

Antimicrobial Agents

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

Gram-negative bacteria possess an outer membrane, with its characteristic asymmetric bilayer as a hallmark. The outer membrane bilayer consists of an inner monolayer containing phospholipids (primarily phosphatidyl ethanolamine) and an outer monolayer that is mainly composed of a single glycolipid, lipopolysaccharide (LPS). There is an immense diversity of LPS structures in the bacterial kingdom and the LPS structure may be modified in response to prevailing environmental conditions. The stability of the LPS layer and interaction between different LPS molecules is mainly achieved by the electrostatic interaction of divalent ions (Mg^{2+} , Ca^{2+}) with the anionic components of the LPS molecule (phosphate groups in the lipid A and the inner core and carboxyl groups of KDO). Furthermore, the dense and ordered packing of the hydrophobic moiety of lipid A, favored by the absence of unsaturated fatty acids, forms a rigid structure with high viscosity. This makes it less permeable for lipophilic molecules and confers additional stability to the outer membrane (OM).

Various types of agents having bactericidal or bacteriostatic activity are known, e.g. antibiotics, endolysins, antimicrobial peptides and defensins. Increasingly microbial resistance to antibiotics, however, is creating difficulties in treating more and more infections

caused by bacteria. Particular difficulties arise with infections caused by Gram-negative bacteria like *Pseudomonas aeruginosa* and Enterobacteriaceae.

Endolysins are peptidoglycan hydrolases encoded by bacteriophages (or bacterial viruses).

5 They are synthesized during late gene expression in the lytic cycle of phage multiplication and mediate the release of progeny virions from infected cells through degradation of the bacterial peptidoglycan. They are either $\beta(1,4)$ -glycosylases (lysozymes), transglycosylases, amidases or endopeptidases. Antimicrobial application of endolysins was already suggested in 1991 by Gasson (GB2243611). Although the killing capacity of endolysins has been known
10 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
15 this need. In 2001, Fischetti and coworkers demonstrated for the first time the therapeutic potential of bacteriophage C1 endolysin towards group A streptococci (Nelson et al., 2001). Since then many publications have established endolysins as an attractive and complementary alternative to control bacterial infections, particularly by Gram positive bacteria. Subsequently different endolysins against other Gram positive pathogens such as
20 *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
25 currently prevents the expansion of the range of effective endolysins to important Gram-negative pathogens.

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

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

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

Thus, there is a need for new antimicrobial agents.

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

The term "protein" as used herein refers synonymously to the term "polypeptide". The term "protein" as used herein refers to a linear polymer of amino acid residues linked by peptide bonds in a specific sequence. The amino-acid residues of a protein may be modified by e.g.

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

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

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

The term “peptide” as used herein refers to short polypeptides consisting of from about 2 to about 100 amino acid residues, more preferably from about 4 to about 50 amino acid residues, more preferably to about 5 to 30 amino acid residues, wherein the amino group of one amino acid residue is linked to the carboxyl group of another amino acid residue by a peptide bond.

A peptide may have a specific function. A peptide can be a naturally occurring peptide or a synthetically designed and produced peptide. The peptide can be, for example, derived or removed from a native protein by enzymatic or chemical cleavage, or can be prepared using conventional peptide synthesis techniques (e.g., solid phase synthesis) or molecular biology techniques (see Sambrook, J. et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Press, Cold Spring Harbor, N.Y. (1989)). Examples of naturally occurring peptides are antimicrobial peptides, defensins, sushi peptides. Examples of synthetically produced peptides are polycationic, amphiphatic or hydrophobic peptides. A peptide in the meaning of

the present invention does not refer to His-tags, Strep-tags, thioredoxin or maltose binding proteins (MBP) or the like, which are used to purify or locate proteins.

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

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

The term “bacteriocin” as used herein refers to protein-like, polypeptide-like or peptide-like
 25 substances which are able to inhibit the growth of other bacteria. Preferably said inhibition is specifically by means of absorption of said other bacteria to specific receptors of the bacteriocin. In general, bacteriocins are produced by microorganisms. However, the term “bacteriocin” as used herein refers both to an isolated form by a microorganism or to a synthetically produced form, and refers also to variants which substantially retain the
 30 activities of their parent bacteriocins, but whose sequences have been altered by insertion or deletion of one or more amino acid residues.

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

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

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

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

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

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

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

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

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

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

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

In one aspect of the present invention the enzyme having the activity of degrading the cell wall of Gram-negative bacteria is an endolysin, autolysin or bacteriocin.

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

- 5 Preferred fusion proteins according to the present invention are depicted in SEQ ID NO:36 to 63. The fusion proteins according to SEQ ID NO:36 to 63 may comprise one or more additional amino acid residues on the N-terminus. Preferably the additional amino acid residue is methionine.
- 10 Preferably, the endolysin is 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*),
- 15 *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*,
- 20 *Streptobacillus*, *Bacteroidaceae* (*Bacteroides*, *Fusobacterium*, *Prevotella*, *Porphyromonas*), *Acinetobacter*, especially *A. baumannii*.

