysin variant PKPSP3gp10. The lane LMW pertains to a size marker (LMW ladder). The following three lanes pertain to protein fractions of the purified protein in Elution Buffer (20 mM NaH₂P0₄-NaOH pH7.4; 0.5 M NaCl; 500 mM imidazole) after Ni²⁺ affinity chromatography. The lane FT pertains to the flow through and the lane W to waste fractions. Only minor secondary bands are visible in the purified protein fractions, indicating the high purity of the recombinant proteins (>90%).

Figures 5 A to D show in a graphic representation the antibacterial activities of unmodified PSP3gp10 and the modified PKPSP3gp10 in different compositions on several exponential growing Gram-negative bacteria after an incubation at room temperature and without shaking. Each species of Gram-negative bacteria was incubated for 30 minutes with a composition comprising 0.5 mM EDTA but no endolysin, with a composition comprising 1.315 μ M unmodified PSP3gp10 but no EDTA, with a composition comprising 1.315 μ M modified PKPSP3gp10 but no EDTA, with a composition comprising 1.315 μ M unmodified PSP3gp10 and 0.5 mM EDTA and with a composition comprising 1.315 μ M modified PKPSP3gp10 and

0.5 mM EDTA. In Figure 5 A the antibacterial activity on *P. aeruginosa* PAO1p cells is represented, in Figure 5 B the antibacterial activity on *P. aeruginosa* Br667 cells, in Figure 5 C the antibacterial activity on *E.coli* WK 6 cells and in Figure 5 D the antibacterial activity on *Salmonella typhimurium* cells. “Δ” gives the difference of activity between the respective PSP3gp10 and PKPSP3gp10 samples. The error bars render the standard deviations of the mean.

Figure 6 shows a picture of a Coomassie-stained SDS-PAGE showing the results of the expression and purification of the unmodified endolysin P2gp09 and its modified endolysin variant PKP2gp09. The lane LMW pertains to a size marker (LMW ladder). The following three lanes pertain to protein fractions of the purified protein in Elution Buffer (20 mM NaH₂P0₄-NaOH pH7.4; 0.5 M NaCl; 500 mM imidazole) after Ni²⁺ affinity chromatography. The lane FT pertains to the flow through and the lane W to waste fractions. Only minor secondary bands are visible in the purified protein fractions, indicating the high purity of the recombinant protein (>95%).

Figures 7 A to F show in a graphic representation the antibacterial activities of unmodified P2gp09 and the modified PKP2gp09 in different compositions on several exponential growing Gram-negative bacteria after an incubation at room temperature and without shaking. Each species of Gram-negative bacteria was incubated for 30 minutes with a composition comprising 0.5 mM EDTA but no endolysin, with a composition comprising 1.315 μM unmodified P2gp09 but no EDTA, with a composition comprising 1.315 μM modified PKP2gp09 but no EDTA, with a composition comprising 1.315 μM unmodified P2gp09 and 0.5 mM EDTA and with a composition comprising 1.315 μM modified PKP2gp09 and 0.5 mM EDTA. In Figure 7 A the antibacterial activity on *P. aeruginosa* PAO1p cells is represented, in Figure 7 B the antibacterial activity on *P. aeruginosa* Br667 cells, in Figure 7 C the antibacterial activity on *E.coli* WK 6 cells, in Figure 7 D the antibacterial activity on *Burkholderia pseudomallei* cells, in Figure 7 E the antibacterial activity on *Pseudomonas putida* G1 cells and in Figure 7 F the antibacterial activity on *Salmonella typhimurium* LT2 (SGSC N° 2317) cells. “Δ” gives the difference of activity between the respective P2gp09 and PKP2gp09 samples. The error bars render the standard deviations of the mean.

Figure 8 shows a picture of a Coomassie-stained SDS-PAGE showing the results of the expression and purification of the unmodified endolysin OBPgpLYS and its modified endolysin variant PKOBPgpLYS. The lane LMW pertains to a size marker (LMW ladder). The following three lanes pertain to protein fractions of the purified protein in Elution Buffer (20 mM NaH₂P0₄-NaOH pH7.4; 0.5 M NaCl; 500 mM imidazole) after Ni²⁺ affinity chromatography. The lane FT pertains to the flow through and the lane W to waste fractions. Only minor secondary bands are visible in the purified protein fractions, indicating the high purity of the recombinant proteins (>90%).

Figures 9 A to F show in a graphic representation the antibacterial activities of different compositions of unmodified OBPgpLYS and the modified PKOBPgpLYS on several exponential growing Gram-negative bacteria after an incubation at room temperature and without shaking. Each species of Gram-negative bacteria was incubated for 30 minutes with a composition comprising 0.5 mM EDTA but no endolysin, with a composition comprising 1.315 µM unmodified OBPgpLYS but no EDTA, with a composition comprising 1.315 µM modified PKOBPgpLYS but no EDTA, with a composition comprising 1.315 µM unmodified OBPgpLYS and 0.5 mM EDTA and with a composition comprising 1.315 µM modified PKOBPgpLYS and 0.5 mM EDTA. In Figure 9 A the antibacterial activity on *Escherichia coli* WK6 cells is represented, in Figure 9 B the antibacterial activity on *Salmonella typhimurium* LT2 (SGSC N° 2317) cells, in Figure 9 C the antibacterial activity on *Pseudomonas aeruginosa* PAO1p cells, in Figure 9 D the antibacterial activity on *Pseudomonas aeruginosa* Br667 cells, in Figure 9 E the antibacterial activity on *Pseudomonas putida* G1 cells and in Figure 9 F the antibacterial activity on *Burkholderia pseudomallei* cells. “Δ” gives the difference of activity between the respective OBPgpLYS and PKOBPgpLYS samples. The error bars render the standard deviations of the mean.

Figures 10 A and B show in a graphic representation the biofilm reducing activities of PolyKZ144 (Art-014) on a mucoid growing clinical isolate of *Pseudomonas aeruginosa* 2573 (Source Uniklinikum Regensburg, nicht näher definiert). *Pseudomonas aeruginosa* 2573 was grown at least 24 hours at 37°C in a polystyrene microtiter plate to allow biofilm formation. To visualize the biofilm content crystal violet staining was performed. In Figure 10 A, the biofilm was then incubated with either Alginate lyase (10u/ml) or PolyKZ144 (50µg/ml). The effect of both enzymes was compared to either biofilm untreated or biofilm washed and

reincubated in LB-medium as controls. In Figure 10 B the effect of PolyKZ144 was compared to either biofilm untreated or biofilm washed and reincubated in LB-medium as controls and a non mucoid growing *E.coli* lab strains to indicated unspecific background staining.

- 5 Figures 11 A to G show in a graphic representation the biofilm reducing activities of several fusion proteins on different Gram-positive and Gram-negative bacterial strains. *Staphylococcus aureus* KS13 (A and B), *Listeria monocytogenes* Scott A (C), *Acinetobacter baumannii* DSMZ30007 (D), *Pseudomonas aeruginosa* 2572 (E, F) and *Pseudomonas aeruginosa* 2573 (G) was grown at least 24 hours at 37°C in a polystyrene microtiter plate to
- 10 allow biofilm formation. To visualize the biofilm content crystal violet staining was performed. In Figure 11 A, the biofilm was then incubated with either PK-Peptide (1.25 µg/well) or the endolysin Ply2638 (25 µg/well) or the fusion protein Ply2638-PK (25 µg/well). The effect of the peptide, the endolysin and the fusion protein was compared to biofilm untreated (one part protein buffer to one part 2x LB without NaCl) indicated by LB.
- 15 In Figure 11 B, the biofilm was then incubated with either the bacteriocin Lysostaphin (18 µg/well) or the bacteriocin variant PK-Lysostaphin (18 µg/well). The effect of the bacteriocin and the bacteriocin variant was compared to biofilm untreated (one part protein buffer to one part 2x LB without NaCl) indicated by LB. In Figure 11 C, the biofilm was then incubated with the modified endolysin variant Pentapeptide-Ply511 (25 µg/well). The effect of the
- 20 modified endolysin variant was compared to biofilm untreated (one part protein buffer to one part 2x LB without NaCl) indicated by LB. In Figure 11 D and E, the biofilm was then incubated with either PK-Peptide (1.25 µg/well) or the endolysin OBP (25 µg/well) or the modified endolysin variant PK-OBP (25 µg/well). The effect of the peptide, the endolysin and the modified endolysin variant was compared to biofilm untreated (one part protein buffer to
- 25 one part 2x LB without NaCl) indicated by LB. In Figures 11 F and G, the biofilm was then incubated with either the endolysin KZ144 (50 µg/well) or the modified endolysin variant SMAP29-KZ144 (50 µg/well). The effect of the peptide, the endolysin and the modified endolysin variant was compared to biofilm untreated (one part protein buffer to one part 2x LB without NaCl) indicated by LB.

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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. There are 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.

- 10 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 or by chemical cross-linking. Moreover, the term “fusion protein” as used herein refers to a fusion of a first amino acid sequence, in particular an endolysin, an autolysin or a bacteriocin and/or other peptidoglycan hydrolase, with a second or further
- 15 amino acid sequence. The second or further amino acid sequence is preferably a peptide, in particular a cationic, apolycationic, a hydrophobic, an amphiphatic and/or an antimicrobial peptide. 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.
- 20 The term “modified endolysin variant” is used herein synonymously with the term “endolysin variant”. Both terms refer to a fusion protein comprising an endolysin and a peptide, in particular a cationic, a polycationic, a hydrophobic, an amphiphatic and/or an antimicrobial peptide.
- 25 The term “modified bacteriocin variant” is used herein synonymously with the term “bacteriocin variant”. Both terms refer to a fusion protein comprising a bacteriocin and a peptide, in particular a cationic, a polycationic, a hydrophobic, an amphiphatic and/or an antimicrobial peptide.
- 30 The term “modified autolysin variant” is used herein synonymously with the term “autolysin variant”. Both terms refer to a fusion protein comprising an autolysin and a peptide, in particular a cationic, a polycationic, a hydrophobic, an amphiphatic and/or an antimicrobial peptide.

The term "peptide stretch" as used herein refers to any kind of peptide linked to a protein such as an endolysin, bacteriocin or autolysin. In particular the term "peptide stretch" as used herein refers to a cationic peptide, a polycationic peptide, an amphipathic peptide, a hydrophobic peptide and/or an antimicrobial peptide. However, a peptide stretch in the meaning of the present invention does not refer to His-tags, preferably His₅-tags, His₆-tags, His₇-tags, His₈-tags, His₉-tags, His₁₀-tags, His₁₁-tags, His₁₂-tags, His₁₆-tags and His₂₀-tags, Strep-tags, Avi-tags, Myc-tags, Gst-tags, JS-tags, cystein-tags, FLAG-tags or other tags known in the art, thioredoxin or maltose binding proteins (MBP). The term "tag" in contrast to the term "peptide stretch" as used herein refers to a peptide which can be useful to facilitate expression and/or affinity purification of a polypeptide, to immobilize a polypeptide to a surface or to serve as a marker or a label moiety for detection of a polypeptide e.g. by antibody binding in different ELISA assay formats as long as the function making the tag useful for one of the above listed facilitation is not caused by the positively charge of said peptide. However, the His₆-tag may, depending on the respective pH, also be positively charged, but is used as affinity purification tool as it binds to immobilized divalent cations and is not used as a peptide stretch according to the present invention.

The term "peptide" as used herein refers to short peptides 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)). Preferred synthetically produced peptides are e.g. cationic, polycationic, amphipathic or hydrophobic peptides. Preferred naturally occurring peptides are e.g. antimicrobial peptides.

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. The term "cationic peptide" as used herein refers also to polycationic peptides.

5 The term "polycationic peptide" as used herein refers to a synthetically designed and produced peptide composed of mostly positively charged amino acid residues, in particular lysine, arginine and/or histidine residues, more preferably lysine and/or arginine residues. A peptide is composed of mostly positively charged amino acid residues if at least about 20, 30, 40, 50, 60, 70, 75, 80, 85, 90, 95 or about 100 % of the amino acid residues are positively
10 charged amino acid residues, in particular lysine and/or arginine residues. The amino acid residues being not positively charged amino acid residues can be neutrally charged amino acid residues and/or negatively charged amino acid residues and/or hydrophobic amino acid residues. Preferably the amino acid residues being not positively charged amino acid residues are neutrally charged amino acid residues, in particular serine and/or glycine.

15

The term, "antimicrobial peptide" (AMP) as used herein refers to any naturally occurring peptide that has microbicidal and/or microbistatic activity on for example bacteria, viruses, fungi, yeasts, mycoplasma and protozoa. Thus, the term "antimicrobial peptide" as used herein refers in particular to any peptide having anti-bacterial, anti-fungal, anti-mycotic, anti-
20 parasitic, anti-protozoal, anti-viral, anti-infectious, anti-infective and/or germicidal, algicidal, amoebicidal, microbicidal, bactericidal, fungicidal, parasiticidal, protozoacidal, protozoicidal properties, in particular sushi peptides and defensin. The antimicrobial peptide may be a member of the RNase A super family, a defensin, cathelicidin, granulysin, histatin, psoriasin, dermicidine or hepcidin. The antimicrobial peptide may be naturally occurring in insects, fish,
25 plants, arachnids, vertebrates or mammals.

Preferably the antimicrobial peptide may be naturally occurring in insects, fish, plants, arachnids, vertebrates or mammals. Preferably the antimicrobial peptide may be naturally occurring in radish, silk moth, wolf spider, frog, preferably in *Xenopus laevis*, *Rana* frogs,
30 more preferably in *Rana catesbeiana*, toad, preferably Asian toad *Bufo bufo gargarizans*, fly, preferably in *Drosophila*, more preferably in *Drosophila melanogaster*, in *Aedes aegypti*, in honey bee, bumblebee, preferably in *Bombus pascuorum*, flesh fly, preferably in *Sarcophaga*

peregrine, scorpion, horseshoe crab, catfish, preferably in *Parasilurus asotus*, cow, pig, sheep, porcine, bovine, monkey and human.

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. Preferably, sushi peptides are naturally occurring antimicrobial peptides.

The term "amphiphatic peptide" as used herein refers to synthetic peptides having both hydrophilic and hydrophobic functional groups. Preferably, the term “amphiphatic peptide” as used herein refers to a peptide having a defined arrangement of hydrophilic and hydrophobic groups e.g. amphiphatic 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 acid residues having a hydrophobic side chain interact with one another to generate a nonaqueous environment. Examples of amino acid residues with hydrophobic side chains are valine, isoleucine, leucine, methionine, phenylalanine, tryptophan, cysteine, alanine, tyrosine, histidine, threonine, serine, proline and glycine residues.

The term “endolysin” as used herein refers to an enzyme which is suitable to hydrolyse bacterial cell walls. “Endolysins” comprise at least one “enzymatically active domain” (EAD) having at least one of the following activities: endopeptidase, N-acetyl-muramoyl-L-alanine-amidase (amidase), N-acetyl-muramidase, N-acetyl-glucosaminidase (lysozyme) or transglycosylases. 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 endolysin may contain one, two or more CBDs. However, the term “endolysin” as used herein refers also to enzymes having at least one EAD but no CBDs. Generally, the cell wall binding domain is able to bind different components on the surface of

bacteria. Preferably, the cell wall binding domain is a peptidoglycan binding domain and binds to the bacteria's peptidoglycan.

The term „cell wall“ as used herein refers to all components that form the outer cell enclosure of the Gram-positive and Gram-negative bacteria and thus guarantee their integrity. In particular, the term „cell wall“ as used herein refers to peptidoglycan, the outer membrane of the Gram-negative bacteria with the lipopolysaccharide, the bacterial cell membrane, but also to additional layers deposited on the peptidoglycan as e.g. capsules, outer protein layers or slimes.

The term “autolysins” as used herein refers to enzymes related to endolysins but encoded by bacteria and involved in e.g. cell division and cell wall metabolism. An overview of autolysins 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 produced 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 “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 activity of an endolysin. The EAD can also be composed of more than one enzymatically active module. The term “EAD” is used herein synonymously with the term “catalytic domain”.

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.

5 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.

10 The term “biofilm” as used herein refers to an aggregate of bacterial microorganisms in which bacterial cells adhere to each other and/or to a surface. These adherent cells are often covered with a matrix of extracellular polymeric substance (EPS), which is produced by the cells. Biofilm EPS, is composed of extracellular DNA, proteins, and polysaccharides. These biofilms may form on any living or non-living surfaces, in particular on both on solid surfaces
15 as colonies and on liquid surfaces as pellicles. Microbial cells growing in a biofilm are physiologically distinct from planktonic cells of the same organism.

The present invention relates to methods of eliminating, reducing or preventing of a bacterial biofilm comprising the steps of:

- 20 a) providing a fusion protein comprising an enzyme having the activity of degrading the cell wall of Gram-negative and/or Gram-positive bacteria to which a peptide with membrane or LPS disrupting activity is fused; and
b) contacting a material, liquid, surface or biological material with said fusion protein.

25 Preferably, the present invention relates to methods of eliminating, reducing or preventing of a bacterial biofilm comprising the steps of:

- c) providing a fusion protein comprising an endolysin, autolysin or bacteriocin to which a peptide with membrane or LPS disrupting activity is fused; and
d) contacting a material, liquid, surface or biological material with said fusion protein.

