Antimicrobial Agents

The present invention relates to modified endolysin variants with improved antibacterial action against Gram-negative bacteria. Said modified endolysin variants comprise an endolysin and a cationic peptide fused to the endolysin, thus enhancing the cationicity of said endolysin. The present invention also relates to a microorganism transformed with a nucleic acid comprising a nucleotide sequence encoding a modified endolysin variant with enhanced cationicity. The invention further relates to a method for producing an endolysin variant using a microorganism transformed with a nucleic acid encoding an endolysin variant according to the present invention as production organism.

In particular the present invention relates to endolysin variants comprising an endolysin to which a peptide stretch with membrane or LPS disrupting activity is fused. Moreover, the present invention relates to nucleic acid molecules encoding said modified endolysin variant, 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 a method for producing said endolysin variant. Further, the present invention relates to said modified endolysin variant for use as a medicament, in particular for the treatment or prevention of Gram-negative bacterial infections, as diagnostic means, disinfectant or as cosmetic substance. The present invention also relates to the removal or reduction 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 the use of said endolysin variant as a diagnostic means in medicinal, food or feed or environmental diagnostic. Finally, the present invention relates to a pharmaceutical composition comprising said modified endolysin variant.

Endolysins are peptidoglycan hydrolases encoded by bacteriophages (or bacterial viruses). They are synthesized during late gene expression in the lytic cycle of phage multiplication and mediate the release of progeny virions from infected cells through degradation of the bacterial peptidoglycan. They are either ß(1,4)-glycosylases (lysozymes), transglycosylases,

amidases or endopeptidases. Antimicrobial application of endolysins was already suggested in 1991 by Gasson (GB2243611). Although the killing capacity of endolysins has been known for a long time, the use of these enzymes as antibacterials was ignored due to the success and dominance of antibiotics. Only after the appearance of multiple antibiotic resistant bacteria this simple concept of combating human pathogens with endolysins received interest. A compelling need to develop totally new classes of antibacterial agents emerged and endolysins used as 'enzybiotics' - a hybrid term of 'enzymes' and 'antibiotics' - perfectly met this need. In 2001, Fischetti and coworkers demonstrated for the first time the therapeutic potential of bacteriophage Cl endolysin towards group A streptococci (Nelson et a1., 2001). Since then many publications have established endolysins as an attractive and complementary alternative to control bacterial infections, particularly by Gram-positive bacteria. Subsequently different endolysins against other Gram-positive pathogens such as *Streptococcus pneumoniae* (Loeffler et al., 2001), *Bacillus anthracis* (Schuch et al., 2002), *S. agalactiae* (Cheng et al., 2005) and *Staphylococcus aureus* (Rashel et al, 2007) have proven their efficacy as enzybiotics. 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 endo1ysins to important Gram-negative pathogens.

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). Therefore, the cation-binding sites are essential for the integrity of the outer membrane (Vaara, 1992). Polycationic agents such as poly-L-lysine polymers (of at least 20 residues) increase the outer membrane permeability by displacement of these stabilizing divalent cations. In addition, they exert a so-called 'self-promoted uptake' mechanism (Hancock and Wong, 1984). Due to their bulkiness, they disrupt

the normal barrier function of the outer membrane and create transient cracks, promoting their own uptake (Vaara and Vaara, 1983). Furthermore, the dense and ordered packing of the 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).

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.

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 illustrate the present invention.

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

Figure 2 shows the expression and secretion of POLY-gp144 by *Pichia pastoris*. An amount of 30 µl supernatant of a *P. pastoris* X33 expression culture [after 1 day (square), 3 days (triangle) and 4 days (circle)] is added to 270 µl chloroform-permeabilized *P. aeruginosa* PAO1p cells. The buffer conditions were the optimal enzymatic conditions of POLY-gp144 (KH_2PO_4/K_2HP0_4) I = 120 mM pH 6.2). Subsequently, the optical density was spectrophotometrically recorded. A drop in optical density indicates the secretion of a

muralytic enzyme by *P. pastoris*. As a negative control, *P. pastoris* X33 without expression plasmid is included (diamond).

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

Figure 4 shows a picture of a Coomassie-stained SDS-PAGE showing the results of the expression and purification of the unmodified endolysin PSP3gp10 and its modified endolysin variant PKPSP3gp10. The lane LMW pertains to a size marker (LMW ladder). The following three lanes pertain to protein fractions of the purified protein in Elution Buffer (20 mM NaH_2P0_4-NaOH pH7.4; 0.5 M NaCl; 500 mM imidazole) after Ni^{2+} affinity chromatography. The lane FT pertains to the flow through and the lane W to waste fractions. Only minor secondary bands are visible in the purified protein fractions, indicating the high purity of the recombinant proteins (>90%).

Figures 5 A to D show in a graphic representation the antibacterial activities of unmodified PSP3gp10 and the modified PKPSP3gp10 in different compositions on several exponential growing Gram-negative bacteria after an incubation at room temperature and without shaking. Each species of Gram-negative bacteria was incubated for 30 minutes with a composition comprising 0.5 mM EDTA but no endolysin, with a composition comprising 1.315 µM unmodified PSP3gp10 but no EDTA, with a composition comprising 1.315 µM modified PKPSP3gp10 but no EDTA, with a composition comprising 1.315 µM unmodified PSP3gp10 and 0.5 mM EDTA and with a composition comprising 1.315 µM modified PKPSP3gp10 and 0.5 mM EDTA. In Figure 5 A the antibacterial activity on *P. aeruginosa* PAO1p cells is represented, in Figure 5 B the antibacterial activity on *P. aeruginosa* Br667 cells, in Figure 5 C the antibacterial activity on *E.coli* WK 6 cells and in Figure 5 D the antibacterial activity on *Salmonella typhimurium* cells. "Δ" gives the difference of activity between the respective PSP3gp10 and PKPSP3gp10 samples. The error bars render the standard deviations of the mean.

Figure 6 shows a picture of a Coomassie-stained SDS-PAGE showing the results of the expression and purification of the unmodified endolysin P2gp09 and its modified endolysin variant PKP2gp09. The lane LMW pertains to a size marker (LMW ladder). The following three lanes pertain to protein fractions of the purified protein in Elution Buffer (20 mM NaH_2P0_4-NaOH pH7.4; 0.5 M NaCl; 500 mM imidazole) after Ni^{2+} affinity chromatography. The lane FT pertains to the flow through and the lane W to waste fractions. Only minor secondary bands are visible in the purified protein fractions, indicating the high purity of the recombinant protein (>95%).

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

Figure 8 shows a picture of a Coomassie-stained SDS-PAGE showing the results of the expression and purification of the unmodified endolysin OBPgpLYS and its modified endolysin variant PKOBPgpLYS. The lane LMW pertains to a size marker (LMW ladder). The following three lanes pertain to protein fractions of the purified protein in Elution Buffer (20 mM NaH_2P0_4-NaOH pH7.4; 0.5 M NaCl; 500 mM imidazole) after Ni^{2+} affinity chromatography. The lane FT pertains to the flow through and the lane W to waste fractions. Only minor secondary bands are visible in the purified protein fractions, indicating the high purity of the recombinant proteins (>90%).

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

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

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, in particular an endolysin, autolysin and/or other peptidoglycan hydrolase, with a second or further amino acid sequence. The second or further

amino acid sequence is preferably a peptide stretch, in particular a cationic and/or polycationic 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 "modified endolysin variant" is used herein synonymously with the term "endolysin variant". Both terms refer to a fusion protein comprising an endolysin and a peptide stretch, in particular a cationic and/or polycationic peptide.

The term "peptide stretch" as used herein refers to any kind of peptide linked to a protein such as an endolysin, autolysin and/or peptidoglycan hydrolase. In particular the term "peptide stretch" as used herein refers to a cationic peptide and/or a polycationic peptide. However, a peptide stretch in the meaning of the present invention does not refer to His-tags, Strep-tags, Avi-tags, Myc-tags, Gst-tags, JS-tags, cystein-tags, FLAG-tags or other tags known in the art, thioredoxin or maltose binding proteins (MBP). The term "tag" in contrast to the term "peptide stretch" as used herein refers to a peptide which can be useful to facilitate expression and/or affinity purification of a polypeptide, to immobilize a polypeptide to a surface or to serve as a marker or a label moiety for detection of a polypeptide e.g. by antibody binding in different ELISA assay formats as long as the function making the tag useful for one of the above listed facilitation is not caused by the positively charge of said peptide. However, the His-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 to about 5 to 30 amino acid residues, wherein the amino group of one amino acid residue is linked to the carboxyl group of another amino acid residue by a peptide bond. A peptide may have a specific function. A peptide can be a naturally occurring peptide or a synthetically designed and produced peptide. The peptide can be, for example, derived or removed from a native protein by enzymatic or chemical cleavage, or can be prepared using conventional peptide synthesis techniques (e.g., solid phase synthesis) or molecular biology techniques (see Sambrook, J. et al., Molecular Cloning: A Laboratory Manual, Cold Spring

Harbor Press, Cold Spring Harbor, N.Y. (1989)). Preferred synthetically produced peptides are e.g. cationic or polycationic peptides.

As used herein, the term "cationic peptide" refers to a peptide having positively charged amino acid residues. Preferably a cationic peptide has a pKa-value of 9.0 or greater. Typically, at least four of the amino acid residues of the cationic peptide can be positively charged, for example, lysine or arginine. "Positively charged" refers to the side chains of the amino acid residues which have a net positive charge at about physiological conditions. The term “cationic peptide” as used herein refers also to polycationic peptides.