- Preferably, the autolysin is encoded by Gram-negative bacteria such as Gram-negative bacteria of bacterial groups, families, genera or species comprising strains pathogenic for
- 25 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*,
- 30 *Francisella*, *Bordetella*, *Legionella*, *Bartonella*, *Coxiella*, *Haemophilus*, *Pasteurella*, *Mannheimia*, *Actinobacillus*, *Gardnerella*, *Spirochaetaceae* (*Treponema* and *Borrelia*), *Leptospiraceae*, *Campylobacter*, *Helicobacter*, *Spirillum*, *Streptobacillus*, *Bacteroidaceae*

(*Bacteroides*, *Fusobacterium*, *Prevotella*, *Porphyromonas*), *Acinetobacter*, especially *A. baumannii*.

The bacteriocin is preferably specific for Gram-negative bacteria as listed above, but may also be less specific.

The enzyme according to the present invention has 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. baumannii*.

Specific examples of an endolysin part derived from a phage or that is a wild type endolysin are depicted in the following table:

Table 1:

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

ΦECO32	Savalia, D and Severinov, K	phi32_17	muraminidase (T4-like)
HK022	Juhala , R and Hendrix, R.W.	HK022p54	muraminidase (lambdalike)
HK97	Juhala , R and Hendrix, R.W.	HK97p58	muraminidase (lambdalike)
HK620	Clark, A.J. and Dhillon, T.S.	HK620p36	muraminidase (lambdalike)
E1	Pickard, D. and Dougan, G	VIP0007	muraminidase (lambdalike)
SF6	Casjens, S and Clark, A.J.	Sf6p62	muraminidase (lambdalike)
SFV	Allison, G.E. and Verma, N.K.	R (SfVp40)	muraminidase (lambdalike)
BCEPC6B	Summer, EJ and Young, R.	gp22	muraminidase (lambdalike)
BCEPNAZGUL	Summer, EJ and Young, R.	Nazgul38	muraminidase (lambdalike)
P2	Christie, G.E. and Calender, R.	K (P2p09)	muraminidase (lambdalike)
WΦ	Christie, G.E. and Esposito, D.	K (Wphi09)	muraminidase (lambdalike)
RV5	Kropinski, A.M. and Johnson	rv5_gp085	muraminidase (lambdalike)
JS98	Zuber, S and Denou, E.	EpJS98_gp116	muraminidase (T4-like)
13A	Savalia, D and Molineux, I.	gp3.5	muramoyl-L-alanine amidase
BA14	Savalia, D and Molineux, I.	gp3.5	muramoyl-L-alanine amidase
ECODS1	Savalia, D and Molineux, I.	gp3.5	muramoyl-L-alanine amidase
K1F	Scholl, D and Merril, C	CKV1F_gp16	muramoyl-L-alanine amidase
T3	Pajunen, M.I. and Mollieux, I.J.	T3p18	muramoyl-L-alanine amidase
GH-1	Kropinski, A.M. and Kovalyova, I.V.	gh-1p12	muramoyl-L-alanine amidase
K11	Molineux, I. and Savalia, D.	gp3.5	muramoyl-L-alanine amidase
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 <i>et al.</i> , 2008		PG-binding domain (N) / unknown catalytic domain (C)
Aeh1	Monod, C and Krisch, H.M.	Aeh1p339	muraminidase (T4-like)
YYZ-2008	Kropinski, A.M.	YYZgp45	muraminidase (lambda-like)

Also preferred is the endolysin part deriving from endolysins of the *Pseudomonas aeruginosa* phages Φ KZ and EL, of the *Pseudomonas putida* phage, of the *E. coli* phage N4, of the phage LUZ24, gp61 muramidase, STM0016 endolysin and PSP3 endolysin.

- 5 Further examples for 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 baumannii* endolysin according to SEQ ID NO:5, *E.coli* Phage K1F endolysin according to SEQ ID NO:18, OBPgpLYS according to SEQ ID NO:34, PSP3 *Salmonella*
- 10 endolysin (PSP3gp10) according to SEQ ID NO:20, *E.coli* Phage P2 endolysin (P2gp09) according to SEQ ID NO:21, *Salmonella typhimurium* phage muramidase STM0016 according to SEQ ID NO:22, *E.coli* Phage N4 muramidase N4-gp61 according to SEQ ID NO:23 and N4-gp61 trunc. according to SEQ ID NO:24, KZ144 according to SEQ ID NO:25.
- 15 In another preferred embodiment of the present invention the endolysins, autolysins and bacteriocins of the fusion protein according to the present invention comprise modifications and/or alterations of the amino acid sequences. Such alterations and/or modifications may comprise mutations such as deletions, insertions and additions, substitutions or combinations thereof and/or chemical changes of the amino acid residues, e.g. biotinylation, acetylation,
- 20 pegylation, chemical changes of the amino-, SH- or carboxyl- groups. Said endolysins, autolysins and bacteriocins of the fusion protein according to the present invention exhibit the lytic activity of the respective wild-type endolysin, autolysin and bacteriocins. However, said activity can be the same, higher or lower as the activity of the respective wild-type endolysin. Said activity can be about 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150,
- 25 160, 170, 180, 190 or about 200 % of the activity of the respective wild-type endolysin or even more. The activity can be measured by assays well known in the art by a person skilled in the art as e.g. the plate lysis assay or the liquid lysis assay which are e.g. described in (Briers *et al.*, *J. Biochem. Biophys Methods* 70: 531-533, (2007) or Donovan DM, Lardeo M, Foster-Frey J. *FEMS Microbiol Lett.* 2006 Dec;265(1) or similar publications.