30

The term “providing” a fusion protein according to the present invention refers either to the mere taking and using of the fusion protein according to the present invention or to the

generating and purification of such fusion protein prior to the use according to the present invention.

Preferably, the material is a stone, rocks, soil, sediments, food, feed or cosmetics. Preferably,
5 the liquid is water, such as drinking water, ground water or waste water, hot springs, seas, lakes, rivers, any kind of aqueous systems, cleaning and storage solutions of contact lenses, dentures, implants, prostheses, or braces.

Preferably, the biological material is any substance derived or obtained from a living
10 organism, in particular plants and mammals, preferably humans, e.g. cells, tissues, organs, blood, blood components and body liquids. Preferably, cells are e.g. nucleated cells or anucleated cells. Cells can be derived from any organ in particular hepatocytes, smooth muscle cells, endothelial cells, keratinocytes, islet cells, stem cells (adult and neonatal, various tissues, or species origin), stem cell progenitor cells, cord blood cells, gametes (male
15 and female), gamete progenitor cells, erythroblasts, leukoblasts, and chondroblasts. Tissues are e.g. mucous membranes, nerves, muscles, epithels, connective and supporting tissues, oral soft tissues and teeth. Organs are e.g. heart, heart valves, eye, ear, urinary tract, lungs, liver, kidney, biliary tract, prostate, nose, digestive tract, respiratory tract, gastrointestinal tract, brain and bone marrow. Preferred body liquids are urine, cerebrospinal fluid and lymph fluids.

20

Preferably, surfaces are solid biological or non-biotic surfaces. Preferred examples of surfaces are the surface of medical devices, in particular implants, prostheses, catheters, such as dental implants, urinary tract prostheses, peritoneal membrane and peritoneal dialysis catheters, indwelling catheters for hemodialysis and for chronic administration of chemotherapeutic
25 agents (Hickman catheters), cardiac implants such as pacemakers, prosthetic heart valves, ventricular assist devices, synthetic vascular grafts and stents, internal fixation devices, percutaneous sutures and tracheal and ventilator tubing, as well as surfaces of industrial or potable water system piping and of natural aquatic systems.

30 Biofilms are formed by bacterial microorganisms in which bacterial cells adhere to each other and/or to a surface. Extracellular polymeric substances (EPS) excreted by the bacterial microorganisms of a biofilm form with water hydrogels, so that a slime-like matrix is formed. This matrix may also comprise gas bubbles and anorganic particles. Biofilm EPS, is

composed of extracellular DNA, proteins, and polysaccharides. Besides the bacterial microorganisms other unicellular organisms may be integrated in the biofilm. Biofilms may occur by the settling of bacterial microorganisms to interfaces. Mostly, the biofilm is formed on water surfaces or on an interface to a solid phase. These biofilms may form on any living or non-living surfaces. In general all interfaces may be settled by biofilms. Biofilms are almost present everywhere, in soil and sediments, in ground water, on rocks, in deserts, in hot springs, on and in plants and animals, in particular on mucous membranes. Moreover, biofilms may occur on medical devices, such as implants, catheters, endoscopes, prostheses, instruments, and apparatus but also in cosmetics, food and feed. Biofilms may be also associated with infections, because in most cases the bacterial microorganisms form biofilm to be protected against the immune system. The formation of a biofilm ensures the long-term survival of the bacterial microorganisms. One example of an acute respiratory tract infection is the legionnaire's disease which is caused by swallowing or inhalation of clumps of legionella-biofilms detached from air or water pipes of heating or cooling systems. Also many food bacteria, like *E. coli* 0157:H7, *Listeria monocytogenes*, *Yersinia enterocolitica*, *Salmonella* spp. and *Camphylobacter jejuni* may form on food and devices biofilms which are then highly resistant to biocides, drought, heat, antibiotics and cleaning reagents. The microorganisms responsible for infections of implants, catheters and other medical devices may be coagulase-negative staphylococci, *Staphylococcus aureus*, *Enterococcus faecalis*, *Streptococcus* spp., *Escherichia coli*, *Klebsiella pneumoniae*, *Acinetobacter* spp., *Proteus mirabilis*, *Pseudomonas aeruginosa* and *Candida* spp. which are also associated with a broad spectrum of nosocomial infections. Typical bacterial infections associated with biofilms in humans are: wound infections, in particular wounds associated with diabetes mellitus, tonsillitis, osteomyelitis, bacterial endocarditis, sinusitis, infections of the cornea, urinary tract infection, infection of the biliary tract, infectious kidney stones, urethritis, prostatitis, catheter infections, middle-ear infections, formation of dental plaque, gingivitis, periodontitis, cystic fibrosis, and infections of permanent indwelling devices such as joint prostheses and heart valves.

The presence of biofilms may be determined by various tests, such as by the Tissue culture plate method (TCP) described in Christensen et al., *J Clin Microbiol* 22:996-1006 (1985), or by the Tube method (TM) as previously described by Christensen et al., *Infect Immun* 37:318-26 (1982), or by the Congo red Agar method (CRA) described by Freeman et al., *J Clin*

Pathol 42:872-4 (1989). The biofilm may be quantified by using crystal violet assay (Peeters et al., *J Microbiol Methods* 72: 157-165 (2008)).

5 The fusion protein according to the present invention may influence the interaction of the bacteria forming a biofilm so that the cells are transferred in single planktonic cells which where then lysed by said fusion protein, consequently the biofilm is then degraded in part or totally. The influence of the fusion protein according to the present invention on the bacteria may also directly lyse the bacteria associated in a biofilm and thus, the biofilm is degraded in part or totally. Moreover, the fusion protein according to the present invention may prevent
10 the formation of bacterial biofilms by lysing bacteria which are able to form a biofilm with other bacteria.

The fusion proteins according to the present invention relate preferably to endolysin variants, bacteriocin variants and autolysin variants.

15

Preferred fusion proteins according to the present invention comprise an endolysin, an autolysin or a bacteriocin fused to a peptide with lipopolysachharide (LPS) or in general membrane disrupting activity. LPS is a major component of the outer membrane of Gram-negative bacteria. It increases the negative charge of the cell membrane and protects the
20 membrane from certain kinds of chemical attack. To a certain degree said LPS protects the membrane of Gram-negative bacteria also from endolysins added from outside of the bacteria. However, the LPS can be disrupted by peptides having a LPS disrupting activity as e.g. positively charged peptides. Moreover, said peptides may be involved in the outer membrane protein transport mechanism, a destabilisation of structural outer membrane proteins and/or in
25 lipid-dependent destabilisation. The inventors of the present invention have surprisingly found, that a peptide having LPS disrupting activity or in general membrane disrupting activity promotes the passage of an endolysin, an autolysin or a bacteriocin fused to said peptide through the outer membrane of Gram-negative bacteria. After the promoted pass of the endolysin, autolysin or bacteriocin through the outer membrane of bacteria, the cell wall
30 of the bacterium can be more easily be disrupted or desintegrated by the endolysin due to degradation of the peptidoglycan layer followed by osmotic lysis when the internal cell pressure of the bacterium cannot longer be resisted. The Gram-positive bacteria have a much thicker peptidoglycan layer than Gram-negative bacteria. Here the membrane disrupting

activity of the fusion protein supports the lysis of the bacteria, by acting on the cytoplasmic membrane.

Thus, the present invention refers to methods of eliminating, reducing or preventing bacterial
5 biofilms by means of fusion proteins composed of an enzyme, preferably an endolysin, an autolysin or a bacteriocin, having the activity of degrading the cell wall of Gram-negative and/or Gram-positive bacteria and a peptide with membrane disrupting activity, wherein said peptide is fused to the enzyme at the N- and/or C-terminus. Said fusion proteins according to the present invention are also called modified endolysin variants or simply endolysin variants
10 or modified endolysins, modified autolysin variants or autolysin variants, modified bacteriocins or bacteriocin variants.

The endolysin part of the fusion protein is preferably encoded by bacteriophages specific for Gram-negative bacteria such as Gram-negative bacteria of bacterial groups, families, genera
15 or species comprising strains pathogenic for humans or animals like *Enterobacteriaceae* (*Escherichia*, especially *E. coli*, *Salmonella*, *Shigella*, *Citrobacter*, *Edwardsiella*, *Enterobacter*, *Hafnia*, *Klebsiella*, especially *K. pneumoniae*, *Morganella*, *Proteus*, *Providencia*, *Serratia*, *Yersinia*), *Pseudomonadaceae* (*Pseudomonas*, especially *P. aeruginosa*, *Burkholderia*, *Stenotrophomonas*, *Shewanella*, *Sphingomonas*, *Comamonas*),
20 *Neisseria*, *Moraxella*, *Vibrio*, *Aeromonas*, *Brucella*, *Francisella*, *Bordetella*, *Legionella*, *Bartonella*, *Coxiella*, *Haemophilus*, *Pasteurella*, *Mannheimia*, *Actinobacillus*, *Gardnerella*, *Spirochaetaceae* (*Treponema* and *Borrelia*), *Leptospiraceae*, *Campylobacter*, *Helicobacter*, *Spirillum*, *Streptobacillus*, *Bacteroidaceae* (*Bacteroides*, *Fusobacterium*, *Prevotella*, *Porphyromonas*), *Acinetobacter*, especially *A. baumannii*.

25

In another preferred embodiment, the endolysin of the fusion protein 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, in particular of the phylum Actinobacteria, in particular of the class Actinobacteridae, in
30 particular of the order Actinomycetales, in particular of the families Actinomycineae: Actinomycetaceae (*Actinomyces*, *Mobiluncus*), Corynebacterineae: Mycobacteriaceae (*Mycobacterium*), Nocardiaceae, Corynebacteriaceae, Frankineae: Frankiaceae, Micrococcineae: Brevibacteriaceae and Propionibacteriaceae (*Propionibacterium*) and of the

order Bifidobacteriales, in particular of the families Bifidobacteriaceae (Bifidobacterium, Falcivibrio, Gardnerella) and other subclasses: Acidimicrobidae, Coriobacteridae, Rubrobacteridae, Sphaerobacteridae; and of the phylum Firmicutes, in particular of the class Bacilli, in particular of the order Bacillales, in particular of the families: Bacillaceae (Bacillus), Listeriaceae (Listeria), Staphylococcaceae (Staphylococcus, Gemella, Jeotgalicoccus) and of the order Lactobacillales, in particular of the families: Enterococcaceae (Enterococcus), Lactobacillaceae (Lactobacillus, Pediococcus), Leuconostocaceae (Leuconostoc), Streptococcaceae (Lactococcus, Streptococcus) and of the class Clostridia, in particular of the order: Clostridiales (Clostridium, Peptostreptococcus, Selenomonas), Halanaerobiales and Thermoanaerobacterales, and of the class Tenericutes/Mollicutes, in particular of the order: Mycoplasmatales (Mycoplasma, Ureaplasma), Entomoplasmatales (Spiroplasma), Anaeroplasmatales (Erysipelothrix), Acholeplasmatales (Acholeplasma), Haloplasmatales (Haloplasma).

In another preferred embodiment, the autolysin or the bacteriocin of the fusion protein is encoded by Gram-negative or Gram-positive bacteria such as Gram-negative or Gram-positive bacteria of bacterial groups, families, genera or species comprising strains pathogenic for humans or animals as listed above.

Preferably, the endolysin part derives from a phage or a wild type endolysin as depicted in the following table 1:

phage	publication	Wild type endolysin	predicted function of the endolysin
ΦV10	Perry, L.L. and Applegate, B.M.	PhiV10p30	chitinase
FELS-1	McClelland, M. and Wilson, R.K.	STM0907.Fels0	chitinase
ε15	Kropinski, A.M. and McConnel, M.R.	epsilon15p25	chitinase
YUA	Ceyssens, P. (Laboratory for Gene technology)	YuA20	lytic transglycosylase (C) / 1 transmembranair domain (N)
B3	Braid, M.D. and Kitts, C.L.	ORF23	lytic transglycosylase (C) / 2 transmembranair domains (N)
BCEPμ	Summer, E.J. and Young, R.	BcepMu22	lytic transglycosylase (M) / 1 transmembranair domain (N)
F116	Byrne, M. and Kropinski, A.M.	F116p62	muraminidase (T4-like)
FELS-2	McClelland, M. and Wilson, R.K.	STM2715.S.Fels2	muraminidase (T4-like)
ES18	Casjens, S.R. and Hendrix, R.W.	gp76	muraminidase (T4-like)
SETP3	De Lappe, N and Cormican, M.	SPSV3_gp23	muraminidase (T4-like)
ΦECO32	Savalia, D and Severinov, K	phi32_17	muraminidase (T4-like)

HK022	Juhala , R and Hendrix, R.W.	HK022p54	muraminidase (lambdalike)
HK97	Juhala , R and Hendrix, R.W.	HK97p58	muraminidase (lambdalike)
HK620	Clark, A.J. and Dhillon, T.S.	HK620p36	muraminidase (lambdalike)
E1	Pickard, D. and Dougan, G	VIP0007	muraminidase (lambdalike)
SF6	Casjens, S and Clark, A.J.	Sf6p62	muraminidase (lambdalike)
SFV	Allison, G.E. and Verma, N.K.	R (SfVp40)	muraminidase (lambdalike)
BCEPC6B	Summer, EJ and Young, R.	gp22	muraminidase (lambdalike)
BCEPNAZGUL	Summer, EJ and Young, R.	Nazgul38	muraminidase (lambdalike)
P2	Christie, G.E. and Calender, R.	K (P2p09)	muraminidase (lambdalike)
WΦ	Christie, G.E. and Esposito, D.	K (Wphi09)	muraminidase (lambdalike)
RV5	Kropinski, A.M. and Johnson	rv5_gp085	muraminidase (lambdalike)
JS98	Zuber, S and Denou, E.	EpJS98_gp116	muraminidase (T4-like)
13A	Savalia, D and Molineux, I.	gp3.5	muramoyl-L-alanine amidase
BA14	Savalia, D and Molineux, I.	gp3.5	muramoyl-L-alanine amidase
ECODS1	Savalia, D and Molineux, I.	gp3.5	muramoyl-L-alanine amidase
K1F	Scholl, D and Merrill, C	CKV1F_gp16	muramoyl-L-alanine amidase
T3	Pajunen, M.I. and Mollieux, I.J.	T3p18	muramoyl-L-alanine amidase
GH-1	Kropinski, A.M. and Kovalyova, I.V.	gh-1p12	muramoyl-L-alanine amidase
K11	Mollieux, I. and Savalia, D.	gp3.5	muramoyl-L-alanine amidase
BIP-1	Liu, M and Miller, J.F.	bip-1p02	lysozyme (N) / PG-binding domain (C)
BMP-1	Liu, M and Miller, J.F.	bmp-1p02	lysozyme (N) / PG-binding domain (C)
BPP-1	Liu, M and Miller, J.F.	bpp2	lysozyme (N) / PG-binding domain (C)
ΦCTX	Nakayama, K and Hayashi, T.	ORF12	PG-binding domain (N) / muramidase (C)
BCEP43	Summer, EJ and Young, R.	Bcep43-27	PG-binding domain (N) / muramidase (C)
BCEP781	Summer, EJ and Young, R.	Bcep781-27	PG-binding domain (N) / muramidase (C)
BCEP1	Summer, EJ and Young, R.	Bcep1-28	PG-binding domain (N) / muramidase (C)
BCEPNY3	Summer, EJ and Young, R.	BcepNY3gene26	PG-binding domain (N) / muramidase (C)
ΦE12-2	DeShazer, D and Niernan, W.C.	gp45	PG-binding domain (N) / muramidase (C)
Φ52237	DeShazer, D and Niernan, W.C.	gp28	PG-binding domain (N) / muramidase (C)
ΦP27	Recktenwald, J and Schmidt, H.	P27p30	endopeptidase
RB49	Monod, C and Krisch, H.M.	RB49p102	endopeptidase
Φ1	Arbiol, C. and Comeau, A.M.	phi1-p102	endopeptidase
T5	Pankova, N.V. and Ksenzenko, V.N.	lys (T5.040)	endopeptidase
201phi2-1	Thomas <i>et al.</i> , 2008		PG-binding domain (N) / unknown catalytic domain (C)
Aeh1	Monod, C and Krisch, H.M.	Aeh1p339	muraminidase (T4-like)

YYZ-2008	Kropinski, A.M.	YYZgp45	muraminidase (lambda-like)
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Also preferred is the endolysin part deriving from endolysins of the *Pseudomonas aeruginosa* phages Φ KZ and EL, of the *Pseudomonas putida* phage OBP, of the phage LUZ24, or from T4 lysozyme, gp61 muramidase, PSP3 endolysin, of the *Salmonella* phage, of the
5 *Acinetobacter baumannii* phage, of the *E. coli* Phage P2, of the *E. coli* phage N4 and K1F and of the *Salmonella typhimurium* phage.

Further preferred endolysins of the fusion protein are Listeria phage endolysins PlyA118, PlyA500, PlyPSA, PlyA511, PlyP35, PlyP40, Staphylococcal phage Phi 11 endolysin, Phi
10 MR11 endolysin, LysK, Ply 2638, *Clostridium perfringens* PlyS6, Ply3626, *Clostridium difficile*: CD27L endolysin, Streptococcus: B30 endolysin, phage Dp-1 Pal amidase, C1 endolysin, Cpl-1 endolysin, PlyGBS, Enterococcus: PlyV12, Bacillus anthracis: Phage gamma endolysin PlyG, Propionibacterium phage endolysin PA6-gp20.

15 Preferred autolysins of the fusion protein 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.

20 Preferred bacteriocins are Lysostaphin (degrading *Staphylococcus* cell walls), Mutanolysin (degrading *Streptococcus* cell walls) and Enterolysin (degrading *Enterococcus* cell walls). More preferably, the bacteriocin of the fusion protein according to the present invention comprises an amino acid sequence according to SEQ ID NO: 87.

25 Further examples for the endolysin part of the fusion protein is selected from the group consisting of Cpl-1 according to SEQ ID NO: 84, Ply511 according to SEQ ID NO: 85, LysK according to SEQ ID NO: 86, PA6-gp20 according to SEQ ID NO: 88, phiKZgp144 according to SEQ ID NO: 1, ELgp188 according to SEQ ID NO: 2, *Salmonella* endolysin according to SEQ ID NO: 3, Enterobacteria phage T4 endolysin according to SEQ ID NO: 4,
30 *Acinetobacter baumannii* endolysin according to SEQ ID NO: 5, *E.coli* Phage K1F endolysin according to SEQ ID NO: 6, OBPgpLYS according to SEQ ID NO: 7, PSP3 *Salmonella* endolysin (PSP3gp10) according to SEQ ID NO: 8, *E.coli* Phage P2 endolysin (P2gp09)

according to SEQ ID NO: 9, *Salmonella typhimurium* phage muramidase STM0016 according to SEQ ID NO: 89, *E. coli* Phage N4 muramidase N4-gp61 according to SEQ ID NO: 90, N4-gp61 trunc. according to SEQ ID NO:91 and Ply 2638 according to SEQ ID NO: 92.

- 5 In another preferred embodiment of the present invention methods of eliminating, reducing or preventing bacterial biofilms by means 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
- 10 residues, e.g. biotinylation, acetylation, PEGylation, chemical changes of the amino-, SH- or carboxyl-groups. Said modified and/or altered endolysins exhibit the lytic activity of the respective wild type endolysin. However, said activity can be 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
- 15 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).
- 20 The peptide of the fusion protein according to the present invention may be linked to the enzyme, preferably to the endolysin, autolysin or bacteriocin by additional amino acid residues e.g. due to cloning reasons. Preferably, said additional amino acid residues may be not recognized and/or cleaved by proteases. Preferably said peptide may be linked to the enzyme by at least 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 additional amino acid residues. Preferably, the
- 25 peptide fused on the N-terminus of the enzyme, preferably to the endolysin, autolysin or bacteriocin, of the fusion protein according to the invention further comprises additional amino acids on its N-terminus. Preferably the peptide comprises the amino acid methionine (Met), or methionine, glycine and serine (Met-Gly-Ser) or alanine, methionine and glycine (Ala-Met-Gly). In another preferred embodiment the peptide is linked to the N-terminus of
- 30 the enzyme, preferably to the endolysin, autolysin or bacteriocin, by the additional amino acid residues, in particular glycine and serine (Gly-Ser). In another preferred embodiment the peptide is linked to the C-terminus of the enzyme by the additional amino acid residues, in particular glycine and serine (Gly-Ser).

In one aspect of the present invention the peptide with membrane and/or LPS disrupting activity comprises a positively charged peptide, which comprises one or more of the positively charged amino acids being lysine, arginine and/or histidine. Preferably, more than 80%, preferably more than 90%, preferably 100% of the amino acids in said peptide are positively charged amino acids. Advantageously, the cationic peptide is positioned at the N-terminal and/or the C-terminal end of the fusion protein, thus enhancing the cationicity of the latter proteins. In another embodiment of the invention, the cationic peptide fused to the enzyme, preferably to the endolysin, autolysin or bacteriocin is at least 5, more preferably at least 9 amino acids long.

In a preferred embodiment said 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 at least 20, 30, 40, 50, 60 or 70%, more preferably at least 80%, for instance at least 90% of the said amino acid residues are either arginine or lysine residues. In another preferred embodiment said 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 said amino acid residues are either arginine or lysine residues.

Preferably, the peptide of the fusion protein is fused to the N-terminus and/or to the C-terminus of the enzyme, preferably of the endolysin, autolysin or bacteriocin. In a particular preferred embodiment said peptide is only fused to the N-terminus of the enzyme, preferably the endolysin, autolysin or bacteriocin. However, also preferred are fusion proteins having a peptide both on the N-terminus and on the C-terminus. Said peptides on the N-terminus and on the C-terminus can be the same or distinct peptides.

The peptide of the fusion protein is preferably covalently bound to the enzyme. Preferably, said peptide 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 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 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.

In one aspect of the present invention the peptide is selected from the group of cationic peptides, polycationic peptides, hydrophobic peptides, antimicrobial peptides and amphiphatic peptides.

In one aspect of the present invention the peptide is a cationic and/or polycationic peptide, which comprises one or more of the positively charged amino acid residues of lysine, arginine and/or histidine, in particular of lysine and/or arginine. Preferably, more than about 20, 30, 40, 50, 60, 70, 75, 80, 85, 90, 95 or 99 % of the amino acid residues in said peptide are positively charged amino acid residues, in particular lysine and/or arginine residues. Especially preferred are peptides consisting of about 100 % positively charged amino acid residues, in particular arginine and/or lysine residues, wherein preferably about 60 % to about 70 % of said positively charged amino acid residues are lysine residues and about 30% to about 40 % of said positively charged amino acid residues are arginine residues. More preferred is a peptide consisting of about 100 % positively charged amino acid residues, in particular arginine and/or lysine residues, wherein preferably about 64 % to about 68 % of said positively charged amino acid residues are lysine and about 32 % to about 36 % of said positively charged amino acid residues are arginine. Peptides consisting of either only arginine or only lysine are also preferred.

Especially preferred are cationic and/or polycationic peptides of the fusion protein comprising at least one motive according to SEQ ID NO: 10 (KRKKRK). In particular cationic peptides 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: 10 (KRKKRK) are preferred. More preferred are cationic peptides 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 peptide is a cationic peptide comprising 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 peptides 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 peptides 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 peptides comprising at least one motive according to SEQ ID NO: 32 (KRXKR), wherein X is any other amino acid than lysine, arginine and histidine. Especially preferred are peptides comprising at least one motive according to SEQ ID NO: 33 (KRSKR). More preferred are cationic peptides 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: 32 (KRXKR) or SEQ ID NO: 33 (KRSKR).

Also preferred are peptides of the fusion protein 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 peptides comprising at least one motive according to SEQ ID NO: 34 (KRGSG). More preferred are cationic peptides 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: 34 (KRGSG).

In another preferred embodiment of the present invention the cationic peptide 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 peptides of the fusion protein 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.

5

Especially preferred are peptides of the fusion protein selected from the group consisting of the following sequences presented in Table 2.

Table 2:

Peptide	length	SEQ ID NO:
KRKKRK	6	SEQ ID NO: 10
KRKKRKKRK	9	SEQ ID NO: 11
RRRRRRRRR	9	SEQ ID NO: 12
KKKKKKKK	8	SEQ ID NO: 13
KRKKRKKRKK	10	SEQ ID NO: 14
KRKKRKKRKKRK	12	SEQ ID NO: 15
KRKKRKKRKKRKKR	14	SEQ ID NO: 16
KKKKKKKKKKKKKKKK	16	SEQ ID NO: 17
KRKKRKKRKKRKKRKKRKK	19	SEQ ID NO: 18
RRRRRRRRRRRRRRRRRR	19	SEQ ID NO: 19
KKKKKKKKKKKKKKKKKK	19	SEQ ID NO: 20
KRKKRKKRKRKRKKRKKRK	20	SEQ ID NO: 21
KRKKRKKRKRKRKKRKKRKK	21	SEQ ID NO: 22
KRKKRKKRKKRKKRKKRKKR	21	SEQ ID NO: 23
KRKKRKKRKRKRGSGKRKKRKKR	22	SEQ ID NO: 24
KRKKRKKRKRKRGSGGKRKKRKKR	24	SEQ ID NO: 25
KRKKRKKRKKRKKRKKRKKRKKR	25	SEQ ID NO: 26
KRKKRKKRKRKRKKRKKRKRKRKRKKR	31	SEQ ID NO: 27
KRKKRKKRKRKRGSGGKRKKRKKRKRKRGSGGKRKKRKKR	38	SEQ ID NO: 28
KRKKRKKRKKRKKRKKRKKRKKRKKRKKRKKRKKRKKR	39	SEQ ID NO: 29
KRKKRKKRKRKRKKRKKRKRKRKRKKRKKRKRKRKRKKR	42	SEQ ID NO: 30

10 Preferably, the peptide of the fusion protein is no tag such as a His-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 the fusion protein according to the present invention may comprise in addition such tag or tags.

15 Preferably, the peptide of the fusion protein has the function to lead the fusion protein according to the present invention through the outer membrane of bacteria but has no or only

low activity when administered without being fused to the endolysin, autolysin or bacteriocin. The function to lead the fusion protein through the outer membrane of Gram-negative and/or Gram-positive bacteria is caused by the potential of the outer membrane or LPS disrupting or permeabilising or destabilizing activity of said peptide. Such outer membrane or LPS disrupting or permeabilising or destabilizing activity of the peptide may be determined in a method as follows: The bacteria cells to be treated are cultured in liquid medium or on agar plates. Then the bacteria cell concentration in the liquid medium is determined photometrically at OD600nm or the colonies on the agar plates are counted, respectively. Now, the bacteria cells in liquid medium or on the plates are treated with a fusion protein according to the invention. After incubation the bacteria cell concentration in the liquid medium is determined photometrically at OD600nm or the colonies on the agar plates are counted again. If the fusion protein exhibits such outer membrane or LPS disrupting or permeabilising or destabilizing activity, the bacteria cells are lysed due to the treatment with the fusion protein and thus, the bacteria cell concentration in the liquid medium or the number of the bacteria colonies on the agar plate is reduced. Thus, the reduction in bacteria cell concentration or in the number of bacteria colonies after treatment with fusion protein is indicative for an outer membrane or LPS disrupting or permeabilising or destabilizing activity of the fusion protein.

In a further embodiment of the present invention the peptide is an antimicrobial peptide comprising a positive net charge and around 50% hydrophobic amino acids. The antimicrobial peptides are amphiphatic, with a length of about 12 to about 50 amino acid residues. The antimicrobial peptides are naturally occurring in insects, fish, plants, arachnids, vertebrates or mammals. Preferably the antimicrobial peptide may be naturally occurring in radish, silk moth, wolf spider, frog, preferably in *Xenopus laevis*, *Rana* frogs, more preferably in *Rana catesbeiana*, toad, preferably Asian toad *Bufo bufo gargarizans*, fly, preferably in *Drosophila*, more preferably in *Drosophila melanogaster*, in *Aedes aegypti*, in honey bee, bumblebee, preferably in *Bombus pascuorum*, flesh fly, preferably in *Sarcophaga peregrine*, scorpion, horseshoe crab, catfish, preferably in *Parasilurus asotus*, cow, pig, sheep, porcine, bovine, monkey and human.

In another preferred embodiment the antimicrobial peptide of the fusion protein consists of about 0 % to about 5 %, or about 0 % to about 35 %, or about 10 % to about 35 % or about 15

% to about 45 %, or about 20 % to about 45 % positively charged amino acid residues, in particular lysine and/or arginine residues and of about 50 % to about 80 %, or about 60 % to about 80 %, or about 55 % to about 75 %, or about 70 % to about 90 % 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.

In another preferred embodiment of the present invention the antimicrobial peptide of the fusion protein consists of about 4 % to about 58 % positively charged amino acid residues, in particular lysine and/or arginine residues and of about 33 % to about 89 % 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.

Examples for antimicrobial peptides of the fusion protein according to the present invention are listed in the following table.

Table 3:

Peptide	Amino acid sequence	SEQ ID NO
LL-37	LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLP RTES	SEQ ID NO:93
SMAP-29	RGLRRLGRKIAHGVKKYGPTVLRIRIAG	SEQ ID NO:94
Indolicidin	ILPWKWPWWPWRR	SEQ ID NO:95
Protegrin	RGGRLCYCRRRFCVCVGR	SEQ ID NO:96
Cecropin P1	SWLSKTAKKLENSAKKRISSEGIAIAIQGGPR	SEQ ID NO:97
Magainin	GIGKFLHSAKKFGKAFVGEIMNS	SEQ ID NO:98
Pleurocidin	GWGSFFKKAHVGKHVGKAALTHYL	SEQ ID NO:99
Cecropin A (<i>A. aegypti</i>)	GGLKKLGKKLEGAGKRVFNAAEKALPVVAG AKALRK	SEQ ID NO:100
Cecropin A (<i>D. melanogaster</i>)	GWLKKIGKKIERVGQHTRDATIQGLGIPQQAA NVAATARG	SEQ ID NO:101
Buforin II	TRSSRAGLQFPVGRVHRLLRK	SEQ ID NO:102
Sarcotoxin IA	GWLKKIGKKIERVGQHTRDATIQGLGIAQQAA NVAATAR	SEQ ID NO:103
Apidaecin	ANRPVYIPPPRPPHPRL	SEQ ID NO:104
Ascaphine 5	GIKDWIKGAAKKLIKTVASHIANQ	SEQ ID NO:105
Nigrocin 2	GLLSKVLGVGKKVLCGVSLVC	SEQ ID NO:106
Pseudin 1	GLNTLKKVFQGLHEAIKLINNHVQ	SEQ ID NO:107
Ranalexin	FLGGLIVPAMICAVTKKC	SEQ ID NO:108

Melittin	GIGAVLKVLTTGLPALISWIKRKRQQ	SEQ ID NO:109
Lycotoxin 1	IWLTALKFLGKHAACKLAKQQLSKL	SEQ ID NO: 110
Parasin 1	KGRGKQGGKVRAKAKTRSS	SEQ ID NO: 111
Buforin I	AGRGKQGGKVRAKAKTRSSRAGLQFPVGRVH RLLRKGN	SEQ ID NO: 112
Dermaseptin 1	ALWKTMLKKLGTMLHAGKAALGAAADTIS QGTQ	SEQ ID NO: 113
Bactenecin 1	RLCRIVVIRVCR	SEQ ID NO: 114
Thanatin	GSKKPVPPIYCNRRTGKQCQRM	SEQ ID NO: 115
Brevinin 1T	VNPIILGVLPKVCLITKKC	SEQ ID NO: 116
Ranateurin 1	SMLSVLKNLGKVGLGFVACKINIKQC	SEQ ID NO: 117
Esculentin 1	GIFSKLGRKKIKNLLISGLKNVGKEVGMDVVR TGIKIAGCKIKGEC	SEQ ID NO: 118
Tachyplesin	RWCFRVCYRGICYRKCR	SEQ ID NO: 119
Androctonin	RSVCRQIKICRRRGGCYKCTNRPY	SEQ ID NO: 120
alpha-defensin	DCYCRIPACIAGERRYGTCTIYQGRWAFCC	SEQ ID NO: 121
beta-defensin	NPVSCVRNKGICVPIRCPGSMKQIGTCVGRAV KCCRKK	SEQ ID NO: 122
theta-defensin	GFCRCLCRRGVCRCICTR	SEQ ID NO: 123
defensin (sapecin A)	ATCDLLSGTGINSACAAHCLLRGNRGGYCN GKAVCVCRN	SEQ ID NO: 124
Thionin (crambin)	TTCCPSIVARSNFNVCRIPTPEAICATYTGCIH PGATCPGDYAN	SEQ ID NO: 125
defensin from radish	QKLCQRPSGTWSGVCNNACKNQCIKLEKA RHGSCNYVFPACICIFPC	SEQ ID NO: 126
Drosomycin	DCLSGRYKGPCAVWDNETCRRVCKEEGRSSG HCSPSLKCWCEGC	SEQ ID NO: 127
Hepcidin	DTHFPICIFCCGCCHRSKCGMCKT	SEQ ID NO: 128
Bac 5	RFRPPIRPPPIRPPFYPPFRPPPIRPPPIRPP LGRPPF	SEQ ID NO: 129
PR-39	RRRPRPPYLPRPRPPFFPPRLPPRIPPGFPPRFPP RFP	SEQ ID NO: 130
Pyrrhocoricin	VDKGSYLPRPTPPRIYNRN	SEQ ID NO: 131
Histatin 5	DSHAKRHHGYKRKFHEKHHSRGY	SEQ ID NO: 132

In a further embodiment of the present invention the peptide 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.

5 Especially preferred is the sushi 1 peptide according to SEQ ID NO: 133.

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

5 In a further embodiment of the present invention the peptide is a hydrophobic peptide, which comprises at least 90 % of the hydrophobic amino acid residues of valine, isoleucine, leucine, methionine, phenylalanine, tryptophan, cysteine, alanine, tyrosine, histidine, threonin, serine, proline and/or glycine. In another preferred embodiment the hydrophobic peptide of the fusion protein consist of about 90 % to about 95 %, or of about 90 to about 100%, or of about 95 % to about 100 % of the hydrophobic amino acid residues of valine, isoleucine, leucine, 10 methionine, phenylalanine, tryptophan, cysteine, alanine, tyrosine, histidine, threonin, serine, proline and/or glycine.