The term “polycationic peptide” as used herein refers to a synthetically designed and produced peptide composed of mostly positively charged amino acid residues, in particular lysine, arginine and/or histidine residues, more preferably lysine and/or arginine residues. A peptide is composed of mostly positively charged amino acid residues if at least about 20, 30, 40, 50, 60, 70, 75, 80, 85, 90, 95 or about 100 % of the amino acid residues are positively 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 “endolysin” as used herein refers to an enzyme which is suitable to hydrolyse bacterial cell walls. “Endolysins” comprise of at least one “enzymatically active domain” (EAD) having at least one of the following activities: endopeptidase, N-acetyl-muramoyl-L-alanine-amidase (amidase), N-acetyl-muramidase, N-acetyl-glucosaminidase (lysozyme) or transglycosylases. In addition, the endolysins may contain also regions which are enzymatically inactive, and bind to the cell wall of the host bacteria, the so-called CBDs (cell wall binding domains). The endolysin may contain one, two or more CBDs. However, the term “endolysin” as used herein refers also to enzymes having at least one EAD but no CBDs. Generally, the cell wall binding domain is able to bind different components on the surface of bacteria. Preferably, the cell wall binding domain is a peptidoglycan binding domain and binds to the bacteria’s peptidoglycan.

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

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

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

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

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

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

The present invention relates to improved antibacterial agents against Gram-negative bacteria, *in casu* modified endolysin variants, comprising an endolysin fused to a peptide with lipopolysachharide (LPS) or in general membrane disrupting activity. LPS is a major component of the outer membrane of Gram-negative bacteria. It increases the negative charge of the cell membrane and protects the membrane from certain kinds of chemical attack. To a certain degree said LPS protects the membrane of Gram-negative bacteria also from endolysins added from outside of the bacteria. However, the LPS can be disrupted by peptide

stretches having a LPS disrupting activity as e.g. positively charged peptides. Moreover, said peptide stretches may be involved in the outer membrane protein transport mechanism, a destabilisation of structural outer membrane proteins and/or in lipid-dependent destabilisation. The inventors of the present invention have surprisingly found, that a peptide stretch having LPS disrupting activity or in general membrane disrupting activity promotes the passage of an endolysin fused to said peptide stretch through the outer membrane of Gram-negative bacteria. After the promoted pass of the endolysin through the outer membrane of Gram-negative bacteria, the cell wall of the Gram-negative bacterium can be more easily be disrupted or desintegrated by the endolysin due to degradation of the peptidoglycan layer followed by osmotic lysis when the internal cell pressure of the bacterium cannot longer be resisted.

Thus, the present invention refers to fusion proteins composed of an endolysin having the activity of degrading the cell wall of Gram-negative bacteria and a peptide stretch with membrane disrupting activity, wherein said peptide stretch is fused to the enzyme at the N- and/or C-terminus. Said fusion proteins according to the present invention are also called modified endolysin variants or simply endolysin variants or modified endolysins.

The endolysin part of the modified endolysin variant is preferably encoded by bacteriophages specific for Gram-negative bacteria such as 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. baumanii.*

Moreover, the endolysin has preferably cell wall degrading activity against 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. baumanii.*

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

phage	publication	Wild type endolysin	predicted function of the endolysin
ΦV10	Perry, L.L. and Applegate, B.M.	PhiV10p30	chitinase
FELS-1	McClelland, M. and Wilson, R.K.	STM0907.Fels0	chitinase
ε15	Kropinksi, A.M. and McConnel, M.R.	epsilon15p25	chitinase
YUA	Ceyssens. P. (Laboratory for Gene technology)	YuA20	lytic transglycosylase (C) / 1 transmembranair domain (N)
B3	Braid, M.D. and Kitts, C.L.	ORF23	lytic transglycosylase (C) / 2 transmembranair domains (N)
BCEPμ	Summer, E.J. and Young, R.	BcepMu22	lytic transglycosylase (M) / 1 transmembranair domain (N)
F116	Byrne, M. and Kropinski, A.M.	F116p62	muraminidase (T4-like)
FELS-2	McClelland, M. and Wilson, R.K.	STM2715.S.Fels2	muraminidase (T4-like)
ES18	Casjens, S.R. and Hendrix, R.W.	gp76	muraminidase (T4-like)
SETP3	De Lappe, N and Cormican, M.	SPSV3_gp23	muraminidase (T4-like)
ΦECO32	Savalia, D and Severinov, K	phi32_17	muraminidase (T4-like)
HK022	Juhala , R and Hendrix, R.W.	HK022p54	muraminidase (lambdalike)
HK97	Juhala , R and Hendrix, R.W.	HK97p58	muraminidase (lambdalike)
HK620	Clark, A.J. and Dhillon, T.S.	HK620p36	muraminidase (lambdalike)
E1	Pickard, D. and Dougan, G	VIP0007	muraminidase (lambdalike)
SF6	Casjens, S and Clark, A.J.	Sf6p62	muraminidase (lambdalike)
SFV	Allison, G.E. and Verma, N.K.	R (SfVp40)	muraminidase (lambdalike)
BCEPC6B	Summer, EJ and Young, R.	gp22	muraminidase (lambdalike)
BCEPNAZGUL	Summer, EJ and Young, R.	Nazgul38	muraminidase (lambdalike)
P2	Christie, G.E. and Calender, R.	K (P2p09)	muraminidase (lambdalike)

WΦ	Christie, G.E. and Esposito, D.	K (Wphi09)	muraminidase (lambdalike)
RV5	Kropinski, A.M. and Johnson	rv5_gp085	muraminidase (lambdalike)
JS98	Zuber, S and Denou, E.	EpJS98_gp116	muraminidase (T4-like)
13A	Savalia, D and Molineux, I.	gp3.5	muramoyl-L-alanine amidase
BA14	Savalia, D and Molineux, I.	gp3.5	muramoyl-L-alanine amidase
ECODS1	Savalia, D and Molineux, I.	gp3.5	muramoyl-L-alanine amidase
K1F	Scholl, D and Merril, C	CKV1F_gp16	muramoyl-L-alanine amidase
T3	Pajunen, M.I. and Mollineux, I.J.	T3p18	muramoyl-L-alanine amidase
GH-1	Kropinski, A.M. and Kovalyova, I.V.	gh-1p12	muramoyl-L-alanine amidase
K11	Molineux, I. and Savalia, D.	gp3.5	muramoyl-L-alanine amidase
BIP-1	Liu, M and Miller, J.F.	bip-1p02	lysozyme (N) / PG-binding domain (C)
BMP-1	Liu, M and Miller, J.F.	bmp-1pO2	lysozyme (N) / PG-binding domain (C)
BPP-1	Liu, M and Miller, J.F.	bpp2	lysozyme (N) / PG-binding domain (C)
ΦCTX	Nakayama, K and Hayashi, T.	ORF12	PG-binding domain (N) / muramidase (C)
BCEP43	Summer, EJ and Young, R.	Bcep43-27	PG-binding domain (N) / muramidase (C)
BCEP781	Summer, EJ and Young, R.	Bcep781-27	PG-binding domain (N) / muramidase (C)
BCEP1	Summer, EJ and Young, R.	Bcep1-28	PG-binding domain (N) / muramidase (C)
BCEPNY3	Summer, EJ and Young, R.	BcepNY3gene26	PG-binding domain (N) / muramidase (C)
ΦE12-2	DeShazer, D and Nierman, W.C.	gp45	PG-binding domain (N) / muramidase (C)
Φ52237	DeShazer, D and Nierman, W.C.	gp28	PG-binding domain (N) / muramidase (C)
ΦP27	Recktenwald, J and Schmidt, H.	P27p30	endopeptidase
RB49	Monod, C and Krisch, H.M.	RB49p102	endopeptidase
Φ1	Arbiol, C. and Comeau, A.M.	phi1-p102	endopeptidase
T5	Pankova, N.V. and Ksenzenko, V.N.	lys (T5.040)	endopeptidase
201phi2-1	Thomas *et al.*, 2008		PG-binding domain (N) / unknown catalytic domain (C)
Aeh1	Monod, C and Krisch, H.M.	Aeh1p339	muraminidase (T4-like)
YYZ-2008	Kropinski, A.M.	YYZgp45	muraminidase (lambda-like)

Also preferred is the endolysin part deriving from endolysins of the *Pseudomonas aeruginosa* phages ΦKZ and EL, of the *Pseudomonas putida* phage OBP, of the phage LUZ24, or from T4 lysozyme, gp61 muramidase and PSP3 endolysin.

More preferably, the endolysin part is selected from the group consisting of phiKZgp144 according to SEQ ID NO:1, ELgp188 according to SEQ ID NO:2, *Salmonella* endolysin according to SEQ ID NO:3, Enterobacteria phage T4 endolysin according to SEQ ID NO:4,

Acinetobacter baumanii endolysin according to SEQ ID NO:5, *E.coli* Phage K1F endolysin according to SEQ ID NO:6, OBPgpLYS according to SEQ ID NO: 7, PSP3 *Salmonella* endolysin (PSP3gp10) according to SEQ ID NO: 8 and *E.coli* Phage P2 endolysin (P2gp09) according to SEQ ID NO: 9.