30

Preferably, the peptide stretch of the fusion protein according to the invention is fused to the N-terminus and/or to the C-terminus of the endolysin, autolysin or bacteriocin. In a particular preferred embodiment said peptide stretch is only fused to the N-terminus of the enzyme. In

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

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

Preferably, the peptide stretch is no tag such as a His-tag, Strep-tag, Avi-tag, Myc-tag, Gst-tag, JS-tag, cystein-tag, FLAG-tag or other tags known in the art and no thioredoxin or

maltose binding proteins (MBP). However, the peptide stretch and/or the endolysin, autolysin or bacteriocin according to the present invention may comprise in addition such tag or tags.

More preferably the peptide stretch has the function to lead the fusion protein through the outer membrane but may have activity or may have no or only low activity when administered without being fused to the enzyme. The function to lead the fusion protein through the outer membrane of Gram-negative bacteria is caused by the potential of the outer membrane or LPS disrupting or permeabilising or destabilizing activity of said peptide stretch.

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

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

Preferred amphipatic peptides are Pleurocidin according to SEQ ID NO:6, Cecropin P1 according to SEQ ID NO:7, Buforin II according to SEQ ID NO:8, Buforin I according to SEQ ID NO:19 and Magainin according to SEQ ID NO:9. Further preferred amphipatic peptides are Cathelidicine e.g. LL-37 according to SEQ ID NO:10, Nigrocine 2 according to SEQ ID NO:26 and Ascaphine 5 according to SEQ ID NO:27.

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

Specific examples of antimicrobial peptides according to the present invention are listed in the following table.

Table 2:

Peptid	Sequenz	
LL-37	LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLPRTES	SEQ ID NO:10
SMAP-29	RGLRRLGRKIAHG VKKYGPTVLRIRIAG	SEQ ID NO:11
Indolicidin	ILPWKWPWWPWRR	SEQ ID NO:12
Protegrin	RGGRLCYCRRRFCVCVGR	SEQ ID NO:13
Cecropin P1	SWLSKTAKKLENSAKKRISegIAIAIQGGPR	SEQ ID NO:7
Magainin	GIGKFLHSAKKFGKAFVGEIMNS	SEQ ID NO:9
Pleurocidin	GWGSFFKKAHVGHVGHVGAALHYL	SEQ ID NO:6
Cecropin A (A.aegypti)	GGLKKLGKKLEGAGKRVFNAAEKALPVVAGAKALRK	SEQ ID NO:14
Cecropin A (D. melanogaster)	GWLKKIGKKIERVGGHTRDATIQGLGIPQQAANVAATARG	SEQ ID NO:15
Buforin II	TRSSRAGLQFPVGRVHRLLRK	SEQ ID NO:8
Sarcotoxin IA	GWLKKIGKKIERVGGHTRDATIQGLGIAQQAANVAATAR	SEQ ID NO:16
Apidaecin	ANRPVYIPPPRPPHPRL	SEQ ID NO:28
Ascaphine 5	GKDWIKGAACKLIKTVASHIANQ	SEQ ID NO:27
Nigrocine 2	GLLSKVLGVGKKVLCGVSGLVLC	SEQ ID NO:26
Pseudin 1	GLNTLKKVFQGLHEAIKLNNHVQ	SEQ ID NO:29
Ranalexin	FLGGLIVPAMICAVTKKC	SEQ ID NO:30
Melittin	GIGAVLKVLTTGLPALISWIKRKRQQ	SEQ ID NO:31

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

peptides: structural characterization and mode of action against Gram-negative bacteria. Especially preferred is the sushi 1 peptide according to SEQ ID NO:32.

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

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

10 In a further aspect of the present invention the fused peptide stretch is a hydrophobic peptidee.g. Apidaecine having the amino acid sequence according to SEQ ID NO:28, WLBU2-Variant having the amino acid sequence according to SEQ ID NO:33 and Walmagh1 having the amino acid sequence according to SEQ ID NO:35. The hydrophobic peptide having the amino acid sequence Phe-Phe-Val-Ala-Pro (SEQ ID NO:17) is not part of the
15 present invention.

In another preferred embodiment of the present invention the peptide stretches of the fusion protein according to the present invention comprise modifications and/or alterations of the amino acid sequences. Such alterations and/or modifications may comprise mutations such as
20 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.