Preferred hydrophobic peptides of the fusion protein are Walmagh1 having the amino acid sequence according to SEQ ID NO: 134 and the hydrophobic peptide of the fusion protein 15 having the amino acid sequence Phe-Phe-Val-Ala-Pro (SEQ ID NO: 135).

In a further embodiment of the present invention the peptide is an amphiphatic 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, 20 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 25 amino acid residues in said peptide are hydrophobic amino acid residues. Advantageously, the amphiphatic peptide is fused at the N-terminal and/or the C-terminal end of the enzyme having cell wall degrading activity, thus enhancing the amphiphaticity of the latter proteins.

In another embodiment of the present invention the peptide is an amphiphatic peptide 30 consisting 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 amphiphatic 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 amphiphatic peptide are of the hydrophobic amino acids valine, isoleucine, leucine, methionine, phenylalanine, tryptophan, cysteine, alanine, tyrosine, histidine, threonin, serine, proline and/or glycine.

5

In another preferred embodiment of the present invention the peptide is an amphiphatic peptide comprising 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 amphiphatic peptides consisting of about 10 % to about 50 %, or about 20 % to about 50 %, or about 30 % to about 45 % or about 5 % to about 30 % positively charged amino acid residues, in particular lysine and/or arginine residues and of about 50 % to about 85 %, or about 50 % to about 90 %, or about 55 % to about 90 %, or about 60 % to about 90 %, or about 65 % to about 90 % 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. In another preferred embodiment amphiphatic peptides consisting of 12 % to about 50 % positively charged amino acid residues, in particular lysine and/or arginine residues and of about 50 % to about 85 % 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.

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Preferred amphiphatic peptides of the fusion protein are, α 4-helix of T4 lysozyme according to SEQ ID NO: 136 and WLBU2-Variant having the amino acid sequence according to SEQ ID NO: 137 and Walmagh 2 according to SEQ ID NO: 138.

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In a preferred embodiment of the present invention the fusion protein consists of a peptide according to SEQ ID NO: 10 to 30, 32 to 34 and 93 to 138 and an endolysin according to SEQ ID NO: 1 to 9, 84 to 86 and 88 to 92 or a bacteriocin according to SEQ ID NO: 87. In a preferred embodiment the fusion protein comprises a peptide selected from the group of

peptides according to SEQ ID NO: 10 to 30, 32 to 34 and 93 to 138 and an endolysin selected from the group of endolysins according to SEQ ID NO: 1 to 9, 84 to 86 and 88 to 92 or a bacteriocin according to SEQ ID NO: 87.

- 5 Especially preferred are fusion proteins selected from the group consisting of the following fusion proteins presented in the Table 4.

Table 4:

Fusion protein	SEQ ID NO: (fusion protein)	Endolysin/Bacteriocin part	Peptide (N-terminal unless otherwise indicated)
POLY-gp144	SEQ ID NO: 35	SEQ ID NO: 1	SEQ ID NO: 11
(POLY) ² -gp144	SEQ ID NO: 36	SEQ ID NO: 1	SEQ ID NO: 21
(POLY) ³ -gp144	SEQ ID NO: 37	SEQ ID NO: 1	SEQ ID NO: 27
(POLY) ⁴ -gp144	SEQ ID NO: 38	SEQ ID NO: 1	SEQ ID NO: 30
POLY-gp188	SEQ ID NO: 39	SEQ ID NO: 2	SEQ ID NO: 11
(POLY) ² -gp188	SEQ ID NO: 40	SEQ ID NO: 2	SEQ ID NO: 21
(POLY) ³ -gp188	SEQ ID NO: 41	SEQ ID NO: 2	SEQ ID NO: 27
(POLY) ⁴ -gp188	SEQ ID NO: 42	SEQ ID NO: 2	SEQ ID NO: 30
pKKZ144pET32b	SEQ ID NO: 43	SEQ ID NO: 1	SEQ ID NO: 14
KRK_6_pET32b	SEQ ID NO: 44	SEQ ID NO: 1	SEQ ID NO: 10
KRK_12_pET32b	SEQ ID NO: 45	SEQ ID NO: 1	SEQ ID NO: 15
KRK_14_pET32b	SEQ ID NO: 46	SEQ ID NO: 1	SEQ ID NO: 16
R9_pET32b	SEQ ID NO: 47	SEQ ID NO: 1	SEQ ID NO: 12
K8_pET32b	SEQ ID NO: 48	SEQ ID NO: 1	SEQ ID NO: 13
pK2KZ144_pET32b_mod3	SEQ ID NO: 49	SEQ ID NO: 1	SEQ ID NO: 28
PKPSP3gp10	SEQ ID NO: 53	SEQ ID NO: 8	SEQ ID NO: 11
PKP2gp09	SEQ ID NO: 57	SEQ ID NO: 9	SEQ ID NO: 11
PKOBPgpLYS	SEQ ID NO: 61	SEQ ID NO: 7	SEQ ID NO: 11
pK2KZ144pET32b	SEQ ID NO: 62	SEQ ID NO: 1	SEQ ID NO: 22
pK3KZ144pET32b	SEQ ID NO: 63	SEQ ID NO: 1	SEQ ID NO: 27
pK4KZ144pET32b	SEQ ID NO: 64	SEQ ID NO: 1	SEQ ID NO: 30
KRK_19_pET32b	SEQ ID NO: 66	SEQ ID NO: 1	SEQ ID NO: 18
KRK_21_pET32b	SEQ ID NO: 67	SEQ ID NO: 1	SEQ ID NO: 23

KRK_25_pET32b	SEQ ID NO: 68	SEQ ID NO: 1	SEQ ID NO: 26
KRK_39_pET32b	SEQ ID NO: 69	SEQ ID NO: 1	SEQ ID NO: 29
K19_pET32b	SEQ ID NO: 70	SEQ ID NO: 1	SEQ ID NO: 20
K16_pET32b	SEQ ID NO: 71	SEQ ID NO: 1	SEQ ID NO: 17
pKKZ-144_K2_pET32b	SEQ ID NO: 72	SEQ ID NO: 1	N-terminal: SEQ ID NO: 11 C-terminal: SEQ ID NO: 21
pK2KZ144_pET32b_mod1	SEQ ID NO: 73	SEQ ID NO: 1	SEQ ID NO: 24
pK2KZ144_pET32b_mod2	SEQ ID NO: 74	SEQ ID NO: 1	SEQ ID NO: 25
smi01_KRK9	SEQ ID NO: 75	SEQ ID NO: 5	SEQ ID NO: 11
smi02_KRK9	SEQ ID NO: 76	SEQ ID NO: 4	SEQ ID NO: 11
smi03_KRK9	SEQ ID NO: 77	SEQ ID NO: 6	SEQ ID NO: 11
smi04_KRK9	SEQ ID NO: 78	SEQ ID NO: 3	SEQ ID NO: 11
SMAP-29-KZ144	SEQ ID NO: 139	SEQ ID NO: 1	SEQ ID NO: 94
Ply2638-PK	SEQ ID NO: 140	SEQ ID NO: 92	SEQ ID NO: 11
Pentapeptid-Ply511	SEQ ID NO: 141	SEQ ID NO: 85	SEQ ID NO: 135
PK-Lysostaphin	SEQ ID NO: 142	SEQ ID NO: 87	SEQ ID NO: 11

The fusion proteins according to the present invention, and thus in particular the especially preferred fusion proteins according to SEQ ID NO: 35 to 49, 53, 57, 61 to 64, 66 to 78 and 139 to 142 may additionally comprise a methionine on the N-terminus.

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The fusion proteins according to the present invention, and thus in particular the especially preferred fusion proteins according to SEQ ID NO: 35 to 49, 53, 57, 61 to 64, 66 to 78 and 139 to 142 may additionally comprise a tag e.g. for purification. Preferred is a His₆-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 His₆-tag at its C-terminus

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linked to the fusion protein by the additional amino acid residues lysine and glycine (Lys-Gly) or leucine and glutamic acid (Leu-Glu). Preferably, said additional amino acid residues may be not recognized or cleaved by proteases. In another preferred embodiment the fusion protein comprises a His₆-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 another preferred embodiment the fusion protein comprises a His₆-tag at its N- and C-terminus linked to the enzyme, preferably to the endolysin, autolysin or bacteriocin by the additional amino acid residues lysine and glycine (Lys-Gly) or leucine and glutamic acid (Leu-Glu).

10 In particular, the fusion proteins as used in the examples as described below are preferred. The fusion proteins according to SEQ ID NO: 35 to 42, 53, 57 and 61 as used in the examples comprise a His₆-tag at the C-terminus linked to the fusion protein by the additional amino acid residues lysine and glycine (Lys-Gly). The fusion protein according to SEQ ID NO: 43 to 49, 75, 139, 141 and 142 as used in the examples comprise a His₆-tag at the C-terminus linked to the respective fusion protein by the additional amino acid residues leucine and glutamic acid (Leu-Glu).

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, autolysin or bacteriocin and the respective peptide.

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).

For antimicrobial function of the fusion proteins it may be necessary to remove the additional fusion protein by proteolytic cleavage. Commercially available kits like the pET32 expression

system (Novagen), may need to modify e.g. the N-terminus of the fusion depending on the protease used, like from MGS to AMGS (SEQ ID NO: 31), were the remaining alanine residue results from an introduced Enterokinase cleavage site.

- 5 In another preferred embodiment of the present invention the peptides of the fusion proteins 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
10 amino-, SH- or carboxyl- groups.

The present invention further relates to methods of eliminating, reducing or preventing bacterial biofilms by means of an isolated nucleic acid molecule encoding the fusion protein according to the present invention. The present invention further relates to a vector
15 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 fusion proteins may be obtained from a micro-organism, such as a genetically modified
20 suitable host cell which expresses said fusion proteins. Said host cell may be a micro-organism such as bacteria or yeast or fungi or an animal cell as e.g. a mammalian cell, in particular a human cell. In one embodiment of the present invention the yeast 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-
25 pathological bacteria or yeast, human cells.

In a further aspect the present invention relates to methods of eliminating, reducing or preventing bacterial biofilms by means of a composition, preferably a pharmaceutical composition, comprising a fusion protein according to the present invention and/or a host
30 transformed with a nucleic acid molecule or a vector comprising a nucleotide sequence encoding a fusion protein according to the present invention.

- In a preferred embodiment of the present invention the methods of eliminating, reducing or preventing bacterial biofilms by means of the composition comprises additionally agents permeabilizing the outer membrane of Gram-negative bacteria such metal chelators as e.g. EDTA, TRIS, lactic acid, lactoferrin, polymyxin, citric acid and/or other substances as described e.g. by Vaara (Agents that increase the permeability of the outer membrane. Vaara M. Microbiol Rev. 1992 Sep;56(3):395-441). Also preferred are compositions comprising combinations of the above mentioned permeabilizing agents. Especially preferred is a composition comprising about 10 μ M to about 100 mM EDTA, more preferably about 50 μ M to about 10 mM EDTA, more preferably about 0.5 mM to about 10 mM EDTA, more preferably about 0.5 mM to about 2 mM EDTA, more preferably about 0.5 mM to 1 mM EDTA. However, also compositions comprising about 10 μ M to about 0.5 mM EDTA are preferred. Also preferred is a composition comprising about 0.5 mM to about 2 mM EDTA, more preferably about 1 mM EDTA and additionally about 10 to about 100 mM TRIS.
- 15 The present invention also relates to methods of eliminating, reducing or preventing bacterial biofilms by means of 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.
- 20 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 fusion protein according to the present invention for eliminating, reducing and preventing bacterial biofilms. Preferred is the use wherein the bacteria generating the biofilm cause a disorder, disease or condition detrimental to plants, animals and/or human beings. Preferred is the use wherein the bacteria generating the biofilm may be Gram-negative bacteria of bacterial groups, families, genera or species comprising strains pathogenic for humans or animals like *Enterobacteriaceae* (*Escherichia*, especially *E. coli*, *Salmonella*, *Shigella*, *Citrobacter*, *Edwardsiella*, *Enterobacter*, *Hafnia*, *Klebsiella*, especially *K. pneumoniae*, *Morganella*, *Proteus*, *Providencia*, *Serratia*, *Yersinia*), *Pseudomonadaceae* (*Pseudomonas*, especially *P. aeruginosa*, *Burkholderia*, *Stenotrophomonas*, *Shewanella*, *Sphingomonas*, *Comamonas*), *Neisseria*, *Moraxella*, *Vibrio*, *Aeromonas*, *Brucella*, *Francisella*, *Bordetella*, *Legionella*, *Bartonella*, *Coxiella*, *Haemophilus*, *Pasteurella*, *Mannheimia*, *Actinobacillus*, *Gardnerella*, *Spirochaetaceae*

(*Treponema* and *Borrelia*), *Leptospiraceae*, *Campylobacter*, *Helicobacter*, *Spirillum*, *Streptobacillus*, *Bacteroidaceae* (*Bacteroides*, *Fusobacterium*, *Prevotella*, *Porphyromonas*), *Acinetobacter*, especially *A. baumannii*. Preferably, said disorder, disease or condition may be caused by *Pseudomonas*, in particular *Pseudomonas aeruginosa* and/or *Pseudomonas putida*,
5 *Burkholderia*, in particular *Burkholderia pseudomallei* and/or *Burkholderia solanacearum*, *Salmonella*, in particular *Salmonella typhimurium* and/or *Salmonella Enteritidis*, *Acinetobacter*, in particular *Acinetobacter baumannii*, *Escherichia coli* and/or *Klebsiella*, in particular *Klebsiella pneumoniae*. In particular the treatment and/or prevention of the disorder, disease or condition may be caused by Gram-positive bacteria of bacterial groups,
10 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*,
15 *Mycobacterium tuberculosis*, *Corynebacterium diphtheriae*, *Mycoplasma pneumoniae*, *Actinomyces*.

In a preferred embodiment, the fusion protein, preferably the endolysin variant, autolysin variant or bacteriocin variant

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In a further aspect the present invention relates to a method of treating a disorder, disease or condition associated with bacterial biofilm 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
25 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.

Preferably said method of treatment may be for the treatment and/or prevention of infections
30 caused by Gram-negative and/or Gram-positive bacteria associated with bacterial biofilm, in particular by the Gram-negative and Gram-positive bacteria as listed above. 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-negative and/or Gram-positive bacteria associated with bacterial biofilm, in particular by the Gram-negative and Gram-positive bacteria as listed above.

- 5 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, inhalational, intravenous, intramuscular, intrathecal, intraspinal, endobronchial, intrapulmonal, intraosseous, intracardial, intraarticular, rectal, vaginal or any other route of
- 10 administration. In a preferred embodiment the fusion protein is applied topical to the biological material, preferably the skin, in particular of mammals, preferably of human. In a preferred embodiment the fusion protein is applied systemic to the biological material, preferably the blood, in particular of mammals, preferably of human.
- 15 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,
- 20 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
- 25 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
- 30 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 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 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 Gram-negative and/or Gram-positive bacterial infections associated with bacterial biofilm. A preferred embodiment relates to the use of 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 Gram-negative and/or Gram-positive bacterial infections associated with bacterial biofilm in combination or addition to antibiotics.

In a specific embodiment 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 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 *Pseudomonas* associated with bacterial biofilm, particularly by *Pseudomonas aeruginosa* in particular intestinal affections, in particular in infants, infections of the meninges, e.g. meningitis haemorrhagica, infections of the middle ear, the skin (*Ecthyma gangraenosum*), in particular burns, the urinary tract, rhinitis, bacteremic pneumonia, in particular wherein the patient is suffering from cystic fibrosis or hematologic malignancies such as leukemia, or with neutropenia from immunosuppressive therapy, septicemia, in particular because of long-term intravenous or urinary catheterization, invasive surgical procedures and severe burns, endocarditis, in particular wherein the patient is a intravenous drug user or a patient with complications from open heart surgery, highly destructive ocular infections, in particular after the use of contaminated ophthalmologic solutions or severe facial burns, osteochondritis, in particular as a result of severe trauma or puncture wounds through contaminated clothing.

In another specific embodiment of the present invention the disorder, disease or condition is caused by *Burkholderia pseudomallei* associated with bacterial biofilm, in particular Whitmore's Disease, chronic pneumonia, septicemia, in particular wherein the patient has a traumatized skin lesion. In another specific embodiment of the present invention the disorder, disease or condition is caused by *Salmonella typhimurium* and *Salmonella enteritidis*

associated with bacterial biofilm, in particular acute gastroenteritis and local purulent processes, particularly osteomyelitis, endocarditis, cholecystitis and especially caused by *Salmonella thyphimurium* meningitis, in particular wherein the patient is less than two years old. In another specific embodiment of the present invention the disorder, disease or condition

5 is caused by *Salmonella typhi*, in particular typhus. In another specific embodiment of the present invention the disorder, disease or condition is caused by *Salmonell paratyphi*, in particular paratyphus. In another specific embodiment of the present invention the disorder, disease or condition is caused by *Acinetobacter baumannii* associated with bacterial biofilm, in particular bronchitis, pneumonia, wound infections and septicemia, in particular as a result

10 of intravenous catheterization. In another specific embodiment of the present invention the disorder, disease or condition is caused by *Escherichia coli* associated with bacterial biofilm, in particular extra intestinal infections, particularly appendicitis, purulent cholecystitis, peritonitis, purulent meningitis and infection of the urinary tract, intrainestinal *E. coli* infections, particularly epidemic enteritis, and infectious disease similar to dysentery, septicemia, enterotoxemia, mastitis and dysentery. In another specific embodiment of the

15 present invention the disorder, disease or condition is caused by *Klebsiella pneumoniae* associated with bacterial biofilm, in particular pneumonia, bacteremia, meningitis and infections of the urinary tract. In a specific embodiment 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 fusion protein according to the present invention in the manufacture of a medicament for the

20 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.

25 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

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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.

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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

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specific embodiment of the present invention the disorder, disease or condition is caused by *Clostridium difficile*, in particular pseudomembranous enterocolitis. 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 nosocomial infections, and endocarditis.

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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

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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.

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In another specific embodiment of the present invention the disorder, disease or condition is caused by *Corynebacterium diphtheriae*, 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 malignant diphtheria, skin and wound diphtheria.

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The methods of eliminating, reducing or preventing bacterial biofilms by means of fusion proteins according to the present invention provide a possibility to invade into the bacterial biofilm and eliminate, reduce and prevent the bacterial biofilm.

The methods of eliminating, reducing or preventing bacterial biofilms by means of fusion proteins according to the present invention may be for the treatment and/or prevention of the following infections: wound infections, in particular wounds associated with diabetes mellitus, tonsillitis, osteomyelitis, bacterial endocarditis, sinusitis, infections of the cornea, urinary tract infection, infection of the biliary tract, infectious kidney stones, urethritis, prostatitis, catheter infections, middle-ear infections, formation of dental plaque, gingivitis, periodontitis, cystic fibrosis, and infections of permanent indwelling devices such as joint prostheses and heart valves.

- 10 In another preferred embodiment of eliminating, reducing and preventing bacterial biofilms by means of fusion proteins according to the present invention may be for the treatment and/or prevention of infections associated with foreign matter, like contamination and colonisation of catheters, implants and medical devices, in particular instruments, apparatus, endoscopes, dental devices, dialysis equipment, like peritoneal dialysis catheter, pacemaker, endotracheal tubes, voice prosthesis, cerebrospinal fluid shunts, venous catheter, artificial heart valves and joint prosthesis.

- In another preferred embodiment of eliminating, reducing and preventing bacterial biofilms by means of fusion proteins according to the present invention the bacterial biofilm is formed by *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Candida albicans*.

- In another preferred embodiment of eliminating, reducing and preventing bacterial biofilms by means of fusion proteins according to the present invention may be for the prevention or removal of contaminations in health care, agriculture, and industrial settings, in particular in water pipes of hospitals, in water, plumbing, ventilation, building heating, air conditioning, oil wells, cosmetics and medicaments.

- In another preferred embodiment of eliminating, reducing and preventing bacterial biofilms by means of fusion proteins according to the present invention may be for the prevention of biocorrosion, in particular in cooling circuits, water treatment plants, domestic hot water systems, power plants, production systems and machineries for automobiles, computers, colours, oil and gas.

In another preferred embodiment of eliminating, reducing and preventing bacterial biofilms by means of fusion proteins according to the present invention may be for the prevention of biofouling, in particular on submarine objects, ships, platforms, buoys, sensor systems for scientific or surveillance purposes in the maritime field.

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In a preferred embodiment of the present invention 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 associated with bacterial biofilm, in particular by strains resistant against one or more of the following antibiotics: streptomycin, tetracycline, cephalothin, gentamicin, cefotaxime, cephalosporin, ceftazidime or imipenem.

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Furthermore, in the methods or the use of the present invention the fusion protein, preferably the endolysin variant, the autolysin variant or bacteriocin variant can be used, added or administered in combination or in addition with conventional antibacterial agents, such as antibiotics, lantibiotics, bacteriocins or endolysins. In another preferred embodiment, the antibiotics are added, used or administered in the methods and the use according to the present invention simultaneous with the fusion protein, after or before the administration or addition of fusion protein.

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In a preferred embodiment of the present invention the fusion protein can be used or administered in combination with at least one of the following antibiotics: β -lactams, aminoglycosides, fluoroquinolones, macrolides, novobiocin, rifampicin, oxazolidinones, fusidic acid, mupirocin, pleuromutilins, daptomycin, vancomycin, tetracyclines, sulfonamides, chloramphenicol, trimetoprim, fosfomycin, cycloserine and polymyxin.

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In another preferred embodiment of the present invention the fusion protein can be used in methods of eliminating, reducing or preventing bacterial biofilms of *Staphylococcus aureus* by administering it in combination with at least one of the following antibiotics: β -lactams, aminoglycosides, fluoroquinolones, macrolides, novobiocin, rifampicin, oxazolidinones, fusidic acid, mupirocin, pleuromutilins, daptomycin, vancomycin, tetracyclines, sulfonamides, chloramphenicol, trimetoprim, fosfomycin and cycloserine.

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In another preferred embodiment of the present invention a fusion protein can be used in methods of eliminating, reducing or preventing bacterial biofilms of *Escherichia coli* by administering it in combination with at least one of the following antibiotics: β -lactams, aminoglycosides, fluoroquinolones, tetracyclines, sulfonamides, chloramphenicol, trimetoprim, fosfomycin, cycloserine and polymyxin.

In another preferred embodiment of the present invention a fusion protein can be used in methods of eliminating, reducing or preventing bacterial biofilms of *Pseudomonas aeruginosa* by administering it in combination with at least one of the following antibiotics: β -lactams, aminoglycosides, fluoroquinolones and polymyxin.

The present invention also relates to a pharmaceutical pack for use of eliminating, reducing and preventing bacterial biofilm 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 for use of eliminating, reducing and preventing bacterial biofilm, 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 for use of eliminating, reducing and preventing bacterial biofilm. 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-negative and/or 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-negative and/or Gram-positive bacteria associated with bacterial biofilm. In this respect the fusion protein according to the present invention may be used as a tool to specifically degrade pathogenic bacteria associated with bacterial biofilm, in particular Gram-negative and/or 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 removal, reduction and/or prevention of Gram-negative and/or Gram-positive bacterial contamination associated with bacterial biofilm 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 in methods of eliminating, reducing or preventing bacterial biofilms 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 *Pseudomonas aeruginosa* (FQRP), *Acinetobacter* species and Enterobacteriaceae such as *E.coli*, *Salmonella*, *Shigella*, *Citrobacter*, *Edwardsiella*, *Enterobacter*, *Hafnia*, *Klebsiella*, *Morganella*, *Proteus*,

Providencia, *Serratia* and *Yersinia* species; *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

5 the present invention. Therefore, a fusion protein according to the present invention may be used as a disinfectant to eliminate, reduce and prevent bacterial biofilms also in combination with other ingredients useful in a disinfecting solution like detergents, tensids, solvents, antibiotics, lantibiotics, or bacteriocins.

10 For the use of the fusion protein according to the present invention for eliminating, reducing or preventing bacterial biofilms 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

15 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

20 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.

25 In a further aspect the present invention relates to the use of the fusion protein according to the present invention as a food additive.

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

30 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.

- 5 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.

10 EXAMPLE 1: Cloning, expression and purification of modified phiKZgp144 and ELgpgp188 endolysin variants.

phiKZgp144 as depicted in SEQ ID NO: 1 and ELgp188 as depicted in SEQ ID NO: 2 are modular endolysins originating from *Pseudomonas aeruginosa* phages ϕ KZ and EL with an N-terminal peptidoglycan binding and C-terminal catalytic domain (Briers et al., 2007).

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For the amplification of the open reading frame (ORF) of phiKZgp144 and ELgp188 PCR a standard 5' primer (for phiKZgp144: 5' ATGAAAGTATTACGCAA 3' (SEQ ID NO: 83); for ELgp188 5' ATGAACTTCCGGACGAAG 3' (SEQ ID NO: 65)) and the standard 3' primers according to SEQ ID NO: 81 and 82 were applied (for phiKZgp144: 20 TTTTCTATGTGCTGCAAC (SEQ ID NO: 81); for ELgp188: ATACGAAAT AACGTGACGA (SEQ ID NO: 82)) was used. To extend the 5' end of the open reading frame encoding phiKZgp144 or ELgp188 with a gene fragment encoding nine positively charged residues (Lys-Arg-Lys-Lys-Arg-Lys-Lys-Arg-Lys – SEQ ID NO: 11) a tail PCR with an extended 5' primer (for phiKZgp144: 5' ATGGGATCCAAACGCAAGAAACGTAAGAAA 25 CGCAAAAAGTATTACGCAAAG 3' (SEQ ID NO 79); for ELgp188: 5' ATGGGATCCAAACGCAAGAAACGTAAGAAA CGCAAAAACCTTCCGGACGAAG 3' (SEQ ID NO: 80)) and the standard 3' primers according to SEQ ID NO: 81 and 82 were applied. The PCR product was cloned in the pEXP5CT/TOPO® expression vector (Invitrogen, Carlsbad, CA, USA) according to the protocol of the manufacturer. Arginine 30 triplets were incorporated besides lysine triplets to avoid tRNA depletion and reduce the risk of frameshifts (the only two available triplets for lysine are AAA and AAG, leading to long A-stretches). Insertion of additional polycationic cassettes into the designed BamHI restriction site lengthens the tail with extra cationic residues. This insertion creates an arginine

and serine triplet at each junction site (Figure 1). Up to four polycationic peptides were fused to both phiKZgp144 and ELgp188, designated (POLY)ⁿ-gp144 or (POLY)ⁿ-gp188 (n=1,2,3,4), comprising respectively 9, 19, 29 and 39 positively charged amino acid residues in the N-terminus. Accordingly, the following constructs were expressed in *E. coli* BL21 (DE3) pLysS cells (exponentially growing cells at 37°C, induction using 1mM IPTG, expression for 4 h at 37°C):

Fusion protein	SEQ ID NO:	Number of positively charged amino acid residues
POLY-gp144	SEQ ID NO: 35	9
(POLY) ² -gp144	SEQ ID NO: 36	19
(POLY) ³ -gp144	SEQ ID NO: 37	29
(POLY) ⁴ -gp144	SEQ ID NO: 38	39
POLY-gp188	SEQ ID NO: 39	9
(POLY) ² -gp188	SEQ ID NO: 40	19
(POLY) ³ -gp188	SEQ ID NO: 41	29
(POLY) ⁴ -gp188	SEQ ID NO: 42	39

The modified endolysin variants POLY-gp144 (SEQ ID NO: 35), (POLY)²-gp144 (SEQ ID NO: 36), POLY-gp188 (SEQ ID NO: 39) and (POLY)²-gp188 (SEQ ID NO: 40) have been used for further investigations. Said proteins were purified by Ni²⁺ affinity chromatography using the C-terminal 6xHis-tag (Akta Fast Protein Liquid Chromatography using 1ml His-trap Ni-NTA columns). The total yields per liter *E. coli* expression culture were determined by spectrophotometric measurement of the protein concentration and the total volume of the purified stock solution. The purification of gp188 derivatives was performed under more stringent conditions (65 mM imidazole) compared to gp144 derivatives (50mM imidazole) to ensure high purity. The total yields per liter *E. coli* expression culture are shown in table 5.

Table 5 - Yields of recombinant purification of endolysin derivatives per liter *E. coli* expression culture.

	Fusion	Endolysin	
		phiKZgp144	ELgp188
	POLY	2 mg	48 mg
5	(POLY) ²	0.5 mg	0.06 mg

Purified stock solutions were ~90% pure. Mass spectrometric analysis of purified solutions of POLY-derivatives revealed traces of the *E. coli* 50S ribosomal subunit protein L2 and 16S rRNA uridine-516 pseudo-uridylyate synthase. All phiKZgp144 derivatives showed multimer formation which could be converted to monomers by addition of β -mercaptoethanol, indicating that interdisulfide bonds cause multimerization.

EXAMPLE 2: Antibacterial activity of modified phiKZgp144 and ELgp188 variants

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Exponential ($\sim 10^6$ /ml) *P. aeruginosa* PAO1p cells (Pirnay JP et al. (2003), *J Clin Microbiol.*, 41(3):1192-1202) were 100 x diluted (final density was $\sim 10^6$ /ml) and incubated at room temperature with each 10 μ g undialyzed protein (unmodified endolysins phiKZgp144 (SEQ ID NO: 1) and ELgp188 (SEQ ID NO: 2) and modified endolysin variants POLY-gp144 (SEQ ID NO: 35), (POLY)²-gp144 (SEQ ID NO: 36), POLY-gp188 (SEQ ID NO: 39) and (POLY)²-gp188 (SEQ ID NO: 40) at a final concentration of 100 μ g/ml in buffer (20 mM NaH₂PO₄-NaOH pH7.4; 0.5 M NaCl; 0.5 M imidazole). After 1 hour cell suspensions were diluted in PBS buffer (10e-5, 10e-4 and 10e-3) and plated (standard LB-medium, incubated overnight at 37°C). Additionally, a negative control containing cells in PBS buffer was plated.

25 The residual colonies were counted after an overnight incubation. Based on the counted cell numbers the antibacterial activity as the relative inactivation (%) ($=100-(N_i/N_0)*100$ with N_0 = number of untreated cells and N_i = number of treated cells) and in logarithmic units ($=\log_{10}N_0/N_i$) was calculated (Table 6). All samples were replicated in six fold. Averages/standard deviations are represented. Statistical analysis was performed using a student's t-test.

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Unmodified endolysins phiKZgp144 and ELgp188 do not reduce cell numbers significantly compared to the negative control. This observation illustrates the efficacy of the outer

membrane as a barrier for the endolysin to degrade the cell wall of the Gram-negative bacteria. In contrast as shown in Table 6 the incubation with the modified endolysins POLY-gp144, (POLY)²-gp144, POLY-gp188 and (POLY)²-gp188 causes a significant reduction ($\alpha = 0.05$) of the bacterial cell number (99.85 ± 0.09 % for POLY-gp144 and 98.0 ± 0.2 % for POLY-gp188). An increase of the length of the polycationic peptide further tends to strengthen the antibacterial activity, especially in case of phiKZgp144 (a reduction up to 99.98 ± 0.02 % or 3.7 ± 0.3 log units is achieved within 1 hour for (POLY)²-gp144). Moreover, the experiments demonstrated that the modified endolysins of phiKZgp144 have a higher antibacterial activity than the modified endolysins of ELgp188.

Table 6 - Antibacterial effect of endolysins unmodified and modified phiKZgp144 and ELgp188 variants.

Exponentially growing cells	Endolysins			
	phiKZgp144		ELgp188	
	%	log	%	log
unmodified endolysin	0 ± 15	0.00 ± 0.06	10 ± 13	0.05 ± 0.06
POLY	99.85 ± 0.09	2.9 ± 0.3	98.0 ± 0.2	1.7 ± 0.1
(POLY) ²	99.98 ± 0.02	3.7 ± 0.3	98.9 ± 0.4	2.0 ± 0.2

Thus, the example demonstrated that the addition of a short peptide of nine cationic residues N-terminally to phiKZgp144 (SEQ ID NO: 1) is already sufficient to kill almost 99.9% of the cells within 1 hour. Poly-L-Lysine has intrinsic antibacterial activity as well, although this property is so far only ascribed to polymers of at least 20 residues (Vaara and Vaara, 1983a, 1983b). However, the concerted action of the polycationic peptide and the endolysin kills the cells.

In a further experiment the modified endolysin POLY-gp144 was dialyzed to 50 mM KH₂PO₄/K₂HPO₄ pH 7 and used instead of undialyzed protein solution as described above. Thereby, the inactivation level was additionally increased from 2.9 ± 0.3 log units to 3.9 ± 0.2 log units.

EXAMPLE 3: Expression of modified phiKZgp144 and ELgp188 variants in *Pichia pastoris* as a host for non-toxic recombinant production

The open reading frame encoding POLY-gp144 (SEQ ID NO: 35) was cloned in the pPICZ α A shuttle vector (Invitrogen), which was subsequently integrated in the *P. pastoris* genome by homologous recombination (as indicated by the manufacturer; *P. pastoris* X33 cells, Invitrogen). Gene expression was induced with methanol (1%) in BMMY-medium and the supernatant was analyzed for the presence of enzymatic activity after 1, 3 and 4 days. Therefore, an amount of 30 μ l supernatant of the *P. pastoris* expression culture was added to 270 μ l chloroform-permeabilized *P. aeruginosa* PAO1p cells (Pirnay JP et al. (2003), *J Clin Microbiol.*, 41(3):1192-1202) after 1, 3 and 4 days (buffer condition: KH₂PO₄/K₂HPO₄ I = 120 mM pH 6.2). Subsequently, the optical density was spectrophotometrically recorded (Figure 2). A drop in optical density indicates the secretion of a muralytic enzyme by *P. pastoris*. As a negative control, *P. pastoris* X33 without expression plasmid was included. Thus, the lysis of the substrate upon addition of the supernatants sample is a measure for successful recombinant production and secretion of POLY-gp144 (SEQ ID NO: 35) by *P. pastoris*. After 1 day, a limited enzymatic activity could be detected. The maximum activity was observed after 3 days since no significant increase of activity in the supernatants was observed at the fourth day. No toxic effect on the cell density of *P. pastoris* was observed.

During expression by *P. pastoris* the α -secretion signal of the vector causes secretion of the recombinant protein to the surrounding media, which allows a simplify purification since only a limited number of other proteins is secreted. A BamHI restriction site in the 5' end of the open reading frames enables the addition of more cassettes encoding additional polycationic peptides.

EXAMPLE 4: Further modified endolysin phiKZgp144 variants with different polycationic peptides

To test and to compare the potential of polycationic peptides variants of phiKZgp144 and other endolysin encoding genes were synthesised having different polycationic peptides at the N-terminal end of the protein. Peptide variation concerns length, composition and insertion of

linker sequences. On the one hand further polycationic peptides having N-terminal multiples of the KRK motive were produced. On the other hand polycationic peptides consisting only of arginine (R) or lysine (K) were produced. Moreover, to enhance the translation of long polycationic peptides, polycationic peptides comprising a linker sequence were produced.

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The different products were cloned in the pET32b expression vector (Novagen, Darmstadt, Germany). pET32b was used to reduce potential toxicity of the polycationic peptide against the *E. coli* host. A vector-encoded fusion protein (thioredoxin) masks the polycationic peptide and can be eliminated during the purification process.

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Accordingly, the following modified endolysin variants were expressed in *E. coli* BL21 (DE3) cells at 37°C until an optical density of OD_{600nm}=0.6 was reached. Then protein expression was induced with 1 mM IPTG (final concentration) and expression was performed for four hours. Then *E. coli* cells were harvested by centrifugation for 20 min at 6000g and cell disruption and protein purification was performed according the S-tag purification kit (Novagen, Darmstadt, Germany):

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Modified endolysin variant	peptide length	Sequence of the peptide
phiKZgp144 (SEQ ID NO: 1)	0	-
pKKZ144pET32b (SEQ ID NO: 43)	10	KRKKRKKRKK (SEQ ID NO: 14)
KRK_6_pET32b (SEQ ID NO: 44)	6	KRKKRK (SEQ ID NO: 10)
KRK_12_pET32b (SEQ ID NO: 45)	12	KRKKRKKRKKRK (SEQ ID NO: 15)
KRK_14_pET32b (SEQ ID NO: 46)	14	KRKKRKKRKKRKKR (SEQ ID NO: 16)
R9_pET32b (SEQ ID NO: 47)	9	RRRRRRRRR (SEQ ID NO: 12)
K8_pET32b (SEQ ID NO: 48)	8	KKKKKKKK (SEQ ID NO: 13)
pK2KZ144_pET32b_mod3 (SEQ ID NO: 49)	38	KRKKRKKRKRGS GSGKRKKRKKRKGSGSGKRKKRKKRK (SEQ ID NO: 28)

All proteins were purified using the *S-TagTM rEK Purification Kit* (Novagen, Darmstadt, Germany). Using the pET32b vector, the expressed proteins were not toxic to the host resulting in high yields of produced protein. Purified stock solutions showed high purity.

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Exponential ($\sim 10^6$ /ml) *P. aeruginosa* PAO1p cells (Burn wound isolate, Queen Astrid Hospital, Brussels; Pirnay JP et al. (2003), *J Clin Microbiol.*, 41(3):1192-1202) were 100 x diluted (final density was $\sim 10^6$ /ml) incubated at room temperature with each 10 μ g undialyzed protein as listed above at a final concentration of 100 μ g/ml in buffer (20 mM NaH₂P0₄-NaOH pH7.4; 0.5 M NaCl; 0.5 M imidazole). After 1 hour cell suspensions were diluted 1:100 and plated on LB. Additionally, a negative control was plated using buffer (20 mM NaH₂P0₄-NaOH pH7.4; 0.5 M NaCl; 0.5 M imidazole). The residual colonies were counted after an overnight incubation at 37°C. Based on the counted cell numbers the antibacterial activity as the relative inactivation (%) ($=100-(N_i/N_0)*100$ with N_0 = number of untreated cells and N_i = number of treated cells) was calculated (Table 7). All samples were replicated at least in four fold.

Table 7 - Antibacterial effect of endolysins unmodified and modified phiKZgp144 and ELgp188

Modified endolysin variant	Sequence of the peptide	Reduction [%]
phiKZgp144 (SEQ ID NO: 1)		0
pKKZ144pET32b (SEQ ID NO: 43)	KRKKRKKRKK (SEQ ID NO: 14)	99 – 99.9
KRK_6_pET32b (SEQ ID NO: 44)	KRKKRK (SEQ ID NO: 10)	99.9
KRK_12_pET32b (SEQ ID NO: 45)	KRKKRKKRKKRK (SEQ ID NO: 15)	99 – 99.9
KRK_14_pET32b (SEQ ID NO: 46)	KRKKRKKRKKRKKR (SEQ ID NO: 16)	99.9
R9_pET32b (SEQ ID NO: 47)	RRRRRRRRR (SEQ ID NO: 12)	99
K8_pET32b (SEQ ID NO: 48)	KKKKKKKK (SEQ ID NO: 13)	99
pK2KZ144_pET32b_mod3 (SEQ ID NO: 49)	KRKKRKKRKRGSKGKRKKRKKRKGSGSGKRKKRKKRK (SEQ ID NO: 28)	99.9

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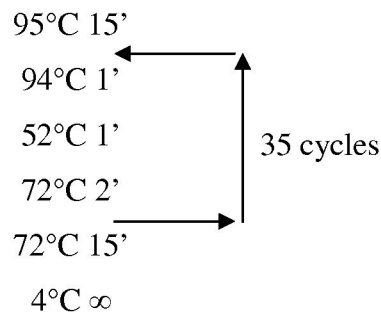
Unmodified phiKZgp144 does not reduce cell numbers significantly compared to the negative control. Beyond that, modified phiKZgp144 variants wearing a polycationic peptide of N-terminal multiples of the KRK motive enhance the antimicrobial effect immensely. However, also variants having a homomer peptide of lysine or arginine show significant reduction of cells compared with unmodified phiKZgp144 as measured. Moreover, also the variant having a polycationic peptide of 38 amino acid residues and comprising a linker sequence enhance the antimicrobial effect immensely.

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EXAMPLE 5: Modified endolysin variants of *Salmonella typhimurium* phage PSP3

PSP3gp10 according to SEQ ID NO: 8 is a globular endolysin with 165 amino acid residues originating from *Salmonella typhimurium* phage PSP3 with a catalytic lambda-like muramidase domain. As predicted by BLASTp and Pfam analysis the PSP3gp10 endolysin comprises its catalytic domain in the range of about amino acid residue 34 to about amino acid residue 152.

Purified genomic DNA of phage PSP3 was used as a template for the amplification of the open reading frame (ORF) of PSP3gp10 in a *Hot Start Taq polymerase PCR* reaction (Qiagen, Germany) using the following PCR parameters:



For said PCR a standard 5' primer (5' ATGGGATCCCCGGTCATTAATACTCACCAG 3' (SEQ ID NO: 50)) and a standard 3' primer (5' TGCCATCACCCCGCCAGCCGTG 3' (SEQ ID NO: 51)) was used. To extend the 5' end of the ORF which encodes PSP3gp10 with a gene fragment encoding the polycationic 9-mer peptide *Lys-Arg-Lys-Lys-Arg-Lys-Lys-Arg-Lys* (SEQ ID NO: 11) a tail PCR (*Hot Start Taq polymerase PCR* with same parameters) with an extended 5' primer (5' ATGGGATCCAAACGCAAGAAACGTAA GAAACGCAAACCGGTCATTAATACTCACCAG 3' (SEQ ID NO: 52)) and the standard 3' primer according to SEQ ID NO: 51 was applied. Both the original unmodified PSP3gp10 PCR fragment and the PK-extended fragment were ligated in the pEXP5CT/TOPO® expression vector (Invitrogen, Carlsbad, CA, USA) by following the TA-cloning protocol of the manufacturer.

Recombinant expression of PSP3gp10 according to SEQ ID NO: 8 and PKPSP3gp10 according to SEQ ID NO: 53 is performed in exponentially growing *E. coli* BL21 (λDE3)

pLysS cells (Invitrogen) after induction with 1 mM IPTG (isopropylthiogalactoside) at 37°C for a period of 4 hours. Both proteins were purified by Ni²⁺ affinity chromatography (Akta FPLC, GE Healthcare) using the C-terminal 6xHis-tag, encoded by the pEXP5CT/TOPO® expression vector. The Ni²⁺ affinity chromatography is performed in 4 subsequent steps, all on room temperature:

1. *Equilibration* of the *Histrap HP 1 ml* column (GE Healthcare) with 10 column volumes of Washing Buffer (60 mM imidazole, 0.5 mM NaCl and 20 mM NaH₂P0₄-NaOH on pH 7.4) at a flow rate of 0.5 ml/min.
2. *Loading* of the total lysate (with wanted endolysin) on the *Histrap HP 1 ml* column at a flow rate of 0.5 ml/min.
3. *Washing* of the column with 15 column volumes of Washing Buffer at a flow rate of 1 ml/min.
4. *Elution* of bounded endolysin from the column with 10 column volumes of Elution Buffer (500 mM imidazole, 5 mM NaCl and 20 mM NaH₂P0₄-NaOH on pH 7.4) at a flow rate of 0.5 ml/min

The total yields of both purified recombinant proteins per liter *E.coli* expression culture shown in Table 8. The values were determined by spectrophotometric measurement of the protein concentration and the total volume of the purified stock solution at a wavelength of 280 nm. Purified stock solutions consisting of PSP3gp10 and PKPSP3gp10, respectively, in Elution Buffer (20 mM NaH₂P0₄-NaOH pH7.4; 0.5 M NaCl; 500 mM imidazole) were at least 90% pure as determined visually on SDS-PAGE gels.

Table 8 - Yields of purified recombinant PSP3gp10 endolysin and its modified variant PKPSP3gp10 per liter E. coli expression culture.

Endolysins	Expression yield
PSP3gp10 (SEQ ID NO: 8)	2.15 mg
PKPSP3gp10 (SEQ ID NO: 53)	5.56 mg

To determine the anti-Gram-negative spectrum of the PKPSP3gp10 endolysin according to SEQ ID NO: 53, a combination of 1.315 µM PKPSP3gp10 endolysin and 0,5 mM EDTA was tested on the clinical *P. aeruginosa* strains PAO1p and Br667, *Escherichia coli* WK6, and *Salmonella typhimurium* (see Table 9). Exponential growing bacterial cells (OD_{600nm} of 0.6)

were 100-fold diluted to a final density of about 10^6 /ml of each strain were incubated for 30 minutes at room temperature without shaking with unmodified endolysin PSP2gp10 (SEQ ID NO: 8) and modified endolysin PKPSP3gp10 (SEQ ID NO: 53) each in combination without and with 0.5 mM EDTA. For incubation, the endolysins were used each in buffer (20 mM $\text{NaH}_2\text{P}_2\text{O}_7$ -NaOH pH7.4; 0.5 M NaCl; 0.5 M imidazole) and the incubation took place at a final concentration of endolysin of 1.315 μM . As a control each strain was also incubated for 30 minutes with 0.5 mM EDTA (in same buffer as outlined above) but no endolysin.

Table 9 – List of used Gram-negative strains

Gram-negative strain	Source	Reference
<i>Pseudomonas aeruginosa</i> PAO1p	Burn wound isolate, Queen Astrid Hospital, Brussels	Pirnay <i>et al.</i> , 2003*
<i>Pseudomonas aeruginosa</i> Br667	Burn wound isolate, Queen Astrid Hospital, Brussels	Pirnay <i>et al.</i> , 2003*
<i>Escherichia coli</i> WK6	Standard laboratory expression strain	Prof. C. Michiels
<i>Salmonella typhimurium</i> LT2	SGSC N° 2317	Prof. C. Michiels

*Pirnay JP *et al.* (2003). Molecular epidemiology of *Pseudomonas aeruginosa* colonization in a burn unit: persistence of a multidrug-resistant clone and a silver sulfadiazine-resistant clone. *J Clin Microbiol.*, 41(3):1192-1202.

After incubation cell suspensions were diluted three times (respectively 10^5 - 10^4 - 10^3 cells/ml) and 100 μl of each dilution was plated out on LB-medium. The residual colonies were counted after an overnight incubation on 37°C. Based on the counted cell numbers the antibacterial activity as the relative inactivation in logarithmic units ($=\log_{10}N_0/N_i$ with N_0 = number of untreated cells and N_i = number of treated cells) was calculated (Table 10).

Table 10 - Antibacterial activity of unmodified endolysin (PSP3gp10) and its modified endolysin variant (PKPSP3gp10) with and without EDTA- Na_2 on different exponential growing Gram-negative species.

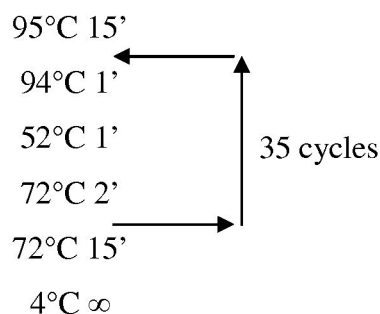
	0.5 mM EDTA	1.315 μ M PSP3gp 10	1.315 μ M PKPSP3 gp10	1.315 μ M PSP3gp10 + 0.5 mM EDTA	1.315 μ M PKPSP3gp10 + 0.5 mM EDTA
<i>P. aeruginosa</i> PAO1p	0.146 +/- 0.002	0.383 +/- 0.015	0.344 +/- 0.163	3.552 +/- 0.536	> 4.146
<i>P. aeruginosa</i> Br667	0.223 +/- 0.038	0.375 +/- 0.056	0.353 +/- 0.086	0.571 +/- 0.035	0.891 +/- 0.118
<i>Salmonella</i> <i>typhimurium</i>	0.104 +/- 0.049	0.283 +/- 0.038	0.327 +/- 0.057	0.690 +/- 0.036	0.850 +/- 0.032
<i>Escherichia</i> <i>coli</i> WK6	0.393 +/- 0.035	0.190 +/- 0.029	0.205 +/- 0.088	0.387 +/- 0.014	0.584 +/- 0.024

All samples were replicated in threefold. Averages +/- standard deviations are represented. The maximal reduction observed is dependent on the detection level of 10 cells/ml and the initial cell density. For PAO1p, EDTA works synergistically with both the unmodified PSP3gp10 endolysin and its modified variant PKPSP3gp10.

EXAMPLE 6: Modified endolysin variants of *Escherichia coli* phage P2

P2gp09 according to SEQ ID NO: 9 is a globular endolysin of 165 amino acid residues originating from *Escherichia coli* phage P2 with a catalytic lambda-like muramidase domain. As predicted by BLASTp and Pfam analysis the P2gp09 endolysin comprises its catalytic domain in the range of about amino acid residue 34 to about amino acid residue 152.

Purified genomic DNA of phage P2 was used as a template for the amplification of the open reading frame (ORF) of P2gp09 in standard PCR reaction with Pfu polymerase (Fermentas) using the following PCR parameters:



For said PCR a standard 5' primer (5' ATGGGATCCCCGGTAATTAACACGCATC 3' (SEQ ID NO: 54)) and a standard 3' primer (5' AGCCGGTACGCCGCCAGCGGTACGC 3' (SEQ ID NO: 55)) was used. To extend the 5' end of the ORF which encodes P2gp09 with a gene fragment encoding the polycationic 9-mer peptide *Lys-Arg-Lys-Lys-Arg-Lys-Lys-Arg-Lys* (SEQ ID NO: 11) a tail PCR (with same parameters as standard PCR above) with an extended 5' primer (5' ATGGGATCCAAACGCAAGAAACGTAAGAAACGC AAACCGGTAATTAACACGCATC 3' (SEQ ID NO: 56) and the standard 3' primer according to SEQ ID NO 55 was applied. Both the original unmodified P2gp09 PCR fragment and the extended fragment were ligated in the pEXP5CT/TOPO® expression vector (Invitrogen, Carlsbad, CA, USA) by following the TA-cloning protocol of the manufacturer. Recombinant expression of P2gp09 according to SEQ ID NO: 9 and PKP2gp09 according to SEQ ID NO: 57 is performed in exponentially growing *E. coli* BL21 (λ DE3) pLysS cells (Invitrogen) after induction with 1 mM IPTG (isopropylthiogalactoside) at 37°C for a period of 4 hours. Both proteins were purified by Ni²⁺ affinity chromatography (Akta FPLC, GE Healthcare) using the C-terminal 6xHis-tag, encoded by the pEXP5CT/TOPO® expression vector. The Ni²⁺ affinity chromatography is performed in 4 subsequent steps, all on room temperature:

1. *Equilibration* of the *Histrap HP 1 ml* column (GE Healthcare) with 10 column volumes of Washing Buffer (60 mM imidazole, 0.5 mM NaCl and 20 mM NaH₂P0₄-NaOH on pH 7.4) at a flow rate of 0.5 ml/min.
2. *Loading* of the total lysate (with wanted endolysin) on the *Histrap HP 1 ml* column at a flow rate of 0.5 ml/min.
3. Washing of the column with 15 column volumes of Washing Buffer at a flow rate of 1 ml/min.
4. Elution of bounded endolysin from the column with 10 column volumes of Elution Buffer (500 mM imidazole, 5 mM NaCl and 20 mM NaH₂P0₄-NaOH on pH 7.4) at a flow rate of 0.5 ml/min

The total yields of both purified recombinant proteins per liter *E.coli* expression culture shown in Table 11. The values were determined by spectrophotometric measurement of the protein concentration and the total volume of the purified stock solution at a wavelength of 280 nm. Purified stock solutions consisting of P2gp09 and PKP2gp09, respectively, in Elution

Buffer (20 mM NaH₂P0₄-NaOH pH7.4; 0.5 M NaCl; 500 mM imidazole) were at least 95% pure as determined visually on SDS-PAGE gels.

Table 11 - Yields of purified recombinant P2gp09 endolysin and its PK-modified derivative PKP2gp09 per liter *E. coli* expression culture.

Endolysins	Expression yield
P2gp09 (SEQ ID NO: 9)	5.52 mg
PKP2gp09 (SEQ ID NO: 57)	3.40 mg

To determine the anti-Gram-negative spectrum of the PK2gp09 endolysin according to SEQ ID NO: 57, a combination of 1.315 µM PK2gp09 endolysin and 0,5 mM EDTA was tested on the clinical *P. aeruginosa* strains PAO1p and Br667, *Burkholderia pseudomallei*, *Pseudomonas putida* G1 and on *Escherichia coli* WK6 (see Table 13). Exponential growing bacterial cells (OD_{600nm} of 0.6) were 100-fold diluted to a final density of about 10⁶/ml of each strain was incubated for 30 minutes at room temperature without shaking with unmodified endolysin P2gp09 (SEQ ID NO: 9) and modified endolysin PKP2gp09 (SEQ ID NO: 57) each in combination without and with 0.5 mM EDTA. For incubation, the endolysins were used each in buffer (20 mM NaH₂P0₄-NaOH pH7.4; 0.5 M NaCl; 0.5 M imidazole) and the incubation took place at a final concentration of endolysin of 1.315 µM. As a control each strain was also incubated for 30 minutes with 0.5 mM EDTA (in same buffer as outlined above) but no endolysin. After incubation cell suspensions were diluted three times (respectively 10⁵-10⁴-10³ cells/ml) and 100 µl of each dilution was plated out on LB-medium. The residual colonies were counted after an overnight incubation on 37°C. Based on the counted cell numbers the antibacterial activity as the relative inactivation in logarithmic units (=log₁₀N₀/N_i with N₀ = number of untreated cells and N_i = number of treated cells, both counted after incubation) was calculated (Table 12).

Table 12 - Antibacterial activity of unmodified endolysin (P2gp09) and its modified endolysin variant (P2gp09) with and without EDTA-Na₂ on different exponential growing Gram-negative species.

	0.5 mM EDTA	1.315 μ M P2gp09	1.315 μ M PKP2gp09	Δ	1.315 μ M P2gp09+ 0.5 mM EDTA	1.315 μ M PKP2gp09 + 0.5 mM EDTA	Δ
<i>P. aeruginosa</i> PA01p	0.330 +/- 0.146	0.374 +/- 0.084	0.326 +/- 0.069	-0.038	2.840 +/- 0.079	3.172 +/- 0.056	0.332
<i>P. aeruginosa</i> Br667	0.003 +/- 0.051	0.246 +/- 0.042	0.300 +/- 0.062	0.054	0.582 +/- 0.074	0.952 +/- 0.213	0.370
<i>P. putida</i> G1	0.072 +/- 0.084	0.419 +/- 0.024	1.014 +/- 0.139	0.595	3.919 +/- 0.118	> 4,386	>0.467
<i>Burkholderia</i> <i>pseudomallei</i>	0.206 +/- 0.151	0.769 +/- 0.110	1.163 +/- 0.073	0.394	3.890 +/- 0.056	4.255 +/- 0.001	0.365
<i>Escherichia</i> <i>coli</i> WK6	0.153 +/- 0.046	0.751 +/- 0.053	1.104 +/- 0.039	0.353	0.784 +/- 0.071	1.545 +/- 0.102	0.749

All samples were replicated in threefold. Averages +/- standard deviations are represented. The maximal reduction observed is dependent on the detection level of 10 cells/ml and the initial cell density.

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Table 13 – List of used Gram-negative strains

Gram-negative strain	Source	Reference
<i>Pseudomonas aeruginosa</i> PA01p	Burn wound isolate, Queen Astrid Hospital, Brussels	Pirnay <i>et al.</i> , 2003*
<i>Pseudomonas aeruginosa</i> Br667	Burn wound isolate, Queen Astrid Hospital, Brussels	Pirnay <i>et al.</i> , 2003*
<i>Burkholderia pseudomallei</i>	Clinical isolate, UZ Gasthuisberg, Leuven	Prof J. Verhaegen
<i>Escherichia coli</i> WK6	Standard laboratory expression strain	Prof C. Michiels
<i>Pseudomonas putida</i> G1	Soil isolate, Moskow	Prof V. Krylov

*Pirnay JP et al., (2003). Molecular epidemiology of *Pseudomonas aeruginosa* colonization in a burn unit: persistence of a multidrug-resistant clone and a silver sulfadiazine-resistant clone. *J Clin Microbiol.*, 41(3):1192-1202.

EXAMPLE 7: Modified endolysin variants of *Pseudomonas putida* phage OBP

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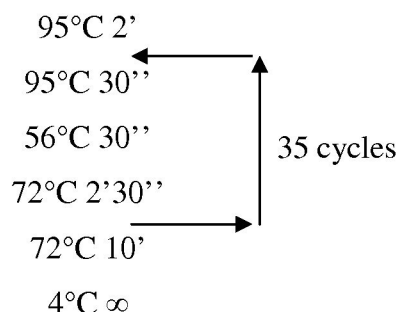
OBPgpLYS according to SEQ ID NO: 7 is a modular endolysin of 328 amino acid residues originating from *Pseudomonas putida* phage OBP with a putative N-terminal peptidoglycan

binding domains and a C-terminal catalytic chitinase domain. As predicted by BLASTp and Pfam analysis the OBPgpLYS endolysin comprises its catalytic domain in the range of about amino acid residue 126 to about amino acid residue 292 and the N-terminal peptidoglycan binding domain in the range of about amino acid residues 7 to 96.

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Purified genomic DNA of phage OBP was used as a template for the amplification of the open reading frame (ORF) of OBPgpLYS in standard PCR reaction with *Pfu* polymerase (Fermentas, Ontario, Canada) using the following PCR parameters:

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Therefore a standard 5' primer (5' ATGAAAAATAGCGAGAAGAAT 3' (SEQ ID NO: 58)) and a standard 3' primer (5' AACTATTCCGAGTGCTTTCTTTGT 3' (SEQ ID NO: 59)) was used. To extend the 5' end of the ORF which encodes OBPgpLYS with a gene fragment encoding the polycationic 9-mer peptide *Lys-Arg-Lys-Lys-Arg-Lys-Lys-Arg-Lys-* (SEQ ID NO: 11) a tail PCR (with same parameters as standard PCR above) with an extended 5' primer (5' ATGGGATCCAAACGCAAGAAACGTAAGAAACGCAAAAAAAAAATAGCGAG AAGAAT 3' (SEQ ID NO: 60)) and the standard 3' primer according to SEQ ID NO 59 was applied. Both the original unmodified OBPgpLYS PCR fragment and the extended fragment were ligated in the pEXP5CT/TOPO® expression vector (Invitrogen, Carlsbad, CA, USA) by following the TA-cloning protocol of the manufacturer.

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Recombinant expression of OBPgpLYS according to SEQ ID NO: 7 and PKOBPgpLYS according to SEQ ID NO: 61 is performed in exponentially growing *E. coli* BL21 (λDE3) pLysS cells (Invitrogen) after induction with 1 mM IPTG (isopropylthiogalactoside) at 37°C for a period of 4 hours. Both proteins were purified by Ni²⁺ affinity chromatography (Akta FPLC, GE Healthcare) using the C-terminal 6xHis-tag, encoded by the pEXP5CT/TOPO®

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

1. *Equilibration* of the *Histrap HP 1 ml* column (GE Healthcare) with 10 column volumes of Washing Buffer (60 mM imidazole, 0.5 mM NaCl and 20 mM NaH_2P_0_4 -NaOH on pH 7.4) at a flow rate of 0.5 ml/min.
2. *Loading* of the total lysate (with wanted endolysin) on the *Histrap HP 1 ml* column at a flow rate of 0.5 ml/min.
3. *Washing* of the column with 15 column volumes of Washing Buffer at a flow rate of 1 ml/min.
4. *Elution* of bounded endolysin from the column with 10 column volumes of Elution Buffer (500 mM imidazole, 5 mM NaCl and 20 mM NaH_2P_0_4 -NaOH on pH 7.4) at a flow rate of 0.5 ml/min

The total yields of both purified recombinant proteins per liter *E. coli* expression culture shown in Table 14. The values were determined by spectrophotometric measurement of the protein concentration and the total volume of the purified stock solution at a wavelength of 280 nm. Purified stock solutions consisting of OBPgpLYS and PKOBPgpLYS, respectively, in Elution Buffer (20 mM NaH_2P_0_4 -NaOH pH7.4; 0.5 M NaCl; 500 mM imidazole) were at least 90% pure as determined visually on SDS-PAGE gels.

Table 14 - Yields of purified recombinant OBPgpLYS endolysin and its PK-modified derivative PKOBPgpLYS per liter E. coli expression culture.

Endolysins	Expression yield
OBPgpLYS (SEQ ID NO: 7)	3.3 mg
PKOBPgpLYS (SEQ ID NO: 61)	4.7 mg

To determine the anti-Gram-negative spectrum of the PKOBPgpLYS endolysin according to SEQ ID NO: 61, a combination of 1.313 μM PK OBPgpLYS endolysin and 0.5 mM EDTA was tested on the clinical multiresistant *P. aeruginosa* strain Br667, *Pseudomonas putida* G1 (host of phage OBP) and a range of other Gram-negative pathogens (*Escherichia coli* WK6, *Salmonella typhimurium* LT2 and *Burkholderia pseudomallei*) (see Table 16). Exponential growing bacterial cells ($\text{OD}_{600\text{nm}}$ of 0.6) were 100-fold diluted to a final density of about

10⁶/ml of each strain was incubated for 30 minutes at room temperature without shaking with unmodified endolysin OBPgpLYS (SEQ ID NO: 7) and modified endolysin PKOBPgpLYS (SEQ ID NO: 61) each in combination without and with 0.5 mM EDTA. For incubation, the endolysins were used each in buffer (20 mM NaH₂P0₄-NaOH pH7.4; 0.5 M NaCl; 0.5 M imidazole) and the incubation took place at a final concentration of endolysin of 1.313 µM. As a control each strain was also incubated for 30 minutes with 0.5 mM EDTA (in same buffer as outlined above) but no endolysin. After incubation cell suspensions were diluted three times (respectively 10⁵-10⁴-10³ cells/ml) and 100 µl of each dilution was plated out on LB-medium. The residual colonies were counted after an overnight incubation on 37°C.

Based on the counted cell numbers the antibacterial activity as the relative inactivation in logarithmic units ($=\log_{10}N_0/N_i$ with N_0 = number of untreated cells and N_i = number of treated cells, both counted after incubation) was calculated (Table 15). All samples were replicated in threefold. Averages +/- standard deviations are represented. The maximal reduction observed is dependent on the detection level of 10 cells/ml and the initial cell density.

Table 15 - Antibacterial activity of unmodified endolysin (OBPgpLYS) and its modified endolysin variant (PKOBPgpLYS) with and without EDTA-Na₂ on different exponential growing Gram-negative species.

	0.5 mM EDTA	1.313 µM OBPgpLYS	1.313 µM PKOBPgpLYS	1.313 µM OBPgpLYS + 0.5 mM EDTA	1.313 µM PKOBPgpLYS + 0.5 mM EDTA
<i>P. aeruginosa</i> PA01p	0.130 +/- 0.023	2.531 +/- 0.173	3.079 +/- 0.015	4.357 +/- 1.857	> 5.687
<i>P. aeruginosa</i> Br667	0.031 +/- 0.023	1.082 +/- 0.083	1.163 +/- 0.063	3.144 +/- 0.223	5.272 +/- 0.573
<i>P. putida</i> G1	0.412 +/- 0.055	0.141 +/- 0.027	0.904 +/- 0.079	4.891 +/- 0.000	> 4.891
<i>Burkholderia pseudomallei</i>	0.220 +/- 0.081	0.997 +/- 0.131	1.806 +/- 0.287	4.08 +/- 0.301	>4.861
<i>Escherichia coli</i> WK6	0.592 +/- 0.113	0.681 +/- 0.032	1.434 +/- 0.018	1.179 +/- 0.200	1.695 +/- 0.147
<i>Salmonella typhimurium</i>	0.054 +/- 0.048	0.076 +/- 0.011	0.127 +/- 0.013	0.774 +/- 0.052	0.908 +/- 0.037

Table 16 – List of used Gram-negative strains

Gram-negative strain	Source	Reference
<i>Pseudomonas aeruginosa</i> PAO1p	Burn wound isolate, Queen Astrid Hospital, Brussels	Pirnay <i>et al.</i> , 2003*
<i>Pseudomonas aeruginosa</i> Br667	Burn wound isolate, Queen Astrid Hospital, Brussels	Pirnay <i>et al.</i> , 2003*
<i>Pseudomonas putida</i> G1	Soil isolate, Moskow	Prof V.Krylov
<i>Burkholderia pseudomallei</i>	Clinical isolate, UZ Gasthuisberg, Leuven	Prof J. Verhaegen
<i>Escherichia coli</i> WK6	Standard laboratory expression strain	Stratagene
<i>Salmonella typhimurium</i> LT2	SGSC N° 2317	Prof C. Michiels

*Pirnay JP, De Vos D, Cochez C, Bilocq F, Pirson J, Struelens M, Duinslaeger L, Cornelis P, Zizi M, Vanderkelen A. (2003). Molecular epidemiology of *Pseudomonas aeruginosa* colonization in a burn unit: persistence of a multidrug-resistant clone and a silver sulfadiazine-resistant clone. *J Clin Microbiol.*, 41(3):1192-1202.

While the global efficacy of the OBPgpLYS treatment is species dependent, the results in table 16 show an added effect of the PKOBPgpLYS compared to unmodified OBPgpLYS for all bacterial species tested, both in the absence as the presence of 0,5 mM EDTA. For *Pseudomonas* and *Burkholderia* species, a clear synergistic effect with EDTA is observed for the PKOBPgpLYS activity.

EXAMPLE 8: Effect of different EDTA concentration on the antibacterial activity of OBPgpLYS and PKOBPgpLYS

To determine the influence of EDTA on the antibacterial activity of unmodified and modified endolysins the antibacterial activity of the unmodified OBPgpLYS endolysin (SEQ ID NO: 7) and the PKOBPgpLYS endolysin (SEQ ID NO: 61) was tested on *Pseudomonas aeruginosa* PAO1p cells (Pirnay JP et al. *J Clin Microbiol.*, 41(3):1192-1202 (2003)) using different concentrations of EDTA and endolysins. Exponential growing bacterial cells (OD_{600nm} of 0.6) were 100-fold diluted to a final density of about 10⁶/ml and incubated for 30 minutes at room temperature without shaking with unmodified endolysin OBPgpLYS (SEQ ID NO: 7) and modified endolysin PKOBPgpLYS (SEQ ID NO: 61). For incubation, the endolysins were used each in buffer (20 mM NaH₂P0₄-NaOH pH7.4; 0.5 M NaCl; 0.5 M imidazole) at final

concentrations of endolysin of 0.013 μ M, 0.131 μ M and 1.315 μ M. Thereby, the following different EDTA concentrations were used: 0 mM, 0.05 mM, 0.5 mM and 10 mM. As a control one sample was also incubated for 30 minutes with no endolysin, instead of there was buffer (20 mM NaH_2PO_4 -NaOH pH7.4; 0.5 M NaCl; 0.5 M imidazole) added. After incubation cell suspensions were diluted three times (respectively 10^5 - 10^4 - 10^3 cells/ml) and 100 μ l of each dilution was plated out on LB-medium. The residual colonies were counted after an overnight incubation on 37°C. Based on the counted cell numbers the antibacterial activity as the relative inactivation in logarithmic units ($=\log_{10}N_0/N_i$ with N_0 = number of untreated cells and N_i = number of treated cells, both counted after incubation) was calculated (Table 17). All samples were replicated in threefold. Averages $\pm$ standard deviations are represented. The maximal reduction observed (5.69 log units) is dependent on the detection level of 10 cells/ml and the initial cell density. “ Δ ” gives the difference of activity between the respective OBPgpLYS and PKOBPgpLYS samples.

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Table 17 - Antibacterial activity of unmodified endolysin (OBPgpLYS) and its modified endolysin variant (PKOBPgpLYS) in combination with different EDTA concentrations on exponential growing *Pseudomonas aeruginosa* PAO1p cells

	Concentration of EDTA- Na_2 (in mM)			
	0	0.05	0.5	10
No endolysin	/	0.028 $\pm$ 0.008	0.130 $\pm$ 0.023	1.827 $\pm$ 0.052
0,013 μ M OBPgpLYS	0.956 $\pm$ 0.110	/	4.626 $\pm$ 0.287	/
0,013 μ M PKOBPgpLYS	0.992 $\pm$ 0.181	/	5.204 $\pm$ 0.000	/
Δ	0.036		0.578	
0,131 μ M OBPgpLYS	2.158 $\pm$ 0.027	/	4.599 $\pm$ 0.275	/
0,131 μ M PKOBPgpLYS	2.529 $\pm$ 0.184	/	5.671 $\pm$ 0.000	/
Δ	0.371		1.072	
1,315 μ M OBPgpLYS	2.531 $\pm$ 0.173	2.762 $\pm$ 0.091	4.357 $\pm$ 1.857	4.888 $\pm$ 0.275

1.315 μM PKOBPgpLYS	3.079 +/- 0.015	4.145 +/- 0.015	> 5.687	>5.687
Δ	0.548	1.383	> 1.330	> 0.799

As shown in Table 17 unmodified endolysin OBPgpLYS reduces cell numbers significantly with more than 2.5 log units for 1.315 μ M and with +/- 1 log unit for 0.013 μ M, compared to the negative control. Modified endolysin PKOBPgpLYS results in an added 0.5 log units reduction for exponentially growing PAO1p cells. The observed antibacterial effect can be increased to more as 5.69 log units reduction (beneath the detection level) by combining PKOBPgpLYS with the outer membrane permeabilizer EDTA- Na_2 at a concentration of 0.5 and 10 mM EDTA. The difference in activity between the unmodified OBPgpLYS and the PK-modified OBPgpLYS increases by raising the amount of added endolysin (from 0.013 – 1.315 μ M endolysin).

EXAMPLE 9: Antibacterial activity of Modified phiKZgp144 variants on different Gram-negative bacteria

To test and to compare the potential of polycationic peptides variants of phiKZgp144 and other endolysins, encoding genes were synthesised having polycationic peptides at the N-terminal end of the protein.

The different products were cloned in the pET32b expression vector (Novagen, Darmstadt, Germany). pET32b was used to reduce potential toxicity of the polycationic peptide against the *E. coli* host. A vector-encoded fusion protein (thioredoxin) masks the polycationic peptide and can be eliminated during the purification process.

The genes encoding smi01 (YP_001712536) and KRK9_smi01 (SEQ ID NO: 75) were fully synthesised (Entelechon, Regensburg, Germany) and cloned into pET32b.

Accordingly, the following modified endolysin variants were expressed in *E. coli* BL21 (DE3) cells at 37°C until an optical density of OD_{600nm}=0.6 was reached: smi01 (YP_001712536), KRK9_smi01 (SEQ ID NO: 75), phiKZgp144 (SEQ ID NO: 1), pKKZ144pET32b (SEQ ID NO: 43) and POLYKZ144 (SEQ ID NO: 35). Protein expression

was induced with 1mM IPTG (final concentration) and expression was performed for four hours. Then *E.coli* cells were harvested by centrifugation for 20 min at 6000g and cell disruption and protein purification was performed using the *S-TagTM rEK Purification Kit* (Novagen, Darmstadt, Germany). Using the pET32b vector, the expressed proteins were not toxic to the host resulting in high yields of produced protein. Purified stock solutions showed high purity.

For testing and as reference for comparison phiKZgp144 and POLYgp144 were synthesized and purified as described in EXAMPLE 1.

Exponential ($\sim 10^6$ /ml) growing cells of *P. aeruginosa* PAO1p (Burn wound isolate, Queen Astrid Hospital, Brussels; Pirnay JP et al. (2003), *J Clin Microbiol.*, 41(3):1192-1202), *Acinetobacter baumannii* (DSMZ 30007) or *Burkholderia solanaceum* (Isolate provided by Prof. C. Michiels) were 100 x diluted (final density was $\sim 10^6$ /ml) incubated at room temperature with each 10 μ g undialyzed protein as listed above at a final concentration of 100 μ g/ml in buffer (20 mM NaH₂P0₄-NaOH pH7.4; 0.5 M NaCl; 0.5 M imidazole). After 1 hour cell suspensions were diluted 1:100 and plated on LB. Additionally, a negative control was plated using buffer (20 mM NaH₂P0₄-NaOH pH7.4; 0.5 M NaCl; 0.5 M imidazole). The residual colonies were counted after an overnight incubation at 37°C. Based on the counted cell numbers the antibacterial activity as the relative inactivation (%) ($=100-(N_i/N_o)*100$ with N_o = number of untreated cells and N_i = number of treated cells) was calculated (Table 18). All samples were replicated at least in four fold.

Table 18 - Antibacterial effect of different modified endolysin variants (NCBI numbers in brackets) on different bacterial species

Protein	Bacterial species	Reduction [%]
smi01 (YP_001712536)	<i>Acinetobacter baumannii</i> DSMZ 30007	0
KRK9_smi01	<i>Acinetobacter baumannii</i> DSMZ 30007	50
phiKZgp144	<i>Pseudomonas aeruginosa</i>	0
pKKZ144pET32b	<i>Pseudomonas aeruginosa</i>	99 - 99,9
phiKZgp144	<i>Acinetobacter baumannii</i> DSMZ 30007	0
pKKZ144pET32b	<i>Acinetobacter baumannii</i> DSMZ 30007	99,9

phiKZgp144	<i>Burkholderia solanacearum</i>	0
POLYKZ144	<i>Burkholderia solanacearum</i>	99 – 99,9

Unmodified endolysins phiKZgp144 and smi01 (YP_001712536) do not reduce cell numbers significantly compared to the negative control. This observation again illustrates the efficacy of the outer membrane as a barrier for the endolysin to degrade the cell wall of the Gram-negative bacteria. In contrast as shown in Table 18 the incubation with the modified endolysins KRK9_smi01, pKKZ144pET32b and POLY-gp144 causes a significant reduction of the bacterial cell number on *Acinetobacter baumannii* (50% for KRK_smi01; 99.9 % for pKKZ144pET32b), *Pseudomonas aeruginosa* (90-99.9 % for pKKZ144pET32b) and *Burkholderia solanaceum* (90 – 99.9 % for POLYKZ144).

These experiments demonstrate the applicability of the cationic/polycationic fusion approach for other endolysins. Moreover, the experiments demonstrated that the modified endolysins are active on a variety of bacteria.

EXAMPLE 10: Reduction of *Pseudomonas aeruginosa* biofilm

In the present experiment the antimicrobial activity of the modified endolysin variants SMAP29-KZ144 and PK-OBP, of the endolysins OBP and KZ144 and of the peptide PK was tested against the biofilm of the *Pseudomonas aeruginosa* strains 2572 and 2573.

Biofilm reduction was quantified using crystal violet assay (Peeters et al., J Microbiol Methods 72: 157-165 (2008)).

Biofilm formation:

Overnight liquid cultures of a mucoid strain of, *Pseudomonas aeruginosa* 2572 (patient isolate), *Pseudomonas aeruginosa* 2573 and a non-mucoid *E. coli* BL21(DE3) were diluted to OD600 = 0.1. A polystyrene 96-well plate was inoculated with 100 µl culture/well. After 4 h incubation at 37°C supernatant was discarded and adherent bacteria were washed using 100 µl physiological saline (PS). Inoculated wells were filled with 100 µl liquid LB media and incubated for an additional 24 h period. After discarding the supernatant the developed biofilm was washed again with 100 µl PS.

Biofilm treatment:

Biofilm was treated using 50 µg/well PKKZ144 or 20 u/well alginate lyase or 50 µg/well SMAP29-KZ144 and KZ144 or 25 µg/well PK-OBP and OBP or 1,25 µg PK-Peptide (all in buffer with 500mM NaCl) diluted one part to one part 2x LB (without NaCl) media and incubated for 12 h. Untreated series were done as negative controls (one part protein buffer to one part 2x LB without NaCl). After discarding the supernatant the developed biofilm was washed again with 100 µl PS.

Biofilm quantification:

The washed biofilm was fixed with 300 µl methanol (99%; 15 min) and air-dried. Staining was done using 100 µl 0.3% crystal violet. After 20 min wells were rinsed with tap water and 300 µl 33% acetic acid was used dissolving the bound crystal violet out of extracellular matrix of the biofilm. After 20 min 1:10 dilution was made and absorption (590 nm) was measured.

Statistical analysis showed a massive reduction of detected biofilm using PKKZ144 compared to alginate lyase-treated or untreated inoculates. Using PKKZ144 it was possible to reduce the biofilm to the level of a non-mucoid *E. coli* lab strain.

Also the modified endolysin variants SMAP29-KZ144 and PK-OBP showed massive reduction of the *Pseudomonas aeruginosa* biofilm compared to the endolysins OBP and KZ144. Contrarily, the PK-peptide seems to enhance the formation of the *Pseudomonas aeruginosa* biofilm.

EXAMPLE 11: Reduction of *Acinetobacter baumannii* biofilm

In the present experiment the antimicrobial activity of the modified endolysin variant PK-OBP, of the endolysin OBP and of the peptide PK was tested against the biofilm of the *Acinetobacter baumannii* strain DSMZ30007.

Biofilm reduction was quantified using crystal violet assay (Peeters et al., J Microbiol Methods 72: 157-165 (2008)).

The biofilm formation, treatment and quantification were performed as described in Example 10.

5 The modified endolysin variant PK-OBP showed massive reduction of the *Acinetobacter baumannii* biofilm compared to the endolysin OBP. Contrarily, the PK-peptide seems to enhance the formation of the *Acinetobacter baumannii* biofilm.

EXAMPLE 12: Reduction of *Staphylococcus aureus* biofilm

10 In the present experiment the antimicrobial activity of the fusion proteins Ply2638-PK and PK-Lysostaphin, of the enzymes Lysostaphin and Ply2638 and of the peptide PK was tested against the biofilm of the *Staphylococcus aureus* strain KS13.

Biofilm reduction was quantified using crystal violet assay (Peeters et al., J Microbiol Methods 72: 157-165 (2008)).

15 The biofilm formation, treatment and quantification were performed as described in Example 10. Except that for the biofilm treatment, 25 µg/well Ply2638A-PK and Ply2638A or 18µg/well PK-Lysostaphin and Lysostaphin or 1,25 µg PK-Peptide was used

20 The fusion proteins PK-Lysostaphin and PK-Ply2638 showed massive reduction of the *Staphylococcus aureus* biofilm compared to the enzymes Lysostaphin and Ply2638. Contrarily, the PK-peptide seems to enhance the formation of the *Staphylococcus aureus* biofilm.

EXAMPLE 13: Reduction of *Listeria monocytogenes* biofilm

25 In the present experiment the antimicrobial activity of the modified endolysin variant Pentapeptide-Ply511 was tested against the biofilm of the *Listeria monocytogenes* strain ScottA.

30 Biofilm reduction was quantified using crystal violet assay (Peeters et al., J Microbiol Methods 72: 157-165 (2008)).

The biofilm formation, treatment and quantification were performed as described in Example 10. Except that for the biofilm treatment, 25 µg/well Pentapeptide-Ply511 was used.

The modified endolysin variant PK-Ply511 showed massive reduction of the *Listeria monocytogenes* biofilm.

CLAIMS

1. An in vitro method of eliminating or reducing a bacterial biofilm comprising
 - a) providing a fusion protein comprising an endolysin or bacteriocin to which a peptide is fused, wherein said peptide fused to the endolysin or bacteriocin is a synthetic cationic, a synthetic polycationic, a synthetic hydrophobic, a synthetic amphiphatic or a naturally occurring antimicrobial peptide; and
 - b) contacting a material, liquid, surface or biological material with said fusion protein.
2. The method according to claim 1, wherein said peptide fused to the endolysin, or bacteriocin is a synthetic or naturally occurring peptide having a length of 6 to 39 amino acid residues.
3. The method according to any one of the preceding claims, wherein said peptide is fused to the N- and/or the C-terminus of the endolysin.
4. The method according to any one of the preceding claims, wherein said endolysin or bacteriocin has the activity of degrading the cell wall of Gram-negative and/or Gram-positive bacteria.
5. The method according to claim 4, wherein the cell wall of the Gram-negative bacteria is selected from the group consisting of *Enterobacteriaceae* (*Escherichia*, especially *E. coli*, *Salmonella*, *Shigella*, *Citrobacter*, *Edwardsiella*, *Enterobacter*, *Hafnia*, *Klebsiella*, especially *K. pneumoniae*, *Morganella*, *Proteus*, *Providencia*, *Serratia*, *Yersinia*), *Pseudomonadaceae* (*Pseudomonas*, especially *P. aeruginosa*, *Burkholderia*, *Stenotrophomonas*, *Shewanella*, *Sphingomonas*, *Comamonas*), *Neisseria*, *Moraxella*, *Vibrio*, *Aeromonas*, *Brucella*, *Francisella*, *Bordetella*, *Legionella*, *Bartonella*, *Coxiella*, *Haemophilus*, *Pasteurella*, *Mannheimia*, *Actinobacillus*, *Gardnerella*, *Spirochaetaceae* (*Treponema* and *Borrelia*), *Leptospiraceae*, *Campylobacter*, *Helicobacter*, *Spirillum*, *Streptobacillus*, *Bacteroidaceae* (*Bacteroides*, *Fusobacterium*, *Prevotella*, *Porphyromonas*), *Acinetobacter*, especially *A. baumannii* and wherein the Gram-positive bacteria are selected from the group consisting of

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 diphtheriae*, *Mycoplasma pneumoniae*, *Actinomyces*.

6. The method according to any one of the preceding claims, wherein said endolysin is selected from the group consisting of phiKZgp144 according to SEQ ID NO: 1, ELgp188 according to SEQ ID NO: 2, *Salmonella endolysin* according to SEQ ID NO: 3, *Enterobacteria phage T4 endolysin* according to SEQ ID NO: 4, *Acinetobacter baumannii endolysin* according to SEQ ID NO: 5, *E.coli* Phage K1F endolysin according to SEQ ID NO: 6, OBPgpLys according to SEQ ID NO: 7, PSP3 salmonella endolysin according to SEQ ID NO: 8, *E.coli* Phage P2 endolysin according to SEQ ID NO: 9, Ply511 according to SEQ ID NO: 85, Ply2638 according to SEQ ID NO: 92 or wherein said bacteriocin is according to SEQ ID NO: 87.
7. The method according to any one of the preceding claims, wherein the cationic/ polycationic peptide exhibits an amino acid sequence according to SEQ ID NO: 10 to 30 and 32 to 34, or wherein the antimicrobial peptide exhibits an amino acid sequence according to SEQ ID NO: 93 to 133, or wherein the hydrophobic peptide exhibits an amino acid sequence according to SEQ ID NO: 134 and 135; or wherein the amphiphatic peptide exhibits an amino acid sequence according to SEQ ID NO: 136 to 138.
8. The method according to any one of the preceding claims, wherein said endolysin variant or bacteriocin variant comprises an amino acid sequence selected from the group consisting of SEQ ID NO: 35 to 49, 53, 57, 62 to 64, 66 to 78 and 139 to 142.
9. The method according to any one of the preceding claims, wherein the material contacted with the fusion protein is a stone, rocks, soil, sediments, food, feed or cosmetics.

10. The method according to any one of the preceding claims, wherein the liquid contacted with the fusion protein is water.
11. The method according to claim 10, wherein water is drinking water, ground water or waste water, hot spring, sea, lake, river, any kind of aqueous system, cleaning and storage solutions of contact lenses, dentures, implants, prothesis or braces.
12. The method according to any one of the preceding claims, wherein the liquid contacted with the fusion protein is any substance derived or obtained from a living organism.
13. The method according to any one of the preceding claims, wherein the liquid contacted with the fusion protein is surface of medical devices, of industrial or potable water system piping and of natural aquatic systems.
14. The method according to any one of the preceding claims, wherein in combination or in addition to the fusion protein antibiotics can be added.
15. The use of an endolysin variant or a bacteriocin variant comprising an endolysin or bacteriocin to which a peptide is fused in the preparation of a medicament for the treatment of Gram-negative and/or Gram-positive bacterial infections associated with bacterial biofilm, wherein said peptide fused to the endolysin or bacteriocin is a synthetic cationic, a synthetic polycationic, a synthetic hydrophobic, a synthetic amphiphatic or a naturally occurring antimicrobial peptide.
16. The use of an endolysin variant or a bacteriocin variant according to claim 15, wherein the endolysin variant or bacteriocin variant is used in combination or in addition to antibiotics.
17. The use of an endolysin variant or a bacteriocin variant comprising an endolysin or bacteriocin to which a peptide is fused for the removal and/or reduction of Gram-negative and/or Gram-positive bacterial contamination associated with bacterial biofilm of foodstuff, of food processing equipment, of food processing plants, of surfaces coming into contact with foodstuff, of medical devices, of surfaces in

hospitals or surgeries, wherein said peptide fused to the endolysin or bacteriocin is a synthetic cationic, a synthetic polycationic, a synthetic hydrophobic, a synthetic amphiphatic or a naturally occurring antimicrobial peptide.

18. The use of an endolysin variant or a bacteriocin variant comprising an endolysin or bacteriocin to which a peptide is fused as a diagnostic means in medicinal, food or feed or environmental diagnostics of bacteria infection associated with bacterial biofilm, as disinfectant or cosmetic substance, wherein said peptide fused to the endolysin or bacteriocin is a synthetic cationic, a synthetic polycationic, a synthetic hydrophobic, a synthetic amphiphatic or a naturally occurring antimicrobial peptide.
19. The use according to claims 17 and 18, wherein the endolysin variant or bacteriocin variant is used in combination or in addition to antibiotics.