In another preferred embodiment of the present invention the endolysins or the modified endolysin variants according to the present invention comprise modifications and/or alterations of the amino acid sequences. Such alterations and/or modifications may comprise mutations such as deletions, insertions and additions, substitutions or combinations thereof and/or chemical changes of the amino acid residues, e.g. biotinylation, acetylation, PEGylation, chemical changes of the amino-, SH- or carboxyl-groups. Said modified and/or altered endolysins exhibit the lytic activity of the respective wild type endolysin. However, said activity can be higher or lower as the activity of the respective wild type endolysin. Said activity can be about 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190 or about 200 % of the activity of the respective wild-type endolysin or even more. The activity can be measured by assays well known in the art by a person skilled in the art as e.g. the plate lysis assay or the liquid lysis assay which are e.g. described in *(Briers et al., J. Biochem. Biophys Methods 70: 531-533, (2007)).*

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

In a preferred embodiment the endolysin variant comprises an endolysin and a peptide fused thereto said peptide comprising about 3 to about 50, more preferably about 5 to about 20, for instance about 5 to about 15 amino acid residues and at least 20, 30, 40, 50, 60 or 70%, more preferably at least 80%, for instance at least 90% of the said amino acid residues are either arginine or lysine residues. In another preferred embodiment the endolysin variant comprises

an endolysin and a peptide fused thereto said peptide comprising about 3 to about 50, more preferably about 5 to about 20, for instance about 5 to about 15 amino acid residues and said amino acid residues are either arginine or lysine residues.

Preferably, the peptide stretch of the modified endolysin variant is fused to the N-terminus and/or to the C-terminus of the endolysin. In a particular preferred embodiment said peptide stretch is only fused to the N-terminus of the endolysin. However, also preferred are modified endolysin variants having a peptide stretch both on the N-terminus and on the C-terminus. Said peptide stretches on the N-terminus and on the C-terminus can be the same or distinct peptide stretches.

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

In one aspect of the present invention the fused peptide stretch is a cationic and/or polycationic peptide, which comprises one or more of the positively charged amino acid residues of lysine, arginine and/or histidine, in particular of lysine and/or arginine. Preferably, more than about 20, 30, 40, 50, 60, 70, 75, 80, 85, 90, 95 or 99 % of the amino acid residues in said peptide 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: 10 (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: 10 (KRKKRK) are preferred. More preferred are cationic peptide stretches comprising at least one KRK motive (lys-arg-lys), preferable at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32 or 33 KRK motives.

In another preferred embodiment of the present invention the cationic peptide stretch comprises beside the positively charged amino acid residues, in particular lysine and/or arginine residues, neutrally charged amino acid residues, in particular glycine and/or serine residues. Preferred are cationic peptide stretches consisting of about 70 % to about 100 %, or about 80 % to about 95 %, or about 85 % to about 90 % positively charged amino acid residues, in particular lysine, arginine and/or histidine residues, more preferably lysine and/or arginine residues and of about 0 % to about 30 %, or about 5 % to about 20 %, or about 10 % to about 20 % neutrally charged amino acid residues, in particular glycine and/or serine residues. Preferred are polypeptide stretches consisting of about 4 % to about 8 % serine residues, of about 33 % to about 36 % arginine residues and of about 56 % to about 63 % lysine residues. Especially preferred are polypeptide stretches comprising at least one motive according to SEQ ID NO: 32 (KRXKR), wherein X is any other amino acid than lysine, arginine and histidine. Especially preferred are polypeptide stretches comprising at least one motive according to SEQ ID NO: 33 (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: 32 (KRXKR) or SEQ ID NO: 33 (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: 34 (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: 34 (KRGSG).

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

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

peptide stretch	**length**	**SEQ ID NO:**
KRKKRK	6	SEQ ID NO: 10
KRKKRKKRK	9	SEQ ID NO: 11
RRRRRRRRR	9	SEQ ID NO: 12
KKKKKKKK	8	SEQ ID NO: 13
KRKKRKKRKK	10	SEQ ID NO: 14
KRKKRKKRKKRK	12	SEQ ID NO: 15
KRKKRKKRKKRKKR	14	SEQ ID NO: 16
KKKKKKKKKKKKKKKK	16	SEQ ID NO: 17

KRKKRKKRKKRKKRKKRKK	19	SEQ ID NO: 18
RRRRRRRRRRRRRRRRRRR	19	SEQ ID NO: 19
KKKKKKKKKKKKKKKKKKK	19	SEQ ID NO: 20
KRKKRKKRKRSKRKKRKKRK	20	SEQ ID NO: 21
KRKKRKKRKRSKRKKRKKRKK	21	SEQ ID NO: 22
KRKKRKKRKKRKKRKKRKKRK	21	SEQ ID NO: 23
KRKKRKKRKRGSGKRKKRKKRK	22	SEQ ID NO: 24
KRKKRKKRKRGSGSGKRKKRKKRK	24	SEQ ID NO: 25
KRKKRKKRKKRKKRKKRKKRKKRKK	25	SEQ ID NO: 26
KRKKRKKRKRSKRKKRKKRKRSKRKKRKKRK	31	SEQ ID NO: 27
KRKKRKKRKRGSGSGKRKKRKKRKGSGSGKRKKRKKRK	38	SEQ ID NO: 28
KRKKRKKRKKRKKRKKRKKRKKRKKRKKRKKRKKRKK	39	SEQ ID NO: 29
KRKKRKKRKRSKRKKRKKRKRSKRKKRKKRKRSKRKKRKKRK	42	SEQ ID NO: 30

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

Preferably, the peptide stretch has the function to lead the modified endolysin variant 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 enzyme. The function to lead the modified endolysin variant through the outer membrane of Gram-negative bacteria is caused by the potential of the outer membrane or LPS disrupting activity of said peptide stretch.

Especially preferred are modified endolysin variants selected from the group consisting of the following modified endolysin variants:

Modified endolysin variant	**SEQ ID NO: (modified endolysin variant)**	**Endolysin part**	**Peptide stretch (N-terminal unless otherwise indicated)**
POLY-gp144	SEQ ID NO: 35	SEQ ID NO: 1	SEQ ID NO: 11
$(POLY)^2$-gp144	SEQ ID NO: 36	SEQ ID NO: 1	SEQ ID NO: 21
$(POLY)^3$-gp144	SEQ ID NO: 37	SEQ ID NO: 1	SEQ ID NO: 27
$(POLY)^4$-gp144	SEQ ID NO: 38	SEQ ID NO: 1	SEQ ID NO: 30
POLY-gp188	SEQ ID NO: 39	SEQ ID NO: 2	SEQ ID NO: 11
$(POLY)^2$-gp188	SEQ ID NO: 40	SEQ ID NO: 2	SEQ ID NO: 21

$(POLY)^3$-gp188	SEQ ID NO: 41	SEQ ID NO: 2	SEQ ID NO: 27
$(POLY)^4$-gp188	SEQ ID NO: 42	SEQ ID NO: 2	SEQ ID NO: 30
pKKZ144pET32b	SEQ ID NO: 43	SEQ ID NO: 1	SEQ ID NO: 14
KRK_6_pET32b	SEQ ID NO: 44	SEQ ID NO: 1	SEQ ID NO: 10
KRK_12_pET32b	SEQ ID NO: 45	SEQ ID NO: 1	SEQ ID NO: 15
KRK_14_pET32b	SEQ ID NO: 46	SEQ ID NO: 1	SEQ ID NO: 16
R9_pET32b	SEQ ID NO: 47	SEQ ID NO: 1	SEQ ID NO: 12
K8_pET32b	SEQ ID NO: 48	SEQ ID NO: 1	SEQ ID NO: 13
pK2KZ144_pET32b_mod3	SEQ ID NO: 49	SEQ ID NO: 1	SEQ ID NO: 28
PKPSP3gp10	SEQ ID NO: 53	SEQ ID NO: 8	SEQ ID NO: 11
PKP2gp09	SEQ ID NO: 57	SEQ ID NO: 9	SEQ ID NO: 11
PKOBPgpLYS	SEQ ID NO: 61	SEQ ID NO: 7	SEQ ID NO: 11
pK2KZ144pET32b	SEQ ID NO: 62	SEQ ID NO: 1	SEQ ID NO: 22
pK3KZ144pET32b	SEQ ID NO: 63	SEQ ID NO: 1	SEQ ID NO: 27
pK4KZ144pET32b	SEQ ID NO: 64	SEQ ID NO: 1	SEQ ID NO: 30
KRK_19_pET32b	SEQ ID NO: 66	SEQ ID NO: 1	SEQ ID NO: 18
KRK_21_pET32b	SEQ ID NO: 67	SEQ ID NO: 1	SEQ ID NO: 23
KRK_25_pET32b	SEQ ID NO: 68	SEQ ID NO: 1	SEQ ID NO: 26
KRK_39_pET32b	SEQ ID NO: 69	SEQ ID NO: 1	SEQ ID NO: 29
K19_pET32b	SEQ ID NO: 70	SEQ ID NO: 1	SEQ ID NO: 20
K16_pET32b	SEQ ID NO: 71	SEQ ID NO: 1	SEQ ID NO: 17
pKKZ-144_K2_pET32b	SEQ ID NO: 72	SEQ ID NO: 1	N-terminal: SEQ ID NO: 11 C-terminal: SEQ ID NO: 21
pK2KZ144_pET32b_mod1	SEQ ID NO: 73	SEQ ID NO: 1	SEQ ID NO: 24
pK2KZ144_pET32b_mod2	SEQ ID NO: 74	SEQ ID NO: 1	SEQ ID NO: 25
smi01_KRK9	SEQ ID NO: 75	SEQ ID NO: 1	SEQ ID NO: 11
smi02_KRK9	SEQ ID NO: 76	SEQ ID NO: 1	SEQ ID NO: 11
smi03_KRK9	SEQ ID NO: 77	SEQ ID NO: 1	SEQ ID NO: 11
smi04_KRK9	SEQ ID NO: 78	SEQ ID NO: 1	SEQ ID NO: 11

The modified endolysin variants according to the present invention, and thus in particular the especially preferred modified endolysin variants according to SEQ ID NO: 35 to 49, 53, 57, 61 to 64 and 66 to 78, may additional comprise a tag e.g. for purification. Preferred is a His_6-

tag, preferably at the C-terminus of the modified endolysin variant. Said tag can be linked to the modified endolysin variant by additional amino acid residues e.g. due to cloning reasons. Preferably said tag can be linked to the modified endolysin variant by at least 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 additional amino acid residues. In a preferred embodiment the modified endolysin variant comprises a His_6-tag at its C-terminus linked to the modified endolysin variant by the additional amino acid residues lysine and glycine (Lys-Gly) or leucine and glutamic acid (Leu-Glu).