Specific examples of fusion proteins according to the present invention are listed in the
25 following table:

Table 3:

Fusion protein	Fusion protein	Enzyme part	Peptide stretch (N-terminal unless otherwise indicated)
P1-E6	SEQ ID NO: 36	KZ144 (SEQ ID NO:25)	Ascapine 5 (SEQ ID NO:27)
P2-E6	SEQ ID NO: 37	KZ144 (SEQ ID NO:25)	Apiadaecine (SEQ ID NO:28)
P3-E6	SEQ ID NO: 38	KZ144	Nigrocine 2

		(SEQ ID NO:25)	(SEQ ID NO:26)
P4-E6	SEQ ID NO: 39	KZ144 (SEQ ID NO:25)	Pseudin 1 (SEQ ID NO:29)
P7-E6	SEQ ID NO: 40	KZ144 (SEQ ID NO:25)	Ranalexin (SEQ ID NO:30)
P8-E6	SEQ ID NO: 41	KZ144 (SEQ ID NO:25)	WLBU2-Variant (SEQ ID NO:33)
P9-E6	SEQ ID NO: 42	KZ144 (SEQ ID NO:25)	Sushi 1 (SEQ ID NO:32)
P10-E6	SEQ ID NO: 43	KZ144 (SEQ ID NO:25)	Melittin (SEQ ID NO:31)
P11-E6	SEQ ID NO: 44	KZ144 (SEQ ID NO:25)	LL-37 (SEQ ID NO:10)
P12-E6	SEQ ID NO: 45	KZ144 (SEQ ID NO:25)	Indolicidin (SEQ ID NO:12)
P13-E6	SEQ ID NO: 46	KZ144 (SEQ ID NO:25)	SMAP-29 (SEQ ID NO:11)
P14-E6	SEQ ID NO: 47	KZ144 (SEQ ID NO:25)	Protegrin (SEQ ID NO:13)
P15-E6	SEQ ID NO: 48	KZ144 (SEQ ID NO:25)	Cecropin P1 (SEQ ID NO:7)
P16-E6	SEQ ID NO: 49	KZ144 (SEQ ID NO:25)	Magainin (SEQ ID NO:9)
P17-E6	SEQ ID NO: 50	KZ144 (SEQ ID NO:25)	Pleurocidin (SEQ ID NO:6)
P18-E6	SEQ ID NO: 51	KZ144 (SEQ ID NO:25)	Cecropin A (A. aegypti) (SEQ ID NO:14)
P19-E6	SEQ ID NO: 52	KZ144 (SEQ ID NO:25)	Cecropin A (A. melanogaster) (SEQ ID NO:15)
P20-E6	SEQ ID NO: 53	KZ144 (SEQ ID NO:25)	Buforin II (SEQ ID NO:8)

P21-E6	SEQ ID NO: 54	KZ144 (SEQ ID NO:25)	Sarcotoxin IA (SEQ ID NO:16)
P1-E3	SEQ ID NO: 55	STM0016 (SEQ ID NO:22)	Ascapphine 5 (SEQ ID NO:27)
	SEQ ID NO: 56	STM0016 (SEQ ID NO:22)	Nigrocline 2 (SEQ ID NO:26)
	SEQ ID NO: 57	STM0016 (SEQ ID NO:22)	SMAP-29 (SEQ ID NO:11)
	SEQ ID NO: 58	STM0016 (SEQ ID NO:22)	Sarcotoxin IA (SEQ ID NO:16)
P10-E4	SEQ ID NO: 59	N4-gp61 (SEQ ID NO:23)	Melittin (SEQ ID NO:31)
	SEQ ID NO: 60	N4-gp61 (SEQ ID NO:23)	SMAP-29 (SEQ ID NO:11)
P10-E5	SEQ ID NO: 61	N4-gp61 trunc. (SEQ ID NO:24)	Melittin (SEQ ID NO:31)
	SEQ ID NO: 62	N4-gp61 trunc. (SEQ ID NO:24)	Cecropin P1 (SEQ ID NO:7)
	SEQ ID NO: 63	N4-gp61 trunc. (SEQ ID NO:24)	SMAP-29 (SEQ ID NO:11)

The fusion protein according to the present invention, and thus in particular the especially preferred fusion proteins according to SEQ ID NO: 36 to 63, may additionally comprise a methionine on the N-terminus.

The fusion protein according to the present invention, and thus in particular the especially preferred fusion proteins according to SEQ ID NO: 36 to 63 may additionally comprise a tag e.g. for purification. Preferred is a His₆-tag, preferably at the C-terminus and/or the N-terminus of the fusion protein. Said tag can be linked to the fusion protein by additional amino acid residues e.g. due to cloning reasons. Preferably said tag can be linked to the fusion protein by at least 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 additional amino acid residues. In a preferred embodiment the fusion protein comprises a His₆-tag at its C-terminus linked to the fusion

protein by the additional amino acid residues lysine and glycine (Lys-Gly) or leucine and glutamic acid (Leu-Glu). In another preferred embodiment the fusion protein comprises a His₆-tag at its N-terminus linked to the fusion protein by the additional amino acid residues lysine and glycine (Lys-Gly) or leucine and glutamic acid (Leu-Glu). In another preferred embodiment the fusion protein comprises a His₆-tag at its N- and C-terminus linked to the fusion protein by the additional amino acid residues lysine and glycine (Lys-Gly) or leucine and glutamic acid (Leu-Glu).

In a more preferred embodiment the fusion protein comprises a His₆-tag at its C-terminus linked to the fusion protein by the additional amino acid residues leucine and glutamic acid (Leu-Glu) and the peptide stretch of the fusion protein according to the invention is linked to the N-terminus of the enzyme by the additional amino acid residues glycine and serine. In another preferred embodiment the fusion protein comprises a His₆-tag at its C-terminus linked to the fusion protein by the additional amino acid residues leucine and glutamic acid (Leu-Glu) and the peptide stretch of the fusion protein according to the invention is linked to the N-terminus of the enzyme by the additional amino acid residues glycine and serine (Gly-Ser) and the fusion protein comprises on the N-terminus the additional amino acid residues methionine (Met) or alanine, methionine and glycine (Ala-Met-Gly) or alanine, methionine, glycine and serine (Ala-Met-Gly-Ser). Preferably the fusion proteins are according to SEQ ID NO: 77 to 90.

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

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

The present invention further relates to an isolated nucleic acid molecule encoding the fusion protein according to the present invention. The present invention further relates to a vector comprising the nucleic acid molecule according to the present invention. Said vector may

provide for the constitutive or inducible expression of said fusion protein according to the present invention.

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

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

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

In a preferred embodiment of the present invention the composition comprises additionally agents permeabilizing the outer membrane of Gram-negative bacteria such metal chelators as
25 e.g. EDTA, TRIS, lactic acid, lactoferrin, polymyxin, citric acid and/or other substances as described e.g. by Vaara (Agents that increase the permeability of the outer membrane. Vaara M. Microbiol. Rev. 1992 Sep; 56 (3):395-441). Also preferred are compositions comprising combinations of the above mentioned permeabilizing agents. Especially preferred is a composition comprising about 10 μ M to about 100 mM EDTA, more preferably about 50 μ M
30 to about 10 mM EDTA, more preferably about 0.5 mM to about 10 mM EDTA, more preferably about 0.5 mM to about 2 mM EDTA, more preferably about 0.5 mM to 1 mM EDTA. However, also compositions comprising about 10 μ M to about 0.5 mM EDTA are

preferred. Also preferred is a composition comprising about 0.5 mM to about 2 mM EDTA, more preferably about 1 mM EDTA and additionally about 10 to about 100 mM TRIS.

The present invention also relates to a fusion protein according to the present invention and/or a host transformed with a nucleic acid comprising a nucleotide sequence encoding a fusion protein according to the present invention for use as a medicament. In a further aspect the present invention relates to the use of a fusion protein according to the present invention and/or a host transformed with a vector comprising a nucleic acid molecule comprising a nucleotide sequence encoding a modified, fusion protein according to the present invention in the manufacture of a medicament for the treatment and/or prevention of a disorder, disease or condition associated with Gram-negative bacteria. In particular the treatment and/or prevention of the disorder, disease or condition may be caused by Gram-negative bacteria of bacterial groups, families, genera or species comprising strains pathogenic for humans or animals like *Enterobacteriaceae* (*Escherichia*, especially *E. coli*, *Salmonella*, *Shigella*, *Citrobacter*, *Edwardsiella*, *Enterobacter*, *Hafnia*, *Klebsiella*, especially *K. pneumoniae*, *Morganella*, *Proteus*, *Providencia*, *Serratia*, *Yersinia*), *Pseudomonadaceae* (*Pseudomonas*, especially *P. aeruginosa*, *Burkholderia*, *Stenotrophomonas*, *Shewanella*, *Sphingomonas*, *Comamonas*), *Neisseria*, *Moraxella*, *Vibrio*, *Aeromonas*, *Brucella*, *Francisella*, *Bordetella*, *Legionella*, *Bartonella*, *Coxiella*, *Haemophilus*, *Pasteurella*, *Mannheimia*, *Actinobacillus*, *Gardnerella*, *Spirochaetaceae* (*Treponema* and *Borrelia*), *Leptospiraceae*, *Campylobacter*, *Helicobacter*, *Spirillum*, *Streptobacillus*, *Bacteroidaceae* (*Bacteroides*, *Fusobacterium*, *Prevotella*, *Porphyromonas*), *Acinetobacter*, especially *A. baumannii*.

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

In a further aspect the present invention relates to a method of treating a disorder, disease or condition in a subject in need of treatment and/or prevention, which method comprises administering to said subject an effective amount of a fusion protein according to the present invention and/or an effective amount of a host transformed with a nucleic acid comprising a nucleotide sequence encoding a fusion protein according to the present invention or a composition according to the present invention. The subject may be a human or an animal.

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

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

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

In a specific embodiment of the present invention the use of a fusion protein according to the present invention and/or a host transformed with a vector comprising a nucleic acid molecule

comprising a nucleotide sequence encoding a fusion protein according to the present invention in the manufacture of a medicament for the treatment and/or prevention of a disorder, disease or condition caused by *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 *Salmonella typhi*, in particular typus.

In another specific embodiment of the present invention the disorder, disease or condition is caused by *Salmonell paratyphi*, in particular paratyphus.

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

- 5 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, intrainestinal *E. coli* infections, particularly epidemic enteritis, and infectious disease similar to dysentery, septicemia, enterotoxemia, mastitis and dysentery.

10

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.

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

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

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

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

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

In a further aspect the present invention relates to the use of the fusion protein according to the present invention for the treatment, removal, 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 such as shelves and food deposit areas and in all other situations, where pathogenic, facultative pathogenic or other undesirable bacteria can potentially infest food material, of medical devices and of all kind of surfaces in hospitals and surgeries.

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

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

Another aspect of the present invention is that the invention can be used like a tool box, i.e. any peptide stretch disclosed above may be fused to any endolysin, autolysin or bacteriocin disclosed herein. Thus, it is possible to combine the respective peptide stretch, which enables the binding of the fusion protein to the respective bacteria and the endolysin, autolysin or

bacteriocin, which inhibit the growth of the respective bacteria. Consequently, it is possible to construct a suitable fusion protein for any bacteria which should be eliminated.

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 Sambrook 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 gp144 and gp188 modified with an amphipathic peptide.

As a proof of principle, the potential of the LPS disrupting activity of amphipathic peptides to lead gp144 and gp188 through the outer membrane and the consequent antibacterial activity against Gram-negative bacteria is demonstrated. Gp144 and gp188 are modular endolysins originating from *Pseudomonas aeruginosa* phages ϕ KZ and EL with an N-terminal peptidoglycan binding and C-terminal catalytic domain (Briers et al., 2007).

To extend the 5' end of the open reading frame encoding gp144 or gp188 with a gene fragment encoding the amphipathic α 4 helix of T4 lysozyme (aa 143-155: Pro-Asn-Arg-Ala-Lys-Arg-Val-Ile-Thr-Thr-Phe-Arg-Thr according to SEQ ID NO: 92) a tail PCR with an extended 5' primer and standard 3' primer was applied. The PCR product was cloned in the pEXP5CT/TOPO® expression vector (Invitrogen, Carlsbad, CA, USA).

Expression of all constructs was performed in *E. coli* BL21 (DE3) pLysS cells. All proteins were purified by Ni²⁺ affinity chromatography using the C-terminal 6xHis-tag. The yields for different purifications are shown in table 4. Remarkably, α 4-KZ144 production was not toxic for the host, in contrast to KZ144, resulting in a significant higher yield.

5

Purified stock solutions were ~90% pure. All gp144 derivatives showed multimer formation which could be converted to monomers by addition of β -mercapto-ethanol, indicating that interdisulfide bonds cause multimerization.

Table 4 - Yields of recombinant purification of endolysins modified with an amphipathic peptide*.

Fusion	Endolysin	
	gp144	gp188
α 4 helix	179 mg	38 mg

* The total yield of purified recombinant protein per liter E. coli expression culture is shown. This value was determined by spectrophotometric measurement of the protein concentration and the total volume of the purified stock solution. The purification of gp188 derivatives was performed under more stringent conditions (65 mM imidazole) compared to gp144 derivatives (50mM imidazole) to ensure high purity.

Characterization of gp144 and gp188 modified with an amphipathic peptide

1.A. Enzymatic activity of gp144 and gp188 modified with an amphipathic peptide

To assess the influence of the modification on the enzymatic activity of gp144 or gp188, the specific activity of the variants was measured on chloroform-permeabilized *Pseudomonas aeruginosa* cells and compared to the corresponding unmodified endolysin. Different incremental amounts of all modified endolysins were tested to determine the corresponding saturation curve. The slope of the linear regression of the linear region of this curve is a measure for the specific activity and was expressed relatively to the slope of unmodified gp144 or gp188 (Table 5).

Table 5 - Enzymatic activity of gp144 or gp188 modified with an amphipathic peptide*.

Fusion	Endolysin	
	gp144	gp188
α 4 helix	23%	146%

* The specific enzymatic activity of the different variants was determined and expressed relatively to the specific activity of the corresponding original endolysin (=100%), which was tested simultaneously. The buffer conditions of the assay were the optimal conditions of the corresponding endolysins (KH₂PO₄/K₂HP0₄ I = 120 mM pH 6.2 and I = 80 mM pH 7.3 for gp144 and gp188, respectively).

1.B. Antibacterial activity of gp144 and gp188 modified with an amphipathic peptide

Exponential ($\sim 10^6$ /ml) *P. aeruginosa* PAO1 cells were incubated at room temperature with unmodified and modified gp144/gp188. After 1 hour, cell suspensions were diluted and plated. The residual colonies were counted after an overnight incubation (Table 6). Unmodified gp144 gp188 does not reduce cell numbers significantly compared to the negative control. This observation illustrates the efficacy of the outer membrane as a barrier. Fusion proteins with the amphipathic α 4-helix inactivate exponential cells with 50 ± 11 and 34 ± 11 % for α 4-KZ144 and α 4-EL188, respectively. When stationary cells with a 100-fold higher density are used, these values are similar (35 ± 18 and $32 \pm 17\%$, respectively). Despite the rather high variability between different replicates, these values differ significantly from the untreated cells ($\alpha = 0.05$). In general, modified gp144 derivatives tend to have a higher antibacterial activity than gp188 derivatives.

Table 6 - Antibacterial effect of endolysins gp144 and gp188 and their derivatives*.

Exponentially growing cells	Endolysins			
	gp144		gp188	
Fusion	%	log	%	log
unmodified	0 ± 15	0.00 ± 0.06	10 ± 13	0.05 ± 0.06
α 4 helix	50 ± 11	0.31 ± 0.09	34 ± 11	0.19 ± 0.07

* Exponentially growing *P. aeruginosa* PAO1 cells were 100 x diluted and incubated (final density was $\sim 10^6$ /ml) with 10 μ g undialyzed protein (final concentration 100 μ g/ml, buffer: 20 mM NaH_2PO_4 -NaOH pH7.4; 0.5 M NaCl; 0.5 M imidazole) for 1 hour at room temperature. Aliquots are diluted and plated. The antibacterial activity is expressed as the relative inactivation (%) ($=100-(N_i/N_0)*100$ with N_0 = number of untreated cells and N_i = number of treated cells) and in logarithmic units ($=\log_{10}N_0/N_i$). All samples were replicated in six fold. Averages/standard deviations are represented. Statistical analysis was performed using a student's t-test.

EXAMPLE 2. Cloning, expression and purification of gp144 and gp188 modified with a hydrophobic peptide.

As a proof of principle, the potential of the LPS disrupting activity of a hydrophobic pentapeptides to lead gp144 and gp188 through the outer membrane and the consequent antibacterial activity against Gram-negative bacteria is demonstrated. Gp144 and gp188 are modular endolysins originating from *Pseudomonas aeruginosa* phages ϕ KZ and EL with an N-terminal peptidoglycan binding and C-terminal catalytic domain (Briers et al., 2007).

To extend the 5' end of the open reading frame encoding gp144 or gp188 with a gene fragment encoding 5 hydrophobic residues (Phe-Phe-Val-Ala-Pro) a tail PCR with an extended 5' primer and standard 3' primer was applied. The PCR product was cloned in the pEXP5CT/TOPO® expression vector (Invitrogen, Carlsbad, CA, USA).

5

Expression of all constructs was performed in E. coli BL21 (DE3) pLysS cells. All proteins were purified by Ni²⁺ affinity chromatography using the C-terminal 6xHis-tag. The yields for different purifications are shown in table 7.

10 Purified stock solutions were ~90% pure. All gp144 derivatives showed multimer formation which could be converted to monomers by addition of β -mercapto-ethanol, indicating that interdisulfide bonds cause multimerization.

Table 7 - Yields of recombinant purification of endolysin derivatives.*

15

Fusion	Endolysin	
	gp144	gp188
Phe-Phe-Val-Ala-Pro	25 mg	85 mg

20 * The total yield of purified recombinant protein per liter E. coli expression culture is shown. This value was determined by spectrophotometric measurement of the protein concentration and the total volume of the purified stock solution. The purification of gp188 derivatives was performed under more stringent conditions (65 mM imidazole) compared to gp144 derivatives (50mM imidazole) to ensure high purity.

25

Characterization of gp144 and gp188 modified with a hydrophobic pentapeptide

2.A. Enzymatic activity of gp144 and gp188 modified with a hydrophobic pentapeptide

30 To assess the influence of the modifications on the enzymatic activity of gp144 or gp188, the specific activity of the variants was measured on chloroform-permeabilized *Pseudomonas aeruginosa* cells and compared to the corresponding unmodified endolysin. Different incremental amounts of all modified endolysins were tested to determine the corresponding saturation curve. The slope of the linear regression of the linear region of this curve is a
 35 measure for the specific activity and was expressed relatively to the slope of unmodified gp144 or gp188 (Table 8).

Table 8 - Enzymatic activity of gp144 or gp188 modified with a hydrophobic peptide*.

Fusion	Endolysin	
	gp144	gp188
Hydrophobic pentapeptide	150%	100%

* The specific enzymatic activity of the different variants was determined and expressed relatively to the specific activity of the corresponding original endolysin (=100%), which was tested simultaneously. The buffer conditions of the assay were the optimal conditions of the corresponding endolysins ($\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$ I = 120 mM pH 6.2 and I = 80 mM pH 7.3 for gp144 and gp188, respectively).

2.B. Antibacterial activity of gp144 and gp188 modified with a hydrophobic pentapeptide

Exponential ($\sim 10^6/\text{ml}$) *P. aeruginosa* PAO1 cells were incubated at room temperature with unmodified and modified gp144/gp188. After 1 hour, cell suspensions were diluted and plated. The residual colonies were counted after an overnight incubation (Table 9). Unmodified gp144 gp188 does not reduce cell numbers significantly compared to the negative control. This observation illustrates the efficacy of the outer membrane as a barrier. Incubation with the hydrophobic pentapeptide fusion proteins causes a significant reduction ($\alpha = 0.05$) of the bacterial cell number (83 ± 7 and $69 \pm 21\%$ for modified gp144 and gp188, respectively). In general, modified gp144 derivatives tend to have a higher antibacterial activity than gp188 derivatives.

Table 9 - Antibacterial effect of endolysins gp144 and gp188 and their derivatives*.

Exponentially growing cells	Endolysins			
	gp144		gp188	
Fusion	%	log	%	log
unmodified	0 ± 15	0.00 ± 0.06	10 ± 13	0.05 ± 0.06
Hydrophobic pentapeptide	83 ± 7	0.9 ± 0.2	69 ± 21	0.7 ± 0.3

* Exponentially growing *P. aeruginosa* PAO1 cells were 100 x diluted and incubated (final density was $\sim 10^6/\text{ml}$) with 10 μg undialyzed protein (final concentration 100 $\mu\text{g}/\text{ml}$, buffer: 20 mM NaH_2PO_4 -NaOH pH7.4; 0.5 M NaCl; 0.5 M imidazole) for 1 hour at room temperature. Aliquots are diluted and plated. The antibacterial activity is expressed as the relative inactivation (%) ($=100-(N_i/N_0)*100$ with N_0 = number of untreated cells and N_i = number of treated cells) and in logarithmic units ($=\log_{10}N_0/N_i$). All samples were replicated in six fold. Averages/standard deviations are represented. Statistical analysis was performed using a student's t-test.

EXAMPLE 3: Cloning, expression and purification of KZ144 and STM0016 modified with various peptide stretches on the N-terminus of the endolysin.

5 KZ144 according to SEQ ID NO: 25 is a modular endolysin originating from *Pseudomonas aeruginosa* phage ϕ KZ with an N-terminal peptidoglycan binding and C-terminal catalytic domain (Briers et al., 2007). The endolysin KZ144 is encoded by the nucleic acid molecule according to SEQ ID NO: 64. The nucleic acid molecule according to SEQ ID NO: 64 was synthetically produced with a BamH I (5'-GGA TCC-3') restriction site at the 5'-end of the
10 nucleic acid molecule and an Xho I (5'-CTC GAG-3') restriction site at the 3'-end of the nucleic acid molecule.

STM0016 is a hypothetical protein with homology to the *E. coli* phage N4 endolysin N4-gp61. The endolysin STM0016 is encoded by the nucleic acid molecule according to SEQ ID
15 NO: 65. The nucleic acid molecule according to SEQ ID NO: 65 was synthetically produced with a BamH I (5'-GGA TCC-3') restriction site at the 5'-end of the nucleic acid molecule and an Xho I (5'-CTC GAG-3') restriction site at the 3'-end of the nucleic acid molecule.

N4-gp61 is an *E. coli* N4 phage endolysin. The endolysin is encoded by the nucleic acid
20 according to SEQ ID NO: 91. The nucleic acid molecule according to SEQ ID NO: 91 was synthetically produced with a BamH I (5'-GGA TCC-3') restriction site at the 5'-end of the nucleic acid molecule and an Xho I (5'-CTC GAG-3') restriction site at the 3'-end of the nucleic acid molecule.

25 The following peptide stretches in table 10 were used for production of fusion proteins with the endolysin KZ144 or STM0016:

Table 10:

Peptide stretch	Nucleic acid molecule encoding the peptide stretch
Pseudin 1 (SEQ ID NO:29)	SEQ ID NO: 66
Ranalexin	SEQ ID NO: 67

(SEQ ID NO:30)	
Sushi 1 (SEQ ID NO:32)	SEQ ID NO: 68
WLBU2-Variant (SEQ ID NO:33)	SEQ ID NO:69
Melittin (SEQ ID NO:31)	SEQ ID NO:70
SMAP-29 (SEQ ID NO:11)	SEQ ID NO:71
Pleurocidin (SEQ ID NO: 6)	SEQ ID NO: 72
Cecropin A (A. aegypti) (SEQ ID NO:14)	SEQ ID NO:73
Cecropin A (A. melanogaster) (SEQ ID NO:15)	SEQ ID NO:74
Buforin II (SEQ ID NO:8)	SEQ ID NO:75
Sarcotoxin IA (SEQ ID NO:16)	SEQ ID NO:76

- The nucleic acid molecules encoding the respective peptide stretches were synthetically produced with a Nde I (5'-CAT ATG-3') restriction site at the 5'-end of the nucleic acid molecule and a BamH I (5'-GGA TCC-3') restriction site at the 3'-end of the nucleic acid molecule, except the nucleic acid molecule encoding the Sushi 1 peptide, which was produced with a Nco I restriction site plus two additional nucleotides (5'-CCA TGG GC-3') at the 5'-end of the nucleic acid molecule.
- 10 Fusion proteins are constructed by linking at least two nucleic acid sequences using standard cloning techniques as described e.g. by Sambrook et al. 2001, Molecular Cloning: A Laboratory Manual. Therefore the nucleic acid molecules encoding the peptide stretches were

cleaved in a digest with the respective restriction enzymes Nde I and BamH I and in case of the nucleic acid molecule encoding the peptide stretch Sushi 1 the digest was performed with the restriction enzymes Nco I and BamH I. Subsequently the cleaved nucleic acids encoding the peptide stretches were ligated into the pET21 b expression vector (Novagen, Darmstadt, Germany), which was also cleaved in a digest with the respective restriction enzymes Nde I and BamH I before. The cleaved nucleic acