

New endolysin OBPgpLYS

The present invention relates to a polypeptide with an amino acid sequence according to SEQ ID NO: 1 and fragments or derivatives thereof. The present invention further relates to fusion proteins comprising said polypeptide and an additional peptide stretch fused to said polypeptide at the N- or C-terminus. Moreover, the present invention relates to nucleic acid molecules encoding said polypeptide or fusion protein, vectors comprising said nucleic acid molecules and host cells comprising either said nucleic acid molecules or said vectors. In addition, the present invention relates to said polypeptide or fusion protein for use as a medicament, in particular for the treatment or prevention of Gram-negative bacterial infections, as diagnostic means, as cosmetic substance or as sanitizing agent. The present invention also relates to the use of said polypeptide or fusion protein for the treatment or prevention of Gram-negative bacterial contamination of foodstuff, of food processing equipment, of food processing plants, of surfaces coming into contact with foodstuff, of medical devices, of surfaces in hospitals and surgeries. Furthermore, the present invention relates to a pharmaceutical composition comprising said polypeptide or fusion protein.

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 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). Furthermore, the dense and ordered packing of the 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).

Various types of agents having bactericidal or bacteriostatic activity are known, e.g. antibiotics, endolysins, antimicrobial peptides and defensins. Increasingly microbial

resistance to antibiotics, however, is creating difficulties in treating more and more infections caused by bacteria. Particular difficulties arise with infections caused by Gram-negative bacteria like *Pseudomonas aeruginosa* and Enterobacteriaceae.

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 $\beta(1,4)$ -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 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.

Antimicrobial peptides (AMPs) represent a wide range of short, cationic or amphipathic, gene encoded peptide antibiotics that can be found in virtually every organism. Different AMPs display different properties, and many peptides in this class are being intensively researched not only as antibiotics, but also as templates for cell penetrating peptides. Despite sharing a few common features (e.g., cationicity, amphipathicity and short size), AMP sequences vary greatly, and at least four structural groups (α -helical, β -sheet, extended and looped) have

been proposed to accommodate the diversity of the observed AMP conformations. Likewise, several modes of action as antibiotics have been proposed, and it was shown e.g. that the primary target of many of these peptides is the cell membrane whereas for other peptides the primary target is cytoplasmic invasion and disruption of core metabolic functions. AMPs may become concentrated enough to exhibit cooperative activity despite the absence of specific target binding; for example, by forming a pore in the membrane, as is the case for most AMPs. However, this phenomenon has only been observed in model phospholipid bilayers, and in some cases, AMP concentrations in the membrane that were as high as one peptide molecule per six phospholipid molecules were required for these events to occur. These concentrations are close to, if not at, full membrane saturation. As the minimum inhibitory concentration (MIC) for AMPs are typically in the low micromolar range, scepticism has understandably arisen regarding the relevance of these thresholds and their importance *in vivo* (Melo et al., Nature reviews, Microbiology, 2009, 245).

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

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

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

The following figures serve to illustrate the invention.

Figure 1 shows the endolysin OBPgpLYS according to the present invention. In (A) the amino acid sequence of the endolysin OBPgpLYS (SEQ ID NO: 1) according to the present

invention is depicted. In (B) the primary structure of the OBPgpLYS comprising an additional His₆-tag is given showing the results of a functional analysis using BLASTp and Pfam analysis. The predicted N-terminal peptidoglycan binding domain (PBD, amino acid residues 7-96) is underlined and the C-terminal catalytic domain (amino acid residues 126 - 292) of the lysozyme-like superfamily is written in italics. The complete amino acid sequence of the OBPgpLYS comprising an additional His₆-tag at the C-terminus shown in (B) is depicted in SEQ ID NO: 47.

Figure 2 shows the nucleotide sequence (SEQ ID NO: 101) of the endolysin of phage OBP.

Figure 3 shows the nucleotide sequence (SEQ ID NO: 3) of the endolysin OBPgpLYS (SEQ ID NO: 1) according to the present invention.

Figure 4 shows a picture of a Coomassie-stained SDS-PAGE showing the results of the expression and purification of the unmodified endolysin OBPgpLYS (SEQ ID NO: 47) and its modified endolysin variant PKOBPgpLYS (SEQ ID NO: 49). 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 (>90%).

Figures 5 A to F show in a graphic representation the antibacterial activities of different compositions of unmodified OBPgpLYS (SEQ ID NO: 47) and the modified PKOBPgpLYS (SEQ ID NO: 49) 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 5 A the antibacterial activity on *Escherichia coli* WK 6 cells is represented, in Figure 5 B the antibacterial activity on *Salmonella typhimurium* LT2 (SGSC N° 2317) cells, in Figure 5 C the antibacterial activity on *Pseudomonas aeruginosa* PAO1p cells, in Figure 5 D the

antibacterial activity on *Pseudomonas aeruginosa* Br667 cells, in Figure 5 E the antibacterial activity on *Pseudomonas putida* G1 cells and in Figure 5 F the antibacterial activity on *Burkholderia pseudomallei* cells. “Δ” gives the difference of antibacterial activity between the respective OBPgpLYS and PKOBPgpLYS samples. The error bars render the standard deviations of the mean.

Figure 6 shows in a graphic representation the host specificity of the unmodified OBPgpLYS (SEQ ID NO: 47) and the modified PKOBPgpLYS (SEQ ID NO: 49). Each species of Gram-negative bacteria was incubated for 30 minutes with a composition comprising each 1.315 μM unmodified OBPgpLYS or modified PKOBPgpLYS. The bar chart gives the antibacterial activities of the unmodified OBPgpLYS and modified OBPgpLYS on *Pseudomonas aeruginosa* PAO1p cells (PAO1), *Escherichia coli* WK6 cells (wk6), *Burkholderia pseudomallei* cells (Burk pseudo), *Pseudomonas aeruginosa* Br667 cells (Br667), *Salmonella typhimurium* LT2 cells (LT2) and *Pseudomonas putida* G1 cells (Ppu G1). The error bars indicate the standard deviations of the mean.

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

The term "fusion protein" as used herein refers to an expression product resulting from the fusion of two nucleic acid sequences. Such a protein may be produced, e.g., in recombinant DNA expression systems. Moreover, the term "fusion protein" as used herein refers to a fusion of a first amino acid sequence as e.g. an endolysin, with a second or further amino acid sequence. The second or further amino acid sequence is preferably a peptide stretch, in particular a cationic peptide, a polycationic peptide, an amphipatic peptide, a sushi peptide, a defensin, a hydrophobic peptide 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.

The term “peptide stretch” as used herein refers to any kind of peptide linked to a protein such as an endolysin. In particular the term “peptide stretch” as used herein refers to a cationic peptide, a polycationic peptide, an amphipathic peptide, a sushi peptide, a defensin, a hydrophobic peptide and/or an antimicrobial peptide. However, a peptide stretch in the meaning of the present invention does not refer to His₆-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 positive 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 polypeptides consisting of from about 2 to about 100 amino acid residues, more preferably from about 4 to about 50 amino acid residues, more preferably from about 5 to about 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 naturally occurring peptides are e.g. antimicrobial peptides, defensins, and sushi peptides. Preferred synthetically produced peptides are e.g. polycationic, amphipathic or hydrophobic peptides. A peptide in the meaning of the present invention does not refer to His-tags, Strep-tags, thioredoxin or maltose binding proteins (MBP) or the like, which are used to purify or locate proteins.

The term “endolysin” as used herein refers to an enzyme which is suitable to hydrolyse bacterial cell walls. “Endolysins” comprise 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 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 structure. The different domains of an endolysin can be connected by a domain linker.

The term “domain linker” as used herein refers to an amino acid sequence functioning to connect single protein domains with one another. As a rule domain linkers form no or only few regular secondary structure like α -helices or β -sheets and can occupy different conformations with the respective structural context. Methods to detect domain linker and properties of linker sequences are well known in the art as e.g. described in Bae et al., 2005, *Bioinformatics*, 21, 2264-2270 or George & Heringa, 2003, *Protein Engineering*, 15, 871-879.

The term “wild type” or “wt” as used herein refers to the amino acid sequence of the endolysin OBPgpLYS as depicted in SEQ ID NO: 86. The nucleic acid sequence encoding the wild type endolysin OBPgpLYS is depicted in SEQ ID NO: 101.

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

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

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

The term „cell wall“ as used herein refers to all components that form the outer cell enclosure of the 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 “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”.

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.

The term “polycationic peptide” as used herein refers to a synthetically produced peptide composed of mostly positively charged amino acid residues, in particular 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 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.

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

amoebicidal, microbicidal, bactericidal, fungicidal, parasiticidal, protozoacidal, protozoicidal properties.

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

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

The term "amphipathic peptide" as used herein refers to peptides having both hydrophilic and hydrophobic functional groups. Preferably, the term "amphipathic peptide" as used herein refers to a peptide having a defined arrangement of hydrophilic and hydrophobic groups e.g. amphipathic 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 present invention relates to new antibacterial agents against Gram-negative bacteria. In particular the present invention relates to a polypeptide comprising an amino acid sequence according to SEQ ID NO: 1 or fragments or derivatives thereof. The polypeptide comprising an amino acid sequence according to SEQ ID NO: 1 is preferably encoded by a nucleotide sequence according to SEQ ID NO: 3.

The endolysin OBPgpLYS having an amino acid sequence according to SEQ ID NO: 1 has a length of 328 amino acids. It comprises a N-terminal cell wall binding domain (CBD) and a C-terminal enzymatic active domain (EAD). The N-terminal CBD is a peptidoglycan binding domain (PGB, aa 7-96) having an amino acid sequence according to SEQ ID NO: 4. The C-terminal EAD is a catalytic domain (aa 126-292) complying with the catalytic domain of the lysozyme-like superfamily and having an amino acid sequence according to SEQ ID NO: 5. The PGB and the catalytic domain of the endolysin OBPgpLYS are connected by a domain linker.

Thus, preferred fragments of the polypeptide according to the present invention are polypeptides comprising an amino acid sequence according to SEQ ID NO: 4 and/or according to SEQ ID NO: 5. Another preferred fragment of the polypeptide according to the present invention comprises an amino acid sequence according to SEQ ID NO: 69. The fragment having an amino acid sequence according to SEQ ID NO: 69 differs from the polypeptide having an amino acid sequence according to SEQ ID NO: 1 in that the starting methionine residue has been deleted.

The derivatives according to the present invention are polypeptides comprising an amino acid sequence according to SEQ ID NO: 1, 4, 5 and/or 69 but having additional modification and/or alterations. Said modifications and/or alterations can be mutations in particular deletions, insertions, additions, substitutions or any combinations thereof and/or chemical changes of the amino acid residues, e.g. biotinylation, acetylation, pegylation, chemical changes of the amino-, SH- or carboxyl- groups. Said derivatives according to the present invention exhibit the lytic activity of the OBPgpLYS (SEQ ID NO: 1) and/or the activity of the fragments according to the present invention. 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 OBPgpLYS and/or the activity of the fragments according to the present invention. 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)).

Preferred derivatives according to the present invention are polypeptides comprising an amino acid sequence according to SEQ ID NO: 86 and 87. Said derivatives differ from the polypeptides having an amino acid sequence according to SEQ ID NO: 1 and SEQ ID NO: 69, respectively, in that the leucine residue has been substituted by a histidine residue at positions 325 and 324, respectively. The polypeptide comprising an amino acid sequence according to SEQ ID NO: 86 is preferably encoded by a nucleotide sequence according to SEQ ID NO: 101.

In a preferred embodiment of the present invention the polypeptide, fragment and/or derivative according to the present invention comprises additionally a tag such as a His₆-tag, Strep-tag, Avi-tag, Myc-tag, Gst-tag, JS-tag, cystein-tag, FLAG-tag or other tags known in the art at the N-terminus or at the C-terminus. In a preferred embodiment of the present invention said tag is linked to the polypeptide, fragment and/or derivative according to the present invention at the C-terminus. Said tag may be linked to said polypeptide, fragment and/or derivative over additional amino acid residues. Said additional amino acid residues may be consist of at least 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 additional amino acid residues. In a preferred embodiment of the present invention the tag is linked to the polypeptide, fragment and/or derivative according to the present invention by the additional amino acid residues Leu-Glu or Lys-Gly.

In a preferred embodiment the present invention relates to polypeptides comprising an amino acid sequence according to SEQ ID NO: 47 or SEQ ID NO: 88. The polypeptide having an amino acid sequence according to SEQ ID NO: 47 and SEQ ID NO: 88, respectively, comprises in comparison to the polypeptide having an amino acid sequence according to SEQ ID NO: 1 and SEQ ID NO: 86, respectively, an additional C-terminal His₆-tag linked to the C-terminus of the polypeptide having an amino acid sequence according to SEQ ID NO: 1 and SEQ ID NO: 86, respectively, by the additional amino acid residues lysine and glycine (Lys-Gly). The polypeptide comprising an amino acid sequence according to SEQ ID NO: 47 is preferably encoded by a nucleotide sequence according to SEQ ID NO: 48. The polypeptide comprising an amino acid sequence according to SEQ ID NO: 88 is preferably encoded by a nucleotide sequence according to SEQ ID NO: 89.

A further aspect of the present invention are fusion proteins composed of an polypeptide, fragment and/or derivative according to the present invention and a peptide stretch fused to the polypeptide, fragment and/or derivative according to the present invention at the N- or C-terminus.

The peptide stretch of the fusion protein according to the present invention is preferably covalently bound to the polypeptide, fragment and/or derivative according to the present invention. Preferably, said peptide stretch consists of at least 5, more preferably at least of 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or at least 100 amino acid residues. Especially preferred is a peptide stretch comprising about 5 to about 100 amino acid residues, about 5 to about 50 or about 5 to about 30 amino acid residues. More preferred is a peptide stretch comprising about 6 to about 42 amino acid residues, about 6 to about 39 amino acid residues, about 6 to about 38 amino acid residues, about 6 to about 31 amino acid residues, about 6 to about 25 amino acid residues, about 6 to about 24 amino acid residues, about 6 to about 22 amino acid residues, about 6 to about 21 amino acid residues, about 6 to about 20 amino acid residues, about 6 to about 19 amino acid residues, about 6 to about 16 amino acid residues, about 6 to about 14 amino acid residues, about 6 to about 12 amino acid residues, about 6 to about 10 amino acid residues or about 6 to about 9 amino acid residues. Preferably, the peptide stretch is no tag such as a His₆-tag, Strep-tag, Avi-tag, Myc-tag, Gst-tag, JS-tag, cystein-tag, FLAG-tag or other tags known in the art and no thioredoxin or maltose binding proteins (MBP). However, the peptide stretch may comprise in addition such tag or tags or the like, which are used to purify or locate proteins.

Preferably, the peptide stretch has the function to lead the fusion protein according to the present invention through the outer membrane of Gram-negative bacteria but has no or only low activity when administered without being fused to the polypeptide, fragment and/or derivative according to the present invention. The function to lead the fusion protein through the outer membrane of Gram-negative bacteria is caused by the potential of the membrane or LPS disrupting activity of said peptide stretch. To determine whether a peptide stretch has membrane or LPS disrupting activity said peptide stretch can be fused to a polypeptide

according to the present invention as e.g. described in the Examples of the present invention. Subsequently, the antibacterial activity of the fusion protein consisting of the polypeptide according to the present invention and the peptide stretch to be tested can be compared to the polypeptide according to the present invention having no peptide stretch as also described in the Examples of the present invention and e.g. shown in Figures 5 A to F and 6. Preferably, said tests may be carried out on *Escherichia coli* WK6 and/or *Pseudomonas aeruginosa* PAO1p cells as used in the Examples of the present invention. In case the fusion protein has an increased antibacterial activity in comparison to the polypeptide according to the present invention without said peptide stretch for at least one of the tested gram-negative bacteria species then said peptide stretch has a membrane or LPS disrupting activity. Preferably, the antibacterial activity (in logarithmic units ($=\log_{10}N_0/N_i$)) of the polypeptide according to the present invention is increased by at least about 5 %, more preferably by at least about 10%, by a peptide stretch having membrane or LPS disrupting activity.

In one aspect of the present invention the fused peptide stretch is an amphipathic peptide, which comprises one or more of the positively charged amino acid residues of lysine, arginine and/or histidine, combined to one or more of the hydrophobic amino acid residues of valine, isoleucine, leucine, methionine, phenylalanine, tryptophan, cysteine, alanine, tyrosine, histidine, threonine, serine, proline and/or glycine. Side chains of the amino acid residues are preferably oriented in order that cationic and hydrophobic surfaces are clustered at opposite sides of the peptide. Preferably, more than about 30, 40, 50, 60 or 70% of the amino acids in said peptide are positively charged amino acids. Preferably, more than about 30, 40, 50, 60 or 70% of the amino acid residues in said peptide are hydrophobic amino acid residues. Advantageously, the amphipathic peptide is fused at the N-terminal and/or the C-terminal end of the polypeptide, fragment and/or derivative according to the present invention having cell wall degrading activity, thus enhancing the amphipathicity of the latter proteins.

In a preferred embodiment at least about 30, 40, 50, 60 or 70% of the said amino acid residues of the amphipathic peptide are either arginine or lysine residues and/or at least about 30, 40, 50, 60 or 70% of the said amino acid residues of the amphipathic peptide are of the hydrophobic amino acid residues valine, isoleucine, leucine, methionine, phenylalanine, tryptophan, cysteine, alanine, tyrosine, histidine, threonine, serine, proline and/or glycine.

Preferred amphipathic peptides are Pleurocidin according to SEQ ID NO: 6, Cecropin P1 according to SEQ ID NO: 7, Buforin II according to SEQ ID NO: 8, Buforin I according to SEQ ID NO: 9 and Magainin according to SEQ ID NO: 10. Further preferred amphipathic peptides are Cathelidicine e.g. LL-37 according to SEQ ID NO: 11.

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

Preferred antimicrobial peptides are listed in the following table.

Peptid	Sequenz	
LL-37	LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLPRTES	SEQ ID NO: 11
SMAP-29	RGLRRLGRKIAHGKKGPTVLRRIIAG	SEQ ID NO: 12
Indolicidin	ILPWKWPWWPWR	SEQ ID NO: 13
Protegrin	RGGRLCYCRRRFCVCGR	SEQ ID NO: 14
Cecropin P1	SWLSKTAKKLENSAKKRISGIAIAIQGGPR	SEQ ID NO: 7
Magainin	GIGKFLHSAKKFGKAFVGEIMNS	SEQ ID NO: 10
Pleurocidin	GWGSFFKKAHVGHVGAALHTYL	SEQ ID NO: 6
Cecropin A (A.egypti)	GGLKKLGKKLEGAGKRVFNAAEKALPVVAGAKALRK	SEQ ID NO: 15
Cecropin A (D. melanogaster)	GWLKKIGKKIERVGQHTRDATIQGLGIPQQAANVAATARG	SEQ ID NO: 16
Buforin II	TRSSRAGLQFPVGRVHRLLRK	SEQ ID NO: 8
Sarcotoxin IA	GWLKKIGKKIERVGQHTRDATIQGLGIAQQAANVAATAR	SEQ ID NO: 17
Ascapine	GKDWIKGAACKLIKTVASHIANQ	SEQ ID NO: 50
Apidaecine	ANRPVYIPPPRPPHRL	SEQ ID NO: 51
Nigrocine	GLLSKVLGVGKKVLCGVSGLVC	SEQ ID NO: 52
Pseudin 1	GLNTLKKVFQGLHEAIKLNNHVQ	SEQ ID NO: 53
Parasin 1	KGRGKQGGKVRKAKTRSS	SEQ ID NO: 72
Lycotoxin	IWLTALKFLGKHAACKLAKQQLSKL	SEQ ID NO: 73
Ranalexin	FLGGLIVPAMICAVTKKC	SEQ ID NO: 117
Melittin	GIGAVLKVL TGLPALISWI KRKRQQ	SEQ ID NO: 119

In a further aspect of the present invention the fused peptide stretch is a sushi peptide which is described by Ding JL, Li P, Ho B Cell Mol Life Sci. 2008 Apr;65(7-8):1202-19. The Sushi peptides: structural characterization and mode of action against Gram-negative bacteria.

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

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

In a further aspect of the present invention the fused peptide stretch is a hydrophobic peptide, preferably having the amino acid sequence Phe-Phe-Val-Ala-Pro (SEQ ID NO:18).

Further preferred peptide stretches are listed in the following table:

Alpha 4	PNRAKRVITTFRT	SEQ ID NO: 68
Artilysin1	GFFIPAVILPSIAFLVP	SEQ ID NO: 70
Artilysin2	GKPGWLIKALVFKKLIRPLKRLA	SEQ ID NO: 71
WLB2 variant	KRWVKRVKRVKRWVKRVVRVVKRWVKR	SEQ ID NO: 118

In one aspect of the present invention the fused peptide stretch is an 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 stretch are positively charged amino acid residues, in particular lysine and/or arginine residues. Especially preferred are peptide stretches 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 stretch 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. Peptide stretches consisting of either only arginine or only lysine are also preferred.

Especially preferred are cationic and/or polycationic peptide stretches comprising at least one motive according to SEQ ID NO: 19 (KRKKRK). In particular cationic peptide stretches comprising at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16 or 17 motives according to SEQ ID NO: 19 (KRKKRK) are preferred. More preferred are cationic peptide stretches comprising at least one KRK motive (lys-arg-lys), preferable at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32 or 33 KRK motives.

In another preferred embodiment of the present invention the cationic peptide stretch comprises beside the positively charged amino acid residues, in particular lysine and/or arginine residues, neutrally charged amino acid residues, in particular glycine and/or serine residues. Preferred are cationic peptide stretches consisting of about 70 % to about 100 %, or about 80 % to about 95 %, or about 85 % to about 90 % positively charged amino acid residues, in particular lysine and/or arginine residues and of about 0 % to about 30 %, or about 5 % to about 20 %, or about 10 % to about 20 % neutrally charged amino acid residues, in particular glycine and/or serine residues. Preferred are polypeptide stretches consisting of about 4 % to about 8 % serine residues, of about 33 % to about 36 % arginine residues and of about 56 % to about 63 % lysine residues. Especially preferred are polypeptide stretches comprising at least one motive according to SEQ ID NO: 40 (KRXKR), wherein X is any other amino acid residue than lysine, arginine and histidine. Especially preferred are polypeptide stretches comprising at least one motive according to SEQ ID NO: 41 (KRSKR). More preferred are cationic stretches comprising at least about 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or about 20 motives according to SEQ ID NO: 40 (KRXKR) or SEQ ID NO: 41 (KRSKR).

Also preferred are polypeptide stretches consisting of about 9 to about 16 % glycine residues, of about 4 to about 11 % serine residues, of about 26 to about 32 % arginine residues and of about 47 to about 55 % lysine residues. Especially preferred are polypeptide stretches comprising at least one motive according to SEQ ID NO: 42 (KRGSG). More preferred are cationic stretches comprising at least about 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or about 20 motives according to SEQ ID NO: 42 (KRGSG).

In another preferred embodiment of the present invention the cationic peptide stretch comprises beside the positively charged amino acid residues, in particular lysine and/or arginine residues, hydrophobic amino acid residues, in particular valine, isoleucine, leucine, methionine, phenylalanine, tryptophan, cysteine, alanine, tyrosine, histidine, threonin, serine, proline and/or glycine residues. Preferred are cationic peptide stretches consisting of about 70 % to about 100 %, or about 80 % to about 95 %, or about 85 % to about 90 % positively charged amino acid residues, in particular lysine and/or arginine residues and of about 0 % to about 30 %, or about 5 % to about 20 %, or about 10 % to about 20 % hydrophobic amino acid residues in particular valine, isoleucine, leucine, methionine, phenylalanine, tryptophan, cysteine, alanine, tyrosine, histidine, threonin, serine, proline and/or glycine residues.

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

Peptide stretch	length	SEQ ID NO:
KRKKRK	6	SEQ ID NO: 19
KRKKRKKRK	9	SEQ ID NO: 20
RRRRRRRRR	9	SEQ ID NO: 21
KKKKKKKK	8	SEQ ID NO: 22
KRKKRKKRKK	10	SEQ ID NO: 23
KRKKRKKRKKRK	12	SEQ ID NO: 24
KRKKRKKRKKRKKR	14	SEQ ID NO: 25
KKKKKKKKKKKKKKKK	16	SEQ ID NO: 26
KRKKRKKRKKRKKRKKRKK	19	SEQ ID NO: 27
RRRRRRRRRRRRRRRRRR	19	SEQ ID NO: 28
KKKKKKKKKKKKKKKKKK	19	SEQ ID NO: 29
KRKKRKKRKRKRKKRKKRK	20	SEQ ID NO: 30
KRKKRKKRKRKRKKRKKRKK	21	SEQ ID NO: 31
KRKKRKKRKKRKKRKKRKKRK	21	SEQ ID NO: 32
KRKKRKKRKRKSGSKRKKRKKRK	22	SEQ ID NO: 33
KRKKRKKRKRKSGSGKRKKRKKRK	24	SEQ ID NO: 34
KRKKRKKRKKRKKRKKRKKRKKRKK	25	SEQ ID NO: 35
KRKKRKKRKRKRKRKKRKKRKRKRKRKKRKKR	31	SEQ ID NO: 36
KRKKRKKRKRKSGSGSKRKKRKKRKRKSGSGSKRKKRKKRK	38	SEQ ID NO: 37
KRKKRKKRKKRKKRKKRKKRKKRKKRKKRKKRKKRKKR	39	SEQ ID NO: 38
KRKKRKKRKRKRKRKKRKKRKRKRKRKRKRKRKRKRKRKR	42	SEQ ID NO: 39

Especially preferred is a fusion protein comprising a polypeptide, fragment and/or derivative according to the present invention and a peptide stretch having an amino acid sequence according to SEQ ID NO: 20. More preferred are fusion proteins having an amino acid sequence according to SEQ ID NO: 43 and SEQ ID NO: 115. Also preferred are fusion proteins an amino acid sequence according to SEQ ID NO: 49 and SEQ ID NO: 116. The fusion proteins having an amino acid sequence according to SEQ ID NO: 49 and SEQ ID NO: 116, respectively, comprises in comparison to the fusion proteins having an amino acid sequence according to SEQ ID NO: 43 and SEQ ID NO: 115, respectively, an additional C-terminal His₆-tag linked to the C-terminus of the fusion protein having an amino acid sequence according to SEQ ID NO: 43 and SEQ ID NO: 115, respectively, by the additional amino acid residues lysine and glycine (Lys-Gly). The fusion proteins having an amino acid sequence according to SEQ ID NO: 43 and SEQ ID NO: 115 and SEQ ID NO: 49 and SEQ ID NO: 116, respectively, differ in that the fusion proteins having an amino acid sequence

according to SEQ ID NO: 115 and SEQ ID NO: 116 has each a substitution of the leucine residue to a histidine residue at position 336.

In another preferred embodiment of the present invention the peptide stretches of the fusion protein according to the present invention comprise modifications and/or alterations of the amino acid sequences. Such alterations and/or modifications may comprise mutations such as deletions, insertions and additions, substitutions or combinations thereof and/or chemical changes of the amino acid residues, e.g. biotinylation, acetylation, pegylation, chemical changes of the amino-, SH- or carboxyl-groups.

A fusion protein according to the present invention as already outlined above is composed of

- (a) an polypeptide, fragment and/or derivative according to the present invention, and
- (b) a peptide stretch fused to said polypeptide, fragment and/or derivative at the N- or C-Terminus, and optionally
- (c) a tag, such as a His₆-tags, Strep-tags, *Avi-tags*, *Myc-tags*, *Gst-tags*, *JS-tags*, *cystein-tags*, *FLAG-tags* or other tags known in the art at the N- or C-Terminus.

In case the peptide stretch is fused to the polypeptide, fragment and/or derivative according to the present invention at the C-Terminus, the fusion protein comprises the additional tag preferably at the N-terminus. In an especially preferred embodiment of the present invention the peptide stretch is fused to the polypeptide, fragment and/or derivative according to the present invention at the N-Terminus. In case said fusion protein comprises an additional tag said tag is preferably at the C-terminus.

The two and three components of the fusion protein, respectively, as outlined above may be linked to each other over additional amino acid residues e.g. due to cloning reasons. Moreover, the peptide stretch may be linked to the starting methionine residue of the fusion protein by additional amino acid residues. Said additional amino acid residues may be consist of at least 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 additional amino acid residues. In a preferred embodiment of the present invention the peptide stretch is linked to the polypeptide, fragment and/or derivative according to the present invention by the additional amino acid residues Gly-Ser or Gly-Gly-Ser. The additional amino acid residues linking the starting methionine

residue and the peptide stretch are preferably Gly-Ser. In case the fusion protein additionally comprises a tag, the polypeptide, fragment and/or derivative according to the present invention is preferably linked to said tag by the additional amino acid residues Leu-Glu or Lys-Gly.

The following table exemplifies the above outlined assembly of specifically preferred fusion proteins according to the present invention listed in the first column starting with the starting methionine residue at the N-terminus in the second column and ending with the optional tag at the C-terminus in the last column:

Fusion protein according to the present invention (SEQ ID NO:)	First amino acid residue (N-term)	additional amino acid residues	peptide stretch (SEQ ID NO:)	additional amino acid residues	polypeptide, fragment, derivative according to the present invention (SEQ ID NO:)	additional amino acid residues	tag (C-term)
SEQ: 43	Met	-	SEQ: 20	Gly-Ser	SEQ: 69	-	-
SEQ: 49	Met	-	SEQ: 20	Gly-Ser	SEQ: 69	Lys-Gly	HIS ₆ -tag
SEQ: 54	Met	-	SEQ: 50	Gly-Ser	SEQ: 87	-	-
SEQ: 55	Met	-	SEQ: 50	Gly-Ser	SEQ: 87	Leu-Glu	HIS ₆ -tag
SEQ: 56	Met	-	SEQ: 51	Gly-Ser	SEQ: 87	-	-
SEQ: 57	Met	-	SEQ: 51	Gly-Ser	SEQ: 87	Leu-Glu	HIS ₆ -tag
SEQ: 58	Met	-	SEQ: 52	Gly-Ser	SEQ: 87	-	-
SEQ: 59	Met	-	SEQ: 52	Gly-Ser	SEQ: 87	Leu-Glu	HIS ₆ -tag
SEQ: 60	Met	-	SEQ: 53	Gly-Ser	SEQ: 87	-	-
SEQ: 61	Met	-	SEQ: 53	Gly-Ser	SEQ: 87	Leu-Glu	HIS ₆ -tag
SEQ: 62	Met	-	SEQ: 17	Gly-Ser	SEQ: 87	-	-
SEQ: 63	Met	-	SEQ: 17	Gly-Ser	SEQ: 87	Leu-Glu	HIS ₆ -tag
SEQ: 64	Met	-	SEQ: 12	Gly-Ser	SEQ: 87	-	-
SEQ: 65	Met	-	SEQ: 12	Gly-Ser	SEQ: 87	Leu-Glu	HIS ₆ -tag
SEQ: 66	Met	-	SEQ: 15	Gly-Ser	SEQ: 87	-	-
SEQ: 67	Met	-	SEQ: 15	Gly-Ser	SEQ: 87	Leu-Glu	HIS ₆ -tag
SEQ: 74	Met	Gly-Ser	SEQ: 68	Gly-Ser-Ser	SEQ: 87	-	-
SEQ: 75	Met	Gly-Ser	SEQ: 68	Gly-Ser-Ser	SEQ: 87	Lys-Gly	HIS ₆ -tag
SEQ: 76	Met	Gly-Ser	SEQ: 69	Gly-Ser-Ser	SEQ: 87	-	-
SEQ: 77	Met	Gly-Ser	SEQ: 69	Gly-Ser-Ser	SEQ: 87	Lys-Gly	HIS ₆ -tag
SEQ: 78	Met	Gly-Ser	SEQ: 70	Gly-Ser-Ser	SEQ: 87	-	-
SEQ: 79	Met	Gly-Ser	SEQ: 70	Gly-Ser-Ser	SEQ: 87	Lys-Gly	HIS ₆ -tag
SEQ: 80	Met	Gly-Ser	SEQ: 71	Gly-Ser-Ser	SEQ: 87	-	-
SEQ: 81	Met	Gly-Ser	SEQ: 71	Gly-Ser-Ser	SEQ: 87	Lys-Gly	HIS ₆ -tag
SEQ: 82	Met	Gly-Ser	SEQ: 72	Gly-Ser-Ser	SEQ: 87	-	-
SEQ: 83	Met	Gly-Ser	SEQ: 72	Gly-Ser-Ser	SEQ: 87	Lys-Gly	HIS ₆ -tag
SEQ: 84	Met	Gly-Ser	SEQ: 73	Gly-Ser-Ser	SEQ: 87	-	-
SEQ: 85	Met	Gly-Ser	SEQ: 73	Gly-Ser-Ser	SEQ: 87	Lys-Gly	HIS ₆ -tag
SEQ: 115	Met	-	SEQ: 20	Gly-Ser	SEQ: 87	-	-
SEQ: 116	Met	-	SEQ: 20	Gly-Ser	SEQ: 87	Lys-Gly	HIS ₆ -tag

The present invention further relates to an isolated nucleic acid molecule encoding the polypeptide, fragment, derivative and/or fusion protein according to the present invention. Especially preferred isolated nucleic acid molecules according to the present invention comprise a nucleic acid sequence according to SEQ ID NO: 2, 3, 48, 89 or 101. The present invention further relates to a vector comprising the nucleic acid molecule according to the present invention. Said vector may provide for the constitutive or inducible expression of said polypeptide, fragment, derivative and/or fusion protein according to the present invention.

The invention also relates to a method for obtaining said polypeptide, fragment, derivative and/or fusion proteins from a micro-organism, such as a genetically modified suitable host cell which expresses said polypeptide, fragment, derivative and/or fusion proteins. Said host cell may be a micro-organism such as bacteria or yeast or an animal cell as e.g. a mammalian cell, in particular a human cell. In one embodiment of the present invention the host cell is an *Escherichia coli* cell. The host may be selected due to mere biotechnological reasons, e.g. yield, solubility, costs, etc. but may be also selected from a medical point of view, e.g. a non-pathological bacteria or yeast or human cells. Another aspect of the present invention is related to a method for genetically transforming a suitable host cell in order to obtain the expression of the polypeptide, fragment, derivative and/or fusion proteins according to the present invention, wherein the host cell is genetically modified by the introduction of a genetic material encoding said polypeptide, fragment, derivative and/or fusion proteins into the host cell and obtain their translation and expression by genetic engineering methods well known by the man skilled in the art.

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

In a preferred embodiment of the present invention 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 about 1 mM EDTA. 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.

The present invention also relates to a polypeptide, fragment, derivative and/or fusion protein according to the present invention and/or a host transformed with a nucleic acid comprising a nucleotide sequence encoding a polypeptide, fragment, derivative and/or fusion protein according to the present invention for use as a medicament. In a further aspect the present invention relates to the use of a polypeptide, fragment, derivative and/or 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 polypeptide, fragment, derivative and/or fusion protein according to the present invention in the manufacture of a medicament for the treatment and/or prevention of a disorder, disease or condition associated with Gram-negative bacteria. In particular the treatment and/or prevention of the disorder, disease or condition may be caused by 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*. In particular, the treatment and/or prevention of the disorder, disease or condition may be caused by *Pseudomonas aeruginosa*, *Pseudomonas putida*, *Burkholderia pseudomallei*, *E. coli* and/or *Salmonella typhimurium*.

The present invention further relates to a medicament comprising a polypeptide, fragment, derivative and/or fusion protein according to the present invention and/or a host transformed

with a nucleic acid comprising a nucleotide sequence encoding a polypeptide, fragment, derivative and/or fusion protein according to the present invention.

In a further aspect the present invention relates to a method of treating a disorder, disease or condition in a subject in need of treatment and/or prevention, which method comprises administering to said subject an effective amount of a polypeptide, fragment, derivative and/or 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 polypeptide, fragment, derivative and/or fusion protein according to the present invention or a composition according to the present invention. The subject may be a human or an animal.

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

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

For application of a polypeptide, fragment, derivative and/or 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 polypeptide, fragment, derivative and/or fusion protein according to the present invention or a composition according to the present invention to a site of infection (or site endangered to be infected) a formulation may be used that protects the active compounds from environmental influences such as proteases, oxidation, immune response etc., until it reaches the site of infection. Therefore, the formulation may be capsule, dragee, pill, suppository, injectable solution or any other medical reasonable galenic formulation. Preferably, the galenic formulation may comprise suitable carriers, stabilizers, flavourings, buffers or other suitable reagents. For example, for topical application the formulation may be a lotion or plaster, for nasopharyngeal application the formulation may be saline solution to be applied via a spray to the nose.

Preferably, a polypeptide, fragment, derivative and/or fusion protein according to the present invention is used for medical treatment, if the infection to be treated (or prevented) is caused by multiresistant bacterial strains, in particular by strains resistant against one or more of the following antibiotics: streptomycin, tetracycline, cephalothin, gentamicin, cefotaxime, cephalosporin, ceftazidime or imipenem. Furthermore, a polypeptide, fragment, derivative and/or fusion protein according to the present invention can be used in methods of treatment by administering it in combination with conventional antibacterial agents, such as antibiotics, lantibiotics, bacteriocins or endolysins, etc.

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

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

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

In a further aspect the present invention relates to the polypeptide, fragment, derivative and/or 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 bacteria. In this respect the polypeptide, fragment, derivative and/or fusion protein according to the present invention may be used as a tool to specifically degrade pathogenic bacteria, in particular Gram-negative pathogenic bacteria. The degradation of the bacterial cells by the polypeptide, fragment, derivative and/or 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 polypeptide, fragment, derivative and/or fusion protein according to the present invention for the treatment or prevention of Gram-negative bacterial contamination of foodstuff, of food processing equipment, of food processing plants, of surfaces coming into contact with foodstuff such as shelves and food deposit areas and in all other situations, where pathogenic, facultative pathogenic or other undesirable bacteria can potentially infest food material, of medical devices and of all kind of surfaces in hospitals and surgeries.

In particular, a polypeptide, fragment, derivative and/or fusion protein of the present invention may be used prophylactically as sanitizing agent. Said sanitizing agent may be used before or after surgery, or for example during hemodialysis. Moreover, premature infants and immunocompromised persons, or those subjects with need for prosthetic devices may be treated with a fusion protein according to the present invention. Said treatment may be either prophylactically or during acute infection. In the same context, nosocomial infections, especially by antibiotic resistant strains like *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 may be treated prophylactically or during acute phase with a polypeptide, fragment, derivative and/or fusion protein of the present invention. Therefore, a

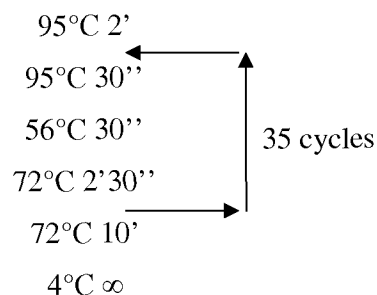
polypeptide, fragment, derivative and/or fusion protein according to the present invention may be used as a disinfectant also in combination with other ingredients useful in a disinfecting solution like detergents, tensids, solvents, antibiotics, lantibiotics, or bacteriocins.

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

EXAMPLE 1: Modified endolysin variants of *Pseudomonas putida* phage OBP

OBPgpLYS having an amino acid sequence according to SEQ ID NO: 1 is a modular endolysin of 332 amino acid residues originating from *Pseudomonas putida* phage OBP with a putative N-terminal peptidoglycan binding domain and a C-terminal catalytic chitinase domain. OPBgpLYS having an amino acid sequence according to SEQ ID NO: 47 comprises in comparison to OBPgpLYS having an amino acid sequence according to SEQ ID NO: 1 an additional C-terminal His₆-tag linked to the C-terminus by the additional amino acid residues lysin and glycine (Lys-Gly)

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:



Therefore a standard 5' primer (5' ATGAAAAATAGCGAGAAGAAT 3' (SEQ ID NO: 44)) and a standard 3' primer (5' AACTATTCCGAGTGCTTTCTTTGT 3' (SEQ ID NO: 45)) 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: 20) a tail PCR (with same parameters as standard PCR above) with an extended 5' primer (5' ATGGGATCCAAACGCAAGAAACGTAAGAAACGCAAAAAAAAAATAGCGAG AAGAAT 3' (SEQ ID NO: 46)) and the standard 3' primer according to SEQ ID NO: 45 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.

Recombinant expression of OBPgpLYS having an amino acid sequence according to SEQ ID NO: 47 and PKOBPgpLYS having an amino acid sequence according to SEQ ID NO: 49 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, 0.5 M 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 is shown in Table 1. 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 NaH₂P0₄-NaOH pH7.4; 0.5 M NaCl; 500 mM imidazole) were at least 90% pure as determined visually on SDS-PAGE gels.

Table 1 - Yields of purified recombinant OBPgpLYS endolysin and its PK-modified PKOBPgpLYS per liter *E. coli* expression culture.

Endolysins	Expression yield
OBPgpLYS (SEQ ID NO: 47)	3.3 mg
PKOBPgpLYS (SEQ ID NO: 49)	4.7 mg

To determine the anti-Gram-negative spectrum of the endolysins OBPgpLYS according to SEQ ID NO: 47 and PKOBPgpLYS according to SEQ ID NO: 49, a combination of 1.313 μ M of each 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 (*P. aeruginosa* PAO1p, *P. aeruginosa* Br667, *P. putida* G1, *Burkholderia pseudomallei*, *Escherichia coli* WK6 and *Salmonella typhimurium*) (see Table 3). Exponential growing bacterial cells (OD_{600nm} of 0.6) were 100-fold diluted to a final density of about 10^6 cells/ml of each strain was incubated for 30 minutes at room temperature without shaking with unmodified endolysin OBPgpLYS (SEQ ID NO: 47) and modified endolysin PKOBPgpLYS (SEQ ID NO: 49) each in combination without and with 0.5 mM EDTA. For incubation, the endolysins were used each in buffer (20 mM NaH₂PO₄-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 μ M. 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 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 at 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 2). 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 2 - Antibacterial activity of unmodified endolysin (OBPgpLYS) and its modified endolysin variant (PKOBPgpLYS) with and without EDTA- Na_2 on different exponential growing Gram-negative species in logarithmic units.

	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> PAO1p	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

While the global efficacy of the OBPgpLYS treatment is species dependent, the results in Table 2 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.

Table 3 – 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, Moscow	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.

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****STANSSENS, P., OPSOMER, C. , MCKEOWNY, M. , KRAMER, W., ZABEAU, M. and FRITZ, H.-J. (1989). Efficient oligonucleotide-directed construction of mutations in expression vectors by the gapped duplex DNA method using alternating selectable markers. *Nucleic Acids Research* 17, 4441-4454 .

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EXAMPLE 2: Effect of different EDTA concentrations 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: 47) and the PKOBPgpLYS endolysin (SEQ ID NO: 49) 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 cells/ml and incubated for 30 minutes at room temperature without shaking with unmodified endolysin OBPgpLYS (SEQ ID NO: 47) and modified endolysin PKOBPgpLYS (SEQ ID NO: 49). 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 µM, 0.131 µM and 1.315 µM. 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 NaH₂P0₄-NaOH pH7.4; 0.5 M NaCl; 0.5 M imidazole) added. 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 at 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 4). All samples were replicated in threefold. Averages +/- 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.

Table 4 - Antibacterial activity of unmodified endolysin (OBPgpLYS) and its modified endolysin variant (PKOBPgpLYS) in combination with different EDTA-Na₂ concentrations on exponential growing Pseudomonas aeruginosa PAO1p cells in logarithmic units

Concentration of EDTA-Na ₂ (in mM)				
	0	0.05	0.5	10
No endolysin	/	0.028 +/- 0.008	0.130 +/- 0.023	1.827 +/- 0.052
0,013 µM OBPgpLYS	0.956 +/- 0.110	/	4.626 +/- 0.287	/
0,013 µM PKOBPgpLYS	0.992 +/- 0.181	/	5.204 +/- 0.000	/
Δ	0.036		0.578	
0,131 µM OBPgpLYS	2.158 +/- 0.027	/	4.599 +/- 0.275	/
0,131 µM PKOBPgpLYS	2.529 +/- 0.184	/	5.671 +/- 0.000	/
Δ	0.371		1.072	
1,315 µM OBPgpLYS	2.531 +/- 0.173	2.762 +/- 0.091	4.357 +/- 1.857	4.888 +/- 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 4 unmodified endolysin OBPgpLYS reduces cell numbers significantly with more than 2.5 log units for 1.315 µM and with +/- 1 log unit for 0.013 µM, 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-Na₂ 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 µM endolysin).

EXAMPLE 3: Cloning, expression and purification of an OBPgpLYS derivative modified with various peptide stretches on the N-terminus of the endolysin.

The OBPgpLYS derivative according to SEQ ID NO:86 is a modular endolysin originating from *Pseudomonas putida* phage OBP with an N-terminal peptidoglycan binding and C-

terminal catalytic domain. The OBPgpLYS derivative is encoded by the nucleic acid molecule according to SEQ ID NO: 101. Purified Plasmid DNA (see Example 1) was used to produce a nucleic acid molecule according to SEQ ID NO: 101 with a BamH I (5'-GGA TCC-3') restriction site at the 5'-end of the nucleic acid molecule and an Xho I (5'-CTC GAG-3') restriction site at the 3'-end of the nucleic acid molecule.

The following peptide stretches in table 5 were used for production of fusion proteins with the endolysin OBPgpLYS derivative. The resulting fusion proteins are also listed in table 5.

Table 5: Peptide stretches and their respective nucleic acid sequence for production of specific fusion proteins

Peptide stretch	Nucleic acid molecule encoding the peptide stretch	Amino acid sequence of resulting fusion protein
Ascapnine (SEQ ID NO: 50)	SEQ ID NO: 90	SEQ ID NO: 55
Apidaecine (SEQ ID NO: 51)	SEQ ID NO: 91	SEQ ID NO: 57
Sarcotoxin IA (SEQ ID NO:17)	SEQ ID NO: 92	SEQ ID NO: 63
SMAP-29 (SEQ ID NO:12)	SEQ ID NO:93	SEQ ID NO:65
Cecropin A (<i>A. aegypti</i>) (SEQ ID NO:15)	SEQ ID NO:94	SEQ ID NO:67

The nucleic acid molecules encoding the respective peptide stretches were synthetically produced with a Nde I (5'-CAT ATG-3') restriction site at the 5'-end of the nucleic acid molecule and a BamH I (5'-GGA TCC-3') restriction site at the 3'-end of the nucleic acid molecule.

Fusion proteins are constructed by linking at least two nucleic acid sequences using standard cloning techniques as described e.g. by Sambrook et al. 2001, Molecular Cloning: A Laboratory Manual. Therefore the nucleic acid molecules encoding the peptide stretches were cleaved in a digest with the respective restriction enzymes Nde I and BamH I. Subsequently

the cleaved nucleic acids encoding the peptide stretches were ligated into the pET21 b expression vector (Novagen, Darmstadt, Germany), which was also cleaved in a digest with the respective restriction enzymes Nde I and BamH I before.

Afterwards, the nucleic acid molecule encoding the endolysin OBPgpLYS derivative was cleaved in a digest with the restriction enzyme BamH I and Xho I, so that the endolysin could be ligated into the pET21b expression vector (Novagen, Darmstadt, Germany).

Thus, the nucleic acid molecule encoding the peptide stretch is ligated into the respective vector at the 5'-end of the nucleic acid molecule encoding the endolysin OBPgpLYS derivative. Moreover, the nucleic acid molecule encoding the endolysin OBPgpLYS derivative is ligated into the respective plasmid, so that a nucleic acid molecule encoding a His₆-tag consisting of six histidine residues is associated at the 3'-end of the nucleic acid molecule encoding the endolysin.

The sequence of the endolysin-peptide-fusions was controlled via DNA-sequencing and correct clones were transformed into *E. coli* T7 Express lysY/Iq (New England Biolabs, Frankfurt, Germany) for protein expression.

Recombinant expression of the fusion proteins according to SEQ ID NO: 55, 57, 63, 65, 67 is performed in *E. coli* T7 Express lysY/Iq (New England Biolabs, Frankfurt, Germany). The cells were growing until an optical density of OD_{600nm} of 0.5-0.8 was reached. Then the expression of the fusion protein was induced with 0.5 mM IPTG (isopropylthiogalactoside) and the expression was performed at 37°C for a period of 4 hours.

Cells were harvested by centrifugation for 15 min at 4000g and disrupted via sonication on ice. Soluble and insoluble fraction of the *E.coli* crude extract were separated by centrifugation (Sorvall, SS34, 30 min, 15000 rpm). All proteins were purified by Ni²⁺ affinity chromatography (Äkta FPLC, GE Healthcare) using the C-terminal 6xHis-tag, encoded by the pET21b vector.

The Ni²⁺ affinity chromatography is performed in 4 subsequent steps, all at room temperature:

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

Purified stock solutions of fusion proteins in Elution Buffer (20 mM Hepes pH 7.4; 0.5 M NaCl; 500 mM imidazole) were at least 60% pure as determined visually on SDS-PAGE gels (data not shown).

EXAMPLE 4: Antimicrobial activity of the endolysin OBPgpLYS derivative modified with various peptide stretches on the N-terminus.

Acinetobacter baumannii DSMZ 30007 and *Pseudomonas aeruginosa* PAO1p cells (Burn wound isolate, Queen Astrid Hospital, Brussels; Pirnay JP et al. (2003), http://www.ncbi.nlm.nih.gov/pubmed/12624051?ordinalpos=3&itool=EntrezSystem2.PEntrez.Pubmed.Pubmed_ResultsPanel.Pubmed_DefaultReportPanel.Pubmed_RVDocSum) *Clin Microbiol.*, 41(3):1192-1202) were used as test strains. Overnight cultures were diluted 10-fold in fresh LB medium and grown to OD₆₀₀=0.6. The culture was spun down and diluted 10-fold in dilution buffer (10 mM HEPES, 0.5 mM EDTA; pH 7.4). Bacteria were incubated at room temperature with each 10 µg undialyzed fusion protein at a final concentration of 100 µg/ml in buffer (20 mM NaH₂PO₄-NaOH pH 7.4; 0.5 M NaCl; 0.5 M imidazole). After 1 hour cell dilution series were made in PBS and plated on LB. Additionally, a negative control was plated using buffer (20 mM NaH₂PO₄-NaOH pH 7.4; 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 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 5). All samples were replicated at least in four fold.

Table 6: Antimicrobial activity of the OBPgpLYS derivative modified with various peptide stretches against gram-negative bacteria

Fusion protein	Enzyme part	Peptide stretch (N-terminal unless otherwise indicated)	Activity against <i>Acinetobacter baumannii</i> DSMZ 30007	Activity against <i>Pseudomonas aeruginosa</i> PAO1p cells
SEQ ID NO: 88	SEQ ID NO:87	-	++	+
SEQ ID NO: 55	SEQ ID NO:87	Ascaphine (SEQ ID NO: 50)	+++	Not determined
SEQ ID NO: 57	SEQ ID NO:87	Apidaecine (SEQ ID NO: 51)	+++	Not determined
SEQ ID NO: 63	SEQ ID NO:87)	Sarcotoxin IA (SEQ ID NO:17)	+++	++
SEQ ID NO: 65	SEQ ID NO:87	SMAP-29 (SEQ ID NO:12)	+++	++
SEQ ID NO: 67	SEQ ID NO:87	Cecropin A (SEQ ID NO:15)	+++	+++

Abbreviations: +: 1 log; ++: 2-3 log; +++: 4 or more logs; not determined means that this strain was not tested with the respective fusion protein.

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

Example 5: N-terminal antibacterial peptide fusion to endolysin of *Pseudomonas putida* phage OBP

OBPgpLys derivative, the modular endolysin of *P. putida* phage OBP, was N-terminally fused to a set of natural antibacterial peptide tags (Table 7) in order to investigate its anti Gram-negative activity.

Table 7: List of antibacterial peptide tags which were fused to the OBPgpLYS derivative

Tag	Description + size	Amino acid sequence	Nucleic acid sequence	Reference
α 4-helix of T4-lysozyme	Amphipathic helix (13 aa)	PNRAKRVITTFRT (SEQ ID NO: 68)	SEQ ID NO: 95	Matthews <i>et al.</i> , 1974*
Pentapeptide (designed)	Hydrophobic (5 aa)	FFVAP (SEQ ID NO: 18)	SEQ ID NO: 96	Briers Y (not published)
Artilysin 1 (designed)	Hydrophobic (18 aa)	GFFIPAVILPSIAFLVP (SEQ ID NO: 70)	SEQ ID NO: 97	Walmagh, M. (Not published)
Artilysin 2 (designed)	Amphipathic helix (25 aa)	GKPGWLIKALVFKKLIRRPLKRLA (SEQ ID NO: 71)	SEQ ID NO: 98	Walmagh, M (Not published)
Parasin 1	Alpha-helical peptide (19 aa)	KGRGKQGGKVRAKAKTRSS (SEQ ID NO: 72)	SEQ ID NO: 99	Park, Y <i>et al.</i> , 1998**
Lycotoxin 1	Amphipathic helix (25 aa)	IWLTALKFLGKHAACKLAKQQLSKL (SEQ ID NO: 73)	SEQ ID NO: 100	Yan & Adams, 1988***

*Matthews, B.W. and Remington, S.J. (1974). The three dimensional structure of the lysozyme from bacteriophage T4. *Proc. Natl. Acad. Sci. USA*, 71: 4178-4182

**In Yup Park, Chan Bae Park, Mi Sun Kim, Sun Chang Kim (1998) . Parasin I, an antimicrobial peptide derived from histone H2A in the *cat ϕ sh*, *Parasilurus asotus*. *FEBS Letters* 437 258-262

***Yan, L and Adams, M.A. (1998). Lycotoxins, Antimicrobial Peptides from Venom of the Wolf Spider, *Lycosa carolinensis* *J. Biol. Chem.*, 273:2059-2066.

Methodology of tag modification of the OBPgpLys derivative

Except for the pentapeptide tag, all antibacterial peptide tags were fused to the ORF which encodes for the OBPgpLYS derivative using an adapted version of the Ligation Independent Cloning (LIC) as e.g. described in Berrow *et al.* 2007. Here fore, an unique *Ecl136II*

restriction site was inserted in front of the WT endolysin gene by a tail PCR with a specific designed 5' primer (5'-GGAATGGGGAGCTCCTCCAAAAATAGCGAGAAG-3'; SEQ ID NO:102) and the standard OBPgpLys derivative reverse primer (5'-AACTATTCCGTGTGCTTTCTTTGT-3'; SEQ ID NO:103) on pure genomic DNA of phage OBP. This extended fragment was then ligated in the pEXP5CT/TOPO® expression vector (Invitrogen, Carlsbad, CA, USA) by following the TA cloning protocol of the manufacturer. Pure plasmid was cutted once in an Ecl136II restriction digest and hybridized peptide cassettes (created by hybridization of primer pairs, see Table 8) were inserted into the cutted plasmid without a necessary ligation step (LIC). For the N-terminal pentapeptide tag fusion a tail PCR with an extended 5' primer which encodes for this pentapeptide (5'-ATGGGATCCTTCTTCGTAGCA CCGGGCTCCTCCAAAAATAGCGAGAAG-3'; SEQ ID NO:104) and the standard OBPgpLys derivative reverse primer (5'-AACTATTCCGTGTGCTTTCTTTGT-3'; SEQ ID NO:103) was applied on phage OBP genomic DNA. Correct insertion of the fragments in the expression vector was verified by sequencing analysis before introducing the construct into a suitable Escherichia coli BL21(DE3)pLysS expression strain.

Table 8: Used primer pairs for hybridization of antibacterial peptide tags to ORF encoding the OBPgpLys derivative

Tag	forward primer	reverse primer
α 4-helix of T4-lysozyme	5'TTGAATGGGGAGCCGAACCGTGCAAAACG TGTAATCA 3'; SEQ ID NO:105	5'TATTTTGGAGGAGCCGGTACGGAAGGTGGTGAT TACACGT 3'; SEQ ID NO:106
Artilysin1 (designed)	5'TTATGGGCTTCTTCATCCCGGCAGTAATCCTGC CCTCCA 3'; SEQ ID NO:107	5'TATTTTGGATCTGCCGCCGGTACGATCAGGAAT GCGATGGAGGGCAGGATT 3'; SEQ ID NO:108
Artilysin 2 (designed)	5'TTATGGGCAAACCGGGCTGGCTGATCAAAAG GCACTGGTATTCAAGA 3' ; SEQ ID NO:109	5'TATTTTGGATCTGCCGCTGCCAGTCTCTTCAGC GGACGACGATCAGTTCTTGAATACCAG 3'; SEQ ID NO:110
Parasin 1	5'TTGAATGGGGAGCAAAGCCGTGGCAAGCA GGGAGGCAAAGTACGTG 3'; SEQ ID NO:111	5'TATTTTGGAGGAGCCTGAGGAACGGGTCTTTGCT TTGCACGTACTTTGC 3'; SEQ ID NO:112
Lycotoxin 1	5'GGAATGGGGAGCATCTGGCTGACCGCACTGA AATTCCTCGCAAACACGCCGCAA 3'; SEQ ID NO:113	5'TATTTTGGAGGAGCCAGTTTGGATAATTGCTGT TTTGCCAGTTTCTTTGCGGCGTGT 3'; SEQ ID NO:114

Large scale recombinant expression of modified OBPgpLYS derivative fusion variants

Standard expression is performed in Lysogeny Broth (LB) in exponentially growing cells (OD_{600nm} = 0.6) induced with 1 mM isopropyl-beta-D-thiogalactopyranoside. Expression

parameters like temperature, time and expression strain varied on a protein specific basis in order to optimize the soluble expression levels of the modified endolysins (see Table 9).

For purification, cells from an expression culture (500-600 ml) are harvested (4500 rpm, 30 min, 4°C) and resuspended in 1/25 volumes of lysis buffer (10 mM imidazole, 20 mM NaH₂PO₄, 0.5 M NaCl, pH 7.4). This suspension is frozen/thawed three times prior to sonication (8 x 30 s, amplitude 40% on a Vibra Cell™, Sonics, Dandurly, CT, USA) and filtered through 0.45 and 0.22 µm Durapore membrane filters (Millipore, Billerica, MA, USA). Purification of the His-tagged fusion protein was performed by a one-step protocol employing Ni²⁺ - affinity chromatography (HisTrap HP 1 ml column, GE Healthcare, Buckinghamshire, UK) according to the manufacturer's instructions. 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 NaH₂PO₄-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, 0.5 M NaCl and 20 mM NaH₂PO₄-NaOH on pH 7.4) at a flow rate of 0.5 ml/min*

The wash buffer included a low imidazole concentration which varied on protein specific base to ensure higher purity of the protein (see Table 9). The total yields of recombinant proteins per liter E. coli expression culture is also shown in Table 3. 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 were at least 60 % pure as determined visually on SDS-PAGE gels.

Table 9: Expression parameters and obtained protein yields per liter expression culture of N-terminal modified endolysins. RP = *E. coli* BL21(DE3)pLysS Codon min RP strain, RIL = *E. coli* BL21(DE3)pLysS Codon Plus RIL strain

Modified endolysin	Temperature/ time	Protein Yield (in mg/l)	[imidazole] (in mM)
α 4-OBPgpLys (SEQ ID NO: 75)	16/overnight	1.28	60
Pentapeptide- OBPgpLys (SEQ ID NO: 77)	16/overnight	1.10	65
Artilysin1-OBPgpLys (SEQ ID NO: 79)	16/overnight	< 0.1	50
Artilysin2-OBPgpLys (SEQ ID NO: 81)	16/overnight	1.32	50
Parasin1-OBPgpLys (SEQ ID NO: 83)	16/overnight	0.38	50
Lycotoxin1- OBPgpLys (SEQ ID NO: 85)	16/overnight	1.71	50

In vitro antibacterial activity and host range of modified OBPgpLys derivative variants

Exponential growing Gram-negative bacterial cells (OD_{600nm} = 0.6) were 100-fold diluted to a final density of about 10⁶ cells/ml and incubated for 30 minutes at room temperature without shaking with the different modified OBPgpLYS derivative variants. After incubation cell suspensions were diluted three times (respectively 10⁵-10⁴-10³ 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 (= log₁₀N₀/N_i with N₀ = number of untreated cells and N_i = number of treated cells, both counted after incubation) is calculated (Table 10). All samples were replicated in threefold. Averages +/- standard deviations are represented.

Table 10: *In vitro* antibacterial activity of different modified OBPgpLYS derivative variants on a range of exponential growing Gram-negative species with 0.5 mM EDTA. Initial density is 10^6 cells/ml and incubation proceeds for 30 minutes without shaking at RT. Protein concentration is 1500 nM, except for Artilysin1-OBPgpLys (800 nM).

	<i>P. aeruginosa</i> PAO1p	<i>P. putida</i> G1	<i>E. coli</i> X1-1	<i>Salmonella</i> <i>typhimurium</i> LT2
1500 nM α 4-OBPgpLys (SEQ ID NO: 75)	++	++	++	+
1500 nM Pentapeptide- OBPgpLys (SEQ ID NO: 77)	++	+++	++	+
800 nM Artilysin1- OBPgpLys (SEQ ID NO: 79)	Not determined	++	+	+
1500 nM Artilysin2- OBPgpLys (SEQ ID NO: 81)	++	++	++	+
1500 nM Parasin1-OBPgpLys (SEQ ID NO: 83)	+++	+++	+++	++
1500 nM Lycotoxin1- OBPgpLys (SEQ ID NO: 85)	++	+++	++	+
1500 nM OBPgpLYS (SEQ ID NO: 88)	+	+	+	+

Abbreviations: +: about 0.5 log; ++: 1-2 log; +++: 3-4 or more logs; not determined means that this strain was not tested with the respective fusion protein.

CLAIMS

1. A polypeptide comprising an amino acid sequence according to SEQ ID NO: 1 or a fragment or derivative thereof.
2. The polypeptide according to claim 1, wherein the fragment comprises an amino acid sequence according to SEQ ID NO: 4, 5 or 69 or 4 and 5.
3. The polypeptide according to claim 1 or 2, wherein the derivative has a deletion, addition, insertion and/or substitution in the amino acid sequence according to SEQ ID NO: 1, 4, 5 and/or 69.
4. The polypeptide according to claim 3, wherein the derivative comprises an amino acid sequence according to SEQ ID NO: 86 or 87.
5. The polypeptide according to any one of the preceding claims comprising additionally a tag, preferably a His₆-tag.
6. The polypeptide according to claim 5, wherein said polypeptide comprises an amino acid sequence according to SEQ ID NO: 47 or 88.
7. A fusion protein comprising a polypeptide according to any one of the preceding claims and a peptide stretch fused to said polypeptide at the N- or C-terminus, wherein said peptide stretch is a cationic peptide, polycationic peptide, amphipatic peptide, sushi peptide, defensin, hydrophobic peptide and/or an antimicrobial peptide.
8. The fusion protein according to claim 7, wherein said peptide stretch comprises about 5 to about 100 amino acid residues, in particular about 5 to 50 amino acid residues, in particular about 5 to 30 amino acid residues.
9. The fusion protein according to claim 7 or 8, wherein said cationic and/or polycationic peptide stretch comprises at least one amino acid residue selected out of the group consisting of arginine, histidine and lysine residues, in particular wherein at least 70%

of the amino acid residues comprised in said peptide stretch are arginine, histidine and/or lysine residues, in particular arginine and/or lysine residues.

10. The fusion protein according to claim 7, wherein the amphipatic peptide comprises at least one positively charged amino acid residues selected out of the group consisting of lysine, arginine and histidine residues, combined to at least one hydrophobic amino acid residue selected out of the group consisting of valine, isoleucine, leucine, methionine, phenylalanine, tryptophan, cysteine, alanine, tyrosine, histidine, threonin, serine, proline and glycine residues, in particular wherein at least about 70% of the said amino acid residues in said amphipatic peptide are either arginine or lysine residues and at least about 30% of the said amino acid residues in said amphipatic peptide are valine, isoleucine, leucine, methionine, phenylalanine, tryptophan, cysteine, alanine, tyrosine, histidine, threonin, serine, proline or glycine residues.
11. The fusion protein according to claim 7, wherein the peptide stretch comprises an amino acid sequence according to SEQ ID NO: 6-39, 50-53, 68, 70-73 or 117-119.
12. The fusion protein according to claim 7, wherein said fusion protein comprises an amino acid sequence according to SEQ ID NO: 43, 49, 54-67, 74-85 or 115-116.
13. An isolated nucleic acid molecule encoding a polypeptide according to any one of claims 1 to 6 or a fusion protein according to any one of claims 7 to 12.
14. A vector comprising the nucleic acid molecule according to claim 13.
15. A host cell comprising the nucleic acid molecule according to claim 13 or the vector according to claim 14.
16. The polypeptide according to any one of claims 1 to 6 or the fusion protein according to any one of claims 7 to 12 for use as human medical, veterinary medical or diagnostic substance, as an antimicrobial in food or on cosmetics, as disinfecting agent or in the environmental field.

17. The polypeptide according to any one of claims 1 to 6 or the fusion protein according to any one of claims 7 to 12 for use as a medicament for treatment or prevention of Gram-negative bacterial infections.
18. The use of the polypeptide according to any one of claims 1 to 6 or the fusion protein according to any one of claims 7 to 12 for the treatment or prevention of Gram-negative bacterial contamination of foodstuff, of food processing equipment, of food processing plants, of surfaces coming into contact with foodstuff, of medical devices, of surfaces in hospitals and surgeries.
19. Pharmaceutical composition comprising the polypeptide according to any one of claims 1 to 6 or the fusion protein according to any one of claims 7 to 12.