In particular, the modified endolysin variants as used in the examples as described below are preferred. The modified endolysin variants according to SEQ ID NO: 35 to 42, 53, 57 and 61 as used in the examples comprise a His_6-tag at the C-terminus linked to the respective modified endolysin variant by the additional amino acid residues lysine and glycine (Lys-Gly). The modified endolysin variants according to SEQ ID NO: 43 to 49 and 75 as used in the examples comprise a His_6-tag at the C-terminus linked to the respective modified endolysin variant by the additional amino acid residues leucine and glutamic acid (Leu-Glu).

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

As some fusion proteins may either be toxic upon expression in bacteria, or not homogenous due to protein degradation, the strategy might be to express these fusion proteins fused or linked to other additional proteins. Example for these other additional protein is Thioredoxin, which was shown to mediate expression of toxic antimicrobial peptides in *E.coli* (TrxA mediating fusion expression of antimicrobial peptide CM4 from multiple joined genes in Escherichia coli. Zhou L, Zhao Z, Li B, Cai Y, Zhang S. Protein Expr Purif. 2009 Apr;64(2):225-230).

For antimicrobial function of the fusion proteins it may be necessary to remove the additional fusion protein by proteolytic cleavage. Commercially available kits like the pET32 expression system (Novagen), may need to modify e.g. the N-terminus of the fusion depending on the

protease used, like from MGS to AMGS (SEQ ID NO: 31), were the remaining alanine residue results from an introduced Enterokinase cleavage site.

In another preferred embodiment of the present invention the peptide stretches of the modified endolysin variant 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.

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

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

Another aspect of the present invention is related to a method for genetically transforming a suitable host cell in order to obtain the expression of the modified endolysin variants according to the invention wherein the host cell is genetically modified by the introduction of a genetic material encoding said modified endolysin variants into the host cell and obtain their translation and expression by genetic engineering methods well known by a person skilled in the art.

In a further aspect the present invention relates to a composition, preferably a pharmaceutical composition, comprising a modified endolysin variant according to the present invention and/or a host transformed with a nucleic acid molecule or a vector comprising a nucleotide sequence encoding a modified endolysin variant 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 µM to about 100 mM EDTA, more preferably about 50 µM to about 10 mM EDTA, more preferably about 0.5 mM to about 10 mM EDTA, more preferably about 0.5 mM to about 2 mM EDTA, more preferably about 0.5 mM to 1 mM EDTA. However, also compositions comprising about 10 µM to about 0.5 mM EDTA are preferred. Also preferred is a composition comprising about 0.5 mM to about 2 mM EDTA, more preferably about 1 mM EDTA and additionally about 10 to about 100 mM TRIS.

The present invention also relates to a modified endolysin variant according to the present invention and/or a host transformed with a nucleic acid comprising a nucleotide sequence encoding a modified endolysin variant according to the present invention for use as a medicament.

In a further aspect the present invention relates to the use of a modified endolysin variant according to the present invention and/or a host transformed with a vector comprising a nucleic acid molecule comprising a nucleotide sequence encoding a modified endolysin variant 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 pathogenic 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. baumanii.* Preferably, said disorder, disease or condition may be caused by Pseudomonas, in particular *Pseudomonas aeruginosa* and/or *Pseudomonas putida*, Burkholderia, in particular *Burkholderia pseudomallei* and/or *Burkholderia solanacearum*, Salmonella, in particular *Salmonella typhimurium* and/or *Salmonella Enteritidis*, Acinetobacter, in particular *Acinetobacter baumannii*, *Escherichia coli* and/or Klebsiella, in particular *Klebsiella pneumoniae.*

The present invention further relates to a medicament comprising a modified endolysin variant according to the present invention and/or a host transformed with a nucleic acid comprising a nucleotide sequence encoding a modified endolysin variant 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 modified endolysin variant according to the present invention and/or an effective amount of a host transformed with a nucleic acid comprising a nucleotide sequence encoding a modified endolysin variant according to the present invention or a composition according to the present invention. The subject may be a human or an animal.

Preferably said method of treatment may be for the treatment and/or prevention of infections caused by Gram-negative bacteria, in particular by the Gram-negative bacteria as listed above. In particular said method of treatment may be for the treatment and/or prevention of infections of the skin, of soft tissues, the respiratory system, the lung, the digestive tract, the eye, the ear, the teeth, the nasopharynx, the mouth, the bones, the vagina, of wounds of bacteraemia and/or endocarditis caused by Gram-negative 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, inhalational, intravenous, intramuscular, intrathecal, intraspinal, endobronchial, intrapulmonal, intraosseous, intracardial, intraarticular, rectal, vaginal or any other route of administration.

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

In a specific embodiment of the present invention the use of a modified endolysin variant according to the present invention and/or a host transformed with a vector comprising a nucleic acid molecule comprising a nucleotide sequence encoding a modified endolysin variant according to the present invention in the manufacture of a medicament for the treatment and/or prevention of a disorder, disease or condition caused by *Pseudomonas*, particularly by *Pseudomonas aeruginosa* in particular intestinal affections, in particular in

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

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

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

In another specific embodiment of the present invention the disorder, disease or condition is caused by *Acinetobacter baumannii*, in particular bronchitis, pneumonia, wound infections and septicemia, in particular as a result of intravenous catheterization.

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

In another specific embodiment of the present invention the disorder, disease or condition is caused by *Klebsiella pneumoniae*, in particular pneumonia, bacteremia, meningitis and infections of the urinary tract.

Preferably, a modified endolysin variant 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 modified endolysin variant 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 modified endolysin variant according to the present invention and/or one or more hosts transformed with a nucleic acid comprising a nucleotide sequence encoding a modified endolysin variant 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 modified endolysin variant according to the present invention and/or one or more hosts transformed with a nucleic acid comprising a nucleotide sequence encoding a modified endolysin variant 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 modified endolysin variant 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 modified endolysin variant 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 modified endolysin variant 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 modified endolysin variant 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 modified endolysin variant according to the present invention for the removal, reduction and/ 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 modified endolysin variant 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 modified endolysin variant 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 modified endolysin variant of the present invention. Therefore, a modified endolysin variant 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.

For the use of the modified endolysin variant according to the present invention as a disinfectant e.g. in hospital, dental surgery, veterinary, kitchen or bathroom, the modified endolysin variant can be prepared in a composition in form of e.g. a fluid, a powder, a gel, or an ingredient of a wet wipe or a disinfection sheet product. Said composition may additionally comprise suitable carrier, additives, diluting agents and/or excipients for its respective use and form, respectively, - but also agents that support the antimicrobial activity like EDTA or agents enhance the antimicrobial activity of the fusion proteins. The fusion protein may also be used with common disinfectant agents like, Alcohols, Aldehydes, Oxidizing agents, Phenolics, Quaternary ammonium compounds or UV-light. For disinfecting for example surfaces, objects and/or devices the modified endolysin variant can be applied on said surfaces, objects and/or devices. The application may occur for instance by wetting the disinfecting composition with any means such as a cloth or rag, by spraying, pouring. The fusion proteins may be used in varying concentration depending on the respective application and the „reaction time“ intended to obtain full antimicrobial activity.

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

The following examples explain the present invention but are not considered to be limiting. Unless indicated differently, molecular biological standard methods were used, as e.g.,

described by Sambrock et al., 1989, Molecular Cloning: A Laboratory Manual, 2nd edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

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

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

For the amplification of the open reading frame (ORF) of phiKZgp144 and ELgp188 PCR a standard 5' primer (for phiKZgp144: 5’ ATGAAAGTATTACGCAAA 3’ (SEQ ID NO: 83); for ELgp188 5’ ATGAACTTCCGGACGAAG 3’ (SEQ ID NO: 65)) and the standard 3' primers according to SEQ ID NO: 81 and 82 were applied (for phiKZgp144: TTTTCTATGTGCTGCAAC (SEQ ID NO: 81); for ELgp188: ATACGAAAT AACGTGACGA (SEQ ID NO: 82)) was used. To extend the 5' end of the open reading frame encoding phiKZgp144 or ELgp188 with a gene fragment encoding nine positively charged residues (Lys-Arg-Lys-Lys-Arg-Lys-Lys-Arg-Lys – SEQ ID NO: 11) a tail PCR with an extended 5' primer (for phiKZgp144: 5’ ATGGGATCCAAACGCAAGAAACGTAAGAAA CGCAAAAAAGTATTACGCAAAG 3’ (SEQ ID NO 79); for ELgp188: 5’ ATGGGATCCAAACGCAAGAAACGTAAGAAA CGCAAAAACTTCCGGACGAAG 3’ (SEQ ID NO: 80)) and the standard 3' primers according to SEQ ID NO: 81 and 82 were applied. The PCR product was cloned in the pEXP5CT/TOPO® expression vector (Invitrogen, Carlsbad, CA, USA) according to the protocol of the manufacturer. Arginine triplets were incorporated besides lysine triplets to avoid tRNA depletion and reduce the risk of frameshifts (the only two available triplets for lysine are AAA and AAG, leading to long A-stretches). Insertion of additional polycationic cassettes into the designed BamHI restriction site lengthens the tail with extra cationic residues. This insertion creates an arginine and serine triplet at each junction site (Figure 1). Up to four polycationic peptide stretches were fused to both phiKZgp144 and ELgp188, designated $(POLY)^n$-gp144 or $(POLY)^n$-gp188 (n=1,2,3,4), comprising respectively 9, 19, 29 and 39 positively charged amino acid residues in the N-terminus. Accordingly, the following constructs were expressed in *E. coli* BL21

(DE3) pLysS cells (exponentially growing cells at 37°C, induction using 1mM IPTG, expression for 4 h at 37°C):

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

The modified endolysin variants POLY-gp144 (SEQ ID NO: 35), $(POLY)^2$-gp144 (SEQ ID NO: 36), POLY-gp188 (SEQ ID NO: 39) and $(POLY)^2$-gp188 (SEQ ID NO: 40) have been used for further investigations. Said proteins were purified by Ni^{2+} affinity chromatography using the C-terminal 6xHis-tag (Akta Fast Protein Liquid Chromatography using 1ml His-trap Ni-NTA columns). The total yields per liter *E.coli* expression culture were determined by spectrophotometric measurement of the protein concentration and the total volume of the purified stock solution. The purification of gp188 derivatives was performed under more stringent conditions (65 mM imidazole) compared to gpl44 derivatives (50mM imidazole) to ensure high purity. The total yields per liter *E. coli* expression culture are shown in table 1.

Table 1 - Yields of recombinant purification of endolysin derivatives per liter E. coli expression culture.

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

Purified stock solutions were ~90% pure. Mass spectrometric analysis of purified solutions of POLY-derivatives revealed traces of the *E. coli 50S* ribosomal subunit protein L2 and 16S

rRNA uridine-516 pseudo-uridylate synthase. All phiKZgp144 derivatives showed multimer formation which could be converted to monomers by addition of ß-mercaptoethanol, indicating that interdisulfide bonds cause multimerization.

EXAMPLE 2: Antibacterial activity of modified phiKZgp144 and ELgp188 variants

Exponential (~10^6/ml) *P. aeruginosa* PAO1p cells (Pirnay JP et al. (2003), *J Clin Microbiol.*, 41(3):1192-1202) were 100 x diluted (final density was ~10^6/ml) and incubated at room temperature with each 10 µg undialyzed protein (unmodified endolysins phiKZgp144 (SEQ ID NO: 1) and ELpg188 (SEQ ID NO: 2) and modified endolysin variants POLY-gp144 (SEQ ID NO:35), $(POLY)^2$-gp144 (SEQ ID NO: 36), POLY-gp188 (SEQ ID NO: 39) and $(POLY)^2$-gp188 (SEQ ID NO: 40) at a final concentration of 100 µg/ml in buffer (20 mM NaH_2P0_4-NaOH pH7.4; 0.5 M NaCl; 0.5 M imidazole). After 1 hour cell suspensions were diluted in PBS buffer (10e-5, 10e-4 and 10e-3) and plated (standard LB-medium, incubated overnight at 37°C). Additionally, a negative control containing cells in PBS buffer was plated. The residual colonies were counted after an overnight incubation. Based on the counted cell numbers the antibacterial activity as the relative inactivation (%) (=100-(N_i/No)*l00 with N_0 = number of untreated cells and N_i = number of treated cells) and in logarithmic units (=$\log_{10}N_0/N_i$) was calculated (Table 2). All samples were replicated in six fold. Averages/standard deviations are represented. Statistical analysis was performed using a student's t-test.

Unmodified endolysins phiKZgp144 and ELgp188 do not reduce cell numbers significantly compared to the negative control. This observation illustrates the efficacy of the outer membrane as a barrier for the endolysin to degrade the cell wall of the Gram-negative bacteria. In contrast as shown in Table 2 the incubation with the modified endolysins POLY-gp144, $(POLY)^2$-gp144, POLY-gp188 and $(POLY)^2$-gp188 causes a significant reduction (α = 0.05) of the bacterial cell number (99.85 ± 0.09 % for POLY-gp144 and 98.0 ± 0.2% for POLY-gp188). An increase of the length of the polycationic peptide stretch further tends to strengthen the antibacterial activity, especially in case of phiKZgp144 (a reduction up to 99.98 ± 0.02 % or 3.7 ± 0.3 log units is achieved within 1 hour for $(POLY)^2$-gp144).

Moreover, the experiments demonstrated that the modified endolysins of phiKZgp144 have a higher antibacterial activity than the modified endolysins of ELgp188.

Table 2 - Antibacterial effect of endolysins unmodified and modified phiKZgp144 and ELgp188 variants.

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

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

In a further experiment the modified endolysin POLY-gp144 was dialyzed to 50 mM KH_2P0_4/K_2HP0_4 pH 7 and used instead of undialyzed protein solution as described above. Thereby, the inactivation level was additionally increased from 2.9 ± 0.3 log units to 3.9 ± 0.2 log units.

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

The open reading frame encoding POLY-gp144 (SEQ ID NO: 35) was cloned in the pPICZαA shuttle vector (Invitrogen), which was subsequently integrated in the *P. pastoris* genome by homologous recombination (as indicated by the manufacturer; *P. pastoris* X33 cells, Invitrogen). Gene expression was induced with methanol (1%) in BMMY-medium and the supernatant was analyzed for the presence of enzymatic activity after 1, 3 and 4 days. Therefore, an amount of 30 µl supernatant of the *P. pastoris* expression culture was added to

270 µl chloroform-permeabilized P. aeruginosa PAO1p cells (Pirnay JP et al. (2003), *J Clin Microbiol.*, 41(3):1192-1202) after 1, 3 and 4 days (buffer condition: KH_2PO_4/K_2HP0_4 I = 120 mM pH 6.2). Subsequently, the optical density was spectrophotometrically recorded (Figure 2). A drop in optical density indicates the secretion of a muralytic enzyme by *P. pastoris*. As a negative control, *P. pastoris* X33 without expression plasmid was included. Thus, the lysis of the substrate upon addition of the supernatants sample is a measure for successful recombinant production and secretion of POLY-gp144 (SEQ ID NO: 35) by *P. pastoris*. After 1 day, a limited enzymatic activity could be detected. The maximum activity was observed after 3 days since no significant increase of activity in the supernatants was observed at the fourth day. No toxic effect on the cell density of *P. pastoris* was observed.

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

EXAMPLE 4: Further modified endolysin phiKZgp144 variants with different polycationic peptide stretches

To test and to compare the potential of polycationic peptides variants of phiKZgp144 and other endolysin encoding genes were synthesised having different polycationic peptides at the N-terminal end of the protein. Peptide stretch variation concerns length, composition and insertion of linker sequences. On the one hand further polycationic peptide stretches having N-terminal multiples of the KRK motive were produced. On the other hand polycationic peptide stretches consisting only of arginine (R) or lysine (K) were produced. Moreover, to enhance the translation of long polycationic peptide stretches, polycationic peptide stretches comprising a linker sequence were produced.

The different products were cloned in the pET32b expression vector (Novagen, Darmstadt, Germany). pET32b was used to reduce potential toxicity of the polycationic peptide against

the *E. coli* host. A vector-encoded fusion protein (thioredoxin) masks the polycationic peptide and can be eliminated during the purification process.

Accordingly, the following modified endolysin variants were expressed in *E. coli* BL21 (DE3) cells at 37°C until an optical density of OD600nm=0.6 was reached. Then protein expression was induced with 1 mM IPTG (final concentration) and expression was preformed for four hours. Then *E. coli* cells were harvested by centrifugation for 20 min at 6000g and cell disruption and protein purification was performed according the S-tag purification kit (Novagen, Darmstadt, Germany):

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

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

Exponential (~10^6/ml) *P. aeruginosa* PAO1p cells (Burn wound isolate, Queen Astrid Hospital, Brussels; Pirnay JP et al. (2003), *J Clin Microbiol.*, 41(3):1192-1202) were 100 x diluted (final density was ~10^6/ml) incubated at room temperature with each 10 µg undialyzed protein as listed above at a final concentration of 100 µg/ml in buffer (20 mM NaH_2P0_4-NaOH pH7.4; 0.5 M NaCl; 0.5 M imidazole). After 1 hour cell suspensions were diluted 1:100 and plated on LB. Additionally, a negative control was plated using buffer (20 mM

NaH_2P0_4-NaOH pH7.4; 0.5 M NaCl; 0.5 M imidazole). The residual colonies were counted after an overnight incubation at 37°C. Based on the counted cell numbers the antibacterial activity as the relative inactivation (%) (=100-(N_i/No)*l00 with N_0 = number of untreated cells and N_i = number of treated cells) was calculated (Table 3). All samples were replicated at least in four fold.

Table 3 - Antibacterial effect of endolysins unmodified and modified phiKZgp144 and ELgp188

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

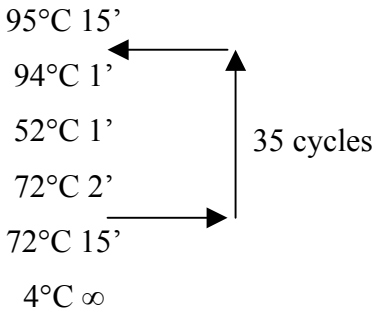
Unmodified phiKZgp144 does not reduce cell numbers significantly compared to the negative control. Beyond that, modified phiKZgp144 variants wearing a polycationic peptide of N-terminal multiples of the KRK motive enhance the antimicrobial effect immensely. However, also variants having a homomer peptide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

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

PSP3gp10 according to SEQ ID NO: 8 is a globular endolysin with 165 amino acid residues originating from *Salmonella typhimurium* phage PSP3 with a catalytic lambda-like

muramidase domain. As predicted by BLASTp and Pfam analysis the PSP3gp10 endolysin comprises its catalytic domain in the range of about amino acid residue 34 to about amino acid residue 152.

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



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

Recombinant expression of PSP3gp10 according to SEQ ID NO: 8 and PKPSP3gp10 according to SEQ ID NO: 53 is performed in exponentially growing *E. coli* BL21 (λDE3) pLysS cells (Invitrogen) after induction with 1 mM IPTG (isopropylthiogalactoside) at 37°C for a period of 4 hours. Both proteins were purified by Ni^{2+} affinity chromatography (Akta FPLC, GE Healthcare) using the C-terminal 6xHis-tag, encoded by the pEXP5CT/TOPO®

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

1. *Equilibration* of the *Histrap HP 1 ml* column (GE Healthcare) with 10 column volumes of Washing Buffer (60 mM imidazole, 0.5 mM NaCl and 20 mM NaH_2P0_4-NaOH on pH 7.4) at a flow rate of 0.5 ml/min.
2. *Loading* of the total lysate (with wanted endolysin) on the *Histrap HP 1 ml* column at a flow rate of 0.5 ml/min.
3. Washing of the column with 15 column volumes of Washing Buffer at a flow rate of 1 ml/min.
4. Elution of bounded endolysin from the column with 10 column volumes of Elution Buffer (500 mM imidazole, 5 mM NaCl and 20 mM NaH_2P0_4-NaOH on pH 7.4) at a flow rate of 0.5 ml/min

The total yields of both purified recombinant proteins per liter *E.coli* expression culture shown in Table 4. The values were determined by spectrophotometric measurement of the protein concentration and the total volume of the purified stock solution at a wavelength of 280 nm. Purified stock solutions consisting of PSP3gp10 and PKPSP3gp10, respectively, in Elution Buffer (20 mM NaH_2P0_4-NaOH pH7.4; 0.5 M NaCl; 500 mM imidazole) were at least 90% pure as determined visually on SDS-PAGE gels.

Table 4 - Yields of purified recombinant PSP3gp10 endolysin and its modified variant PKPSP3gp10 per liter E. coli expression culture.

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

To determine the anti-Gram-negative spectrum of the PKPSP3gp10 endolysin according to SEQ ID NO: 53, a combination of 1.315 µM PKPSP3gp10 endolysin and 0,5 mM EDTA was tested on the clinical *P. aeruginosa* strains PAO1p and Br667, *Escherichia coli* WK6, and *Salmonella typhimurium* (see Table 5). Exponential growing bacterial cells (OD_{600nm} of 0.6) were 100-fold diluted to a final density of about 10^6/ml of each strain were incubated for 30 minutes at room temperature without shaking with unmodified endolysin PSP2gp10 (SEQ ID NO: 8) and modified endolysin PKPSP3gp10 (SEQ ID NO: 53) each in combination without

and with 0.5 mM EDTA. For incubation, the endolysins were used each in buffer (20 mM NaH_2P0_4-NaOH pH7.4; 0.5 M NaCl; 0.5 M imidazole) and the incubation took place at a final concentration of endolysin of 1.315 µM. As a control each strain was also incubated for 30 minutes with 0.5 mM EDTA (in same buffer as outlined above) but no endolysin.

Table 5 – List of used Gram-negative strains

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

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

After incubation cell suspensions were diluted three times (respectively 10^5-10^4-10^3 cells/ml) and 100 µl of each dilution was plated out on LB-medium. The residual colonies were counted after an overnight incubation on 37°C. Based on the counted cell numbers the antibacterial activity as the relative inactivation in logarithmic units ($=\log_{10}N_0/N_i$ with N_0 = number of untreated cells and N_i = number of treated cells) was calculated (Table 6).

Table 6 - Antibacterial activity of unmodified endolysin (PSP3gp10) and its modified endolysin variant (PKPSP3gp10) with and without EDTA-Na_2 on different exponential growing Gram-negative species.

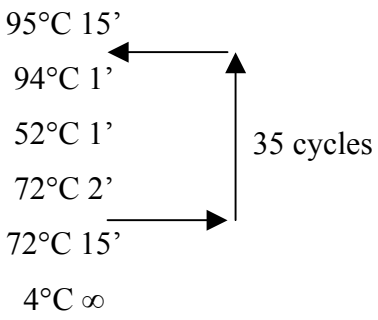
	0.5 mM EDTA	**1.315 µM PSP3gp 10**	**1.315 µM PKPSP3 gp10**	**1.315 µM PSP3gp10+ 0.5 mM EDTA**	**1.315 µM PKPSP3gp10 + 0.5 mM EDTA**
***P. aeruginosa* PAO1p**	0.146 +/- 0.002	0.383 +/- 0.015	0.344 +/- 0.163	3.552 +/- 0.536	> 4.146
***P. aeruginosa* Br667**	0.223 +/- 0.038	0.375 +/- 0.056	0.353 +/- 0.086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

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

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

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

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



For said PCR a standard 5' primer (5’ ATGGGATCCCCGGTAATTAACACGCATC 3’ (SEQ ID NO: 54)) and a standard 3' primer (5’ AGCCGGTACGCCGCCAGCGGTACGC 3’ (SEQ ID NO: 55)) was used. To extend the 5' end of the ORF which encodes P2gp09 with a gene fragment encoding the polycationic 9-mer peptide *Lys-Arg-Lys-Lys-Arg-Lys-Lys-Arg-Lys* (SEQ ID NO: 11) a tail PCR (with same parameters as standard PCR above) with an extended 5' primer (5’ ATGGGATCC**AAACGCAAGAAACGTAAGAAACGC AAA**CCGGTAATTAACACGCATC 3’ (SEQ ID NO: 56) and the standard 3' primer according to SEQ ID NO 55 was applied. Both the original unmodified P2gp09 PCR fragment and the extended fragment were ligated in the pEXP5CT/TOPO® expression vector (Invitrogen, Carlsbad, CA, USA) by following the TA-cloning protocol of the manufacturer.

Recombinant expression of P2gp09 according to SEQ ID NO: 9 and PKP2gp09 according to SEQ ID NO: 57 is performed in exponentially growing *E. coli* BL21 (λDE3) pLysS cells (Invitrogen) after induction with 1 mM IPTG (isopropylthiogalactoside) at 37°C for a period of 4 hours. Both proteins were purified by Ni^{2+} affinity chromatography (Akta FPLC, GE Healthcare) using the C-terminal 6xHis-tag, encoded by the pEXP5CT/TOPO® expression vector. The Ni^{2+} affinity chromatography is performed in 4 subsequent steps, all on room temperature:

1. *Equilibration* of the *Histrap HP 1 ml* column (GE Healthcare) with 10 column volumes of Washing Buffer (60 mM imidazole, 0.5 mM NaCl and 20 mM NaH_2P0_4-NaOH on pH 7.4) at a flow rate of 0.5 ml/min.
2. *Loading* of the total lysate (with wanted endolysin) on the *Histrap HP 1 ml* column at a flow rate of 0.5 ml/min.
3. Washing of the column with 15 column volumes of Washing Buffer at a flow rate of 1 ml/min.
4. Elution of bounded endolysin from the column with 10 column volumes of Elution Buffer (500 mM imidazole, 5 mM NaCl and 20 mM NaH_2P0_4-NaOH on pH 7.4) at a flow rate of 0.5 ml/min

The total yields of both purified recombinant proteins per liter *E.coli* expression culture shown in Table 7. The values were determined by spectrophotometric measurement of the protein concentration and the total volume of the purified stock solution at a wavelength of 280 nm. Purified stock solutions consisting of P2gp09 and PKP2gp09, respectively, in Elution Buffer (20 mM NaH_2P0_4-NaOH pH7.4; 0.5 M NaCl; 500 mM imidazole) were at least 95% pure as determined visually on SDS-PAGE gels.

Table 7 - Yields of purified recombinant P2gp09 endolysin and its PK-modified derivative PKP2gp09 per liter E. coli expression culture.

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

To determine the anti-Gram-negative spectrum of the PK2gp09 endolysin according to SEQ ID NO: 57, a combination of 1.315 µM PK2gp09 endolysin and 0,5 mM EDTA was tested on the clinical *P. aeruginosa* strains PAO1p and Br667 and on *Escherichia coli* WK6 (see Table 9). Exponential growing bacterial cells (OD_{600nm} of 0.6) were 100-fold diluted to a final density of about 10^6/ml of each strain was incubated for 30 minutes at room temperature without shaking with unmodified endolysin P2gp09 (SEQ ID NO: 9) and modified endolysin PKP2gp09 (SEQ ID NO: 57) each in combination without and with 0.5 mM EDTA. For incubation, the endolysins were used each in buffer (20 mM NaH_2P0_4-NaOH pH7.4; 0.5 M NaCl; 0.5 M imidazole) and the incubation took place at a final concentration of endolysin of 1.315 µM. As a control each strain was also incubated for 30 minutes with 0.5 mM EDTA (in same buffer as outlined above) but no endolysin. After incubation cell suspensions were diluted three times (respectively 10^5-10^4-10^3 cells/ml) and 100 µl of each dilution was plated out on LB-medium. The residual colonies were counted after an overnight incubation on 37°C. Based on the counted cell numbers the antibacterial activity as the relative inactivation in logarithmic units ($=\log_{10}N_0/N_i$ with N_0 = number of untreated cells and N_i = number of treated cells, both counted after incubation) was calculated (Table 8).

Table 8 - Antibacterial activity of unmodified endolysin (P2gp09) and its modified endolysin variant (P2gp09) with and without EDTA-Na_2 on different exponential growing Gram-negative species.

	0.5 mM EDTA	**1.315 µM P2gp09**	**1.315 µM PKP2gp09**	**Δ**	**1.315 µM P2gp09+ 0.5 mM EDTA**	**1.315 µM PKP2gp09 + 0.5 mM EDTA**	**Δ**
***P. aeruginosa* PAO1p**	0.330 +/- 0.146	0.374 +/- 0.084	0.326 +/- 0.069	-0.038	2.840 +/- 0.079	3.172 +/- 0.056	0.332
***P. aeruginosa* Br667**	0.003 +/- 0.051	0.246 +/- 0.042	0.300 +/- 0.062	0.054	0.582 +/- 0.074	0.952 +/- 0.213	0.370
P. putida G1	0.072 +/- 0.084	0.419 +/- 0.024	1.014 +/- 0.139	0.595	3.919 +/- 0.118	> 4,386	>0.467
Burkholderia pseudomallei	0.206 +/- 0.151	0.769 +/- 0.110	1.163 +/- 0.073	0.394	3.890 +/- 0.056	4.255 +/- 0,001	0.365
***Escherichia coli* WK6**	0.153 +/- 0.046	0.751 +/- 0.053	1.104 +/- 0.039	0.353	0.784 +/- 0.071	1.545 +/ - 0.102	0.749

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

Table 9 – List of used Gram-negative strains

Gram-negative strain	Source	Reference
Pseudomonas aeruginosa PAO1p	Burn wound isolate, Queen Astrid Hospital, Brussels	Pirnay *et al.*, 2003*
Pseudomonas aeruginosa Br667	Burn wound isolate, Queen Astrid Hospital, Brussels	Pirnay *et al.*, 2003*
Burkholderia pseudomallei	Clinical isolate, UZ Gasthuisberg, Leuven	Prof J. Verhaegen
Escherichia coli WK6	Standard laboratory expression strain	Prof C. Michiels
Pseudomonas putida G1	Soil isolate, Moskow	Prof V.Krylov

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

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

OBPgpLYS according to SEQ ID NO: 7 is a modular endolysin of 328 amino acid residues originating from *Pseudomonas putida* phage OBP with a putative N-terminal peptidoglycan binding domains and a C-terminal catalytic chitinase domain. As predicted by BLASTp and Pfam analysis the OBPgpLYS endolysin comprises its catalytic domain in the range of about amino acid residue 126 to about amino acid residue 292 and the N-terminal peptidoglycan binding domain in the range of about amino acid residues 7 to 96.

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:

95°C 2’
95°C 30’’
56°C 30’’
72°C 2’30’’
35 cycles
72°C 10’
4°C ∞

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

Recombinant expression of OBPgpLYS according to SEQ ID NO: 7 and PKOBPgpLYS according to SEQ ID NO: 61 is performed in exponentially growing *E. coli* BL21 (λDE3) pLysS cells (Invitrogen) after induction with 1 mM IPTG (isopropylthiogalactoside) at 37°C for a period of 4 hours. Both proteins were purified by Ni^{2+} affinity chromatography (Akta FPLC, GE Healthcare) using the C-terminal 6xHis-tag, encoded by the pEXP5CT/TOPO® expression vector. The Ni^{2+} affinity chromatography is performed in 4 subsequent steps, all on room temperature:

1. *Equilibration* of the *Histrap HP 1 ml* column (GE Healthcare) with 10 column volumes of Washing Buffer (60 mM imidazole, 0.5 mM NaCl and 20 mM NaH_2P0_4-NaOH on pH 7.4) at a flow rate of 0.5 ml/min.
2. *Loading* of the total lysate (with wanted endolysin) on the *Histrap HP 1 ml* column at a flow rate of 0.5 ml/min.
3. Washing of the column with 15 column volumes of Washing Buffer at a flow rate of 1 ml/min.
4. Elution of bounded endolysin from the column with 10 column volumes of Elution Buffer (500 mM imidazole, 5 mM NaCl and 20 mM NaH_2P0_4-NaOH on pH 7.4) at a flow rate of 0.5 ml/min

The total yields of both purified recombinant proteins per liter *E. coli* expression culture shown in Table 10. The values were determined by spectrophotometric measurement of the protein concentration and the total volume of the purified stock solution at a wavelength of 280 nm. Purified stock solutions consisting of OBPgpLYS and PKOBPgpLYS, respectively,

in Elution Buffer (20 mM NaH_2P0_4-NaOH pH7.4; 0.5 M NaCl; 500 mM imidazole) were at least 90% pure as determined visually on SDS-PAGE gels.

Table 10 - Yields of purified recombinant OBPgpLYS endolysin and its PK-modified derivative PKOBPgpLYS per liter E. coli expression culture.

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

To determine the anti-Gram-negative spectrum of the PKOBPgpLYS endolysin according to SEQ ID NO: 61, a combination of 1.313 µM PK OBPgpLYS endolysin and 0.5 mM EDTA was tested on the clinical multiresistant *P. aeruginosa* strain Br667, *Pseudomonas putida* G1 (host of phage OBP) and a range of other Gram-negative pathogens (*Escherichia coli* WK6*, Salmonella typhimurium* LT2 and *Burkholderia pseudomallei*) (see Table 12). Exponential growing bacterial cells (OD_{600nm} of 0.6) were 100-fold diluted to a final density of about 10^6/ml of each strain was incubated for 30 minutes at room temperature without shaking with unmodified endolysin OBPgpLYS (SEQ ID NO: 7) and modified endolysin PKOBPgpLYS (SEQ ID NO: 61) each in combination without and with 0.5 mM EDTA. For incubation, the endolysins were used each in buffer (20 mM NaH_2P0_4-NaOH pH7.4; 0.5 M NaCl; 0.5 M imidazole) and the incubation took place at a final concentration of endolysin of 1.313 µM. As a control each strain was also incubated for 30 minutes with 0.5 mM EDTA (in same buffer as outlined above) but no endolysin. After incubation cell suspensions were diluted three times (respectively 10^5-10^4-10^3 cells/ml) and 100 µl of each dilution was plated out on LB-medium. The residual colonies were counted after an overnight incubation on 37°C. Based on the counted cell numbers the antibacterial activity as the relative inactivation in logarithmic units ($=\log_{10}N_0/N_i$ with N_0 = number of untreated cells and N_i = number of treated cells, both counted after incubation) was calculated (Table 11). 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 11 - 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.

	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**
***P. aeruginosa* PAO1p**	0.130 +/- 0.023	2.531 +/- 0.173	3.079 +/- 0.015	4.357 +/- 1.857	> 5.687
***P. aeruginosa* Br667**	0.031 +/- 0.023	1.082 +/- 0.083	1.163 +/- 0.063	3.144 +/- 0.223	5.272 +/- 0.573
***P. putida* G1**	0.412 +/- 0.055	0.141 +/- 0.027	0.904 +/- 0.079	4.891 +/- 0.000	> 4.891
Burkholderia pseudomallei	0.220 +/- 0.081	0.997 +/- 0.131	1.806 +/- 0.287	4.08 +/- 0.301	>4.861
***Escherichia coli* WK6**	0.592 +/- 0.113	0.681 +/- 0.032	1.434 +/- 0.018	1.179 +/- 0.200	1.695 +/- 0.147
Salmonella typhimurium	0.054 +/- 0.048	0.076 +/- 0.011	0.127 +/- 0.013	0.774 +/- 0.052	0.908 +/-0.037

Table 12 – List of used Gram-negative strains

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

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

While the global efficacy of the OBPgpLYS treatment is species dependent, the results in table 11 show an added effect of the PKOBPgpLYS compared to unmodified OBPgpLYS for all bacterial species tested, both in the absence as the presence of 0,5 mM EDTA. For

Pseudomonas and *Burkholderia* species, a clear synergistic effect with EDTA is observed for the PKOBPgpLYS activity.

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

To determine the influence of EDTA on the antibacterial activity of unmodified and modified endolysins the antibacterial activity of the unmodified OBPgpLYS endolysin (SEQ ID NO: 7) and the PKOBPgpLYS endolysin (SEQ ID NO: 61) was tested on *Pseudomonas aeruginosa* PAO1p cells (Pirnay JP et al. *J Clin Microbiol.*, 41(3):1192-1202 (2003)) using different concentrations of EDTA and endolysins. Exponential growing bacterial cells (OD_{600nm} of 0.6) were 100-fold diluted to a final density of about 10^6/ml and incubated for 30 minutes at room temperature without shaking with unmodified endolysin OBPgpLYS (SEQ ID NO: 7) and modified endolysin PKOBPgpLYS (SEQ ID NO: 61). For incubation, the endolysins were used each in buffer (20 mM NaH_2P0_4-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 NaH_2P0_4-NaOH pH7.4; 0.5 M NaCl; 0.5 M imidazole) added. After incubation cell suspensions were diluted three times (respectively 10^5-10^4-10^3 cells/ml) and 100 µl of each dilution was plated out on LB-medium. The residual colonies were counted after an overnight incubation on 37°C. Based on the counted cell numbers the antibacterial activity as the relative inactivation in logarithmic units ($=\log_{10}N_0/N_i$ with N_0 = number of untreated cells and N_i = number of treated cells, both counted after incubation) was calculated (Table 13). 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 13 - Antibacterial activity of unmodified endolysin (OBPgpLYS) and its modified endolysin variant (PKOBPgpLYS) in combination with different EDTA concentrations on exponential growing Pseudomonas aeruginosa PAO1p cells

	Concentration of EDTA-Na_2 (in mM)			
	0	**0.05**	**0.5**	**10**
No endolysin	/	0.028 +/- 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 13 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-Na_2 at a concentration of 0.5 and 10 mM EDTA. The difference in activity between the unmodified OBPgpLYS and the PK-modified OBPgpLYS increases by raising the amount of added endolysin (from 0.013 – 1.315 µM endolysin).

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

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

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

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

Accordingly, the following modified endolysin variants were expressed in *E. coli* BL21 (DE3) cells at 37°C until an optical density of OD600nm=0.6 was reached: smi01 (YP_001712536), KRK9_smi01 (SEQ ID NO: 75), phiKZgp144 (SEQ ID NO: 1), pKKZ144pET32b (SEQ ID NO: 43) and POLYKZ144 (SEQ ID NO: 35). Protein expression was induced with 1mM IPTG (final concentration) and expression was preformed for four hours. Then *E.coli* cells were harvested by centrifugation for 20 min at 6000g and cell disruption and protein purification was performed using the *S-Tag™ rEK Purification Kit* (Novagen, Darmstadt, Germany). Using the pET32b vector, the expressed proteins were not toxic to the host resulting in high yields of produced protein. Purified stock solutions showed high purity.

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

Exponential (~10^6/ml) growing cells of *P. aeruginosa* PAO1p (Burn wound isolate, Queen Astrid Hospital, Brussels; Pirnay JP et al. (2003), *J Clin Microbiol.*, 41(3):1192-1202), *Acinetobacter baumanii* (DSMZ 30007) or *Burkholderia solanaceum* (Isolate provided by Prof. C. Michiels) were 100 x diluted (final density was ~10^6/ml) incubated at room

temperature with each 10 µg undialyzed protein as listed above at a final concentration of 100 µg/ml in buffer (20 mM NaH_2P0_4-NaOH pH7.4; 0.5 M NaCl; 0.5 M imidazole). After 1 hour cell suspensions were diluted 1:100 and plated on LB. Additionally, a negative control was plated using buffer (20 mM NaH_2P0_4-NaOH pH7.4; 0.5 M NaCl; 0.5 M imidazole). The residual colonies were counted after an overnight incubation at 37°C. Based on the counted cell numbers the antibacterial activity as the relative inactivation (%) (=100-(N_i/No)*l00 with N_0 = number of untreated cells and N_i = number of treated cells) was calculated (Table 3). All samples were replicated at least in four fold.

Table 14 - Antibacterial effect of different modified endolysin variants (NCBI numbers in brackets) on different bacterial species

Protein	**Bacterial species**	**Reduction [%]**
smi01 (YP_001712536)	*Acinetobacter baumannii* DSMZ 30007	0
KRK9_smi01	*Acinetobacter baumannii* DSMZ 30007	50
phiKZgp144	*Pseudomonas aeruginosa*	0
pKKZ144pET32b	*Pseudomonas aeruginosa*	99 - 99,9
phiKZgp144	*Acinetobacter baumannii DSMZ 30007*	0
pKKZ144pET32b	*Acinetobacter baumannii DSMZ 30007*	99,9
phiKZgp144	*Burkholderia solanacearum*	0
POLYKZ144	*Burkholderia solanacearum*	99 – 99,9

Unmodified endolysins phiKZgp144 and smi01 (YP_001712536) do not reduce cell numbers significantly compared to the negative control. This observation again illustrates the efficacy of the outer membrane as a barrier for the endolysin to degrade the cell wall of the Gram-negative bacteria. In contrast as shown in Table 14 the incubation with the modified endolysins KRK9_smi01, pKKZ144pET32b and POLY-gp144 causes a significant reduction of the bacterial cell number on *Acinetobacter baumanii* (50% for KRK_smi01; 99.9 % for pKKZ144pET32b), *Pseudomonas aeruginosa* (90-99.9 % for pKKZ144pET32b) and *Burkholderia solanaceum* (90 – 99.9 % for POLYKZ144).

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

CLAIMS

1. An endolysin variant comprising an endolysin to which a cationic peptide stretch with LPS disrupting activity is fused, wherein said cationic peptide stretch comprises 5 to 100 amino acid residues and wherein at least 70% of the amino acid residues comprised in said peptide stretch are arginine and/or lysine and 0% to 30% are serine and/or glycine.

2. The endolysin variant according to claim 1, wherein said peptide stretch comprises 5 to 50 amino acid residues, in particular 5 to 30 amino acid residues.

3. The endolysin variant according to any one of the preceding claims, wherein said peptide stretch is fused to the N- and/or the C-terminus of the endolysin, in particular to the N-terminus of the endolysin.

4. The endolysin variant according to any one of the preceding claims, wherein said endolysin has the activity of degrading the cell wall of Gram-negative bacteria, in particular the cell wall of the Gram-negative bacteria selected from the group consisting of

Enterobacteriaceae,

in particular *Escherichia, Salmonella, Shigella, Citrobacter, Edwardsiella, Enterobacter, Hafnia, Klebsiella, Morganella, Proteus, Providencia, Serratia*, and *Yersinia*,

Pseudomonadaceae,

in particular *Pseudomonas, Burkholderia, Stenotrophomonas, Shewanella, Sphingomonas* and *Comamonas*,

Neisseria, Moraxella, Vibrio, Aeromonas, Brucella, Francisella, Bordetella, Legionella, Bartonella, Coxiella, Haemophilus, Pasteurella, Mannheimia, Actinobacillus, Gardnerella, Spirochaetaceae,

in particular *Treponema and Borrelia,*

Leptospiraceae, Campylobacter, Helicobacter, Spirillum, Streptobacillus, Bacteroidaceae,

in particular *Bacteroides, Fusobacterium, Prevotella* and *Porphyromonas*, and *Acinetobacter*,

in particular *A. baumanii*.

5. The endolysin variant according to any one of the preceding claims, wherein said endolysin is selected from the group consisting of phiKZgp144 according to SEQ ID NO:1, ELgp188 according to SEQ ID NO:2, *Salmonella endolysin* according to SEQ ID NO:3, *Enterobacteria phage T4 endolysin* according to SEQ ID NO:4, *Acinetobacter baumanii endolysin* according to SEQ ID NO:5, *E.coli* Phage K1F endolysin according to SEQ ID NO:6, PSP3 salmonella endolysin according to SEQ ID NO: 8 and E.coli Phage P2 endolysin according to SEQ ID NO: 9.

6. The endolysin variant according to any one of the preceding claims, wherein said peptide stretch comprises at least one KRK motive, in particular wherein said peptide stretch consists of a sequence selected from the group consisting of SEQ ID: 10 to 30.

7. The endolysin variant according to any one of the preceding claims, wherein said endolysin variant comprises an amino acid sequence selected from the group consisting of SEQ ID NO: 35 to 49, 53, 57, 62 to 64 and 66 to 78.

8. An isolated nucleic acid molecule comprising a nucleotide sequence coding an endolysin variant according to any one of claims 1 to 7.

9. A vector comprising the nucleic acid molecule according to claim 8.

10. A host cell comprising a nucleic acid molecule according to claim 8 or a vector according to claim 9, wherein said host cell is in particular a bacterial cell or a yeast cell.

11. A method for the production of an endolysin variant according to any of the claims 1 to 7 comprising the expression of the endolysin variant in a host cell according to claims 10.

12. The endolysin variant according to any one of claims 1 to 7 for use as a medicament.

13. The endolysin variant according to any one of claims 1 to 7 for use as a medicament for the treatment of Gram-negative bacterial infections.

14. The use of the endolysin variant according to any one of claims 1 to 7 as diagnostic means, disinfectant or cosmetic substance.

15. The use of the endolysin variant according to any one of claims 1 to 7 for the removal, reduction and/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, or of surfaces in hospitals and surgeries.

16. The use of the endolysin variant according to any one of claims 1 to 7 as a diagnostic means in medicinal, food or feed or environmental diagnostics.

17. The endolysin variant for use according to claims 12 or 13 or the use according to claim 15, wherein said endolysin variant is used in combination with antibiotics, lantibiotics or bacteriocins.

Abstract

Antimicrobial Agents

The present invention relates to endolysin variants comprising an endolysin to which a peptide stretch with membrane or LPS disrupting activity is fused. Moreover, the present invention relates to nucleic acid molecules encoding said modified endolysin variant, 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 a method for producing said endolysin variant. Further, the present invention relates to said modified endolysin variant for use as a medicament, in particular for the treatment or prevention of Gram-negative bacterial infections, as diagnostic means, disinfectant or as cosmetic substance. The present invention also relates to the removal or reduction 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 the use of said endolysin variant as a diagnostic means in medicinal, food or feed or environmental diagnostic. Finally, the present invention relates to a pharmaceutical composition comprising said modified endolysin variant

Figure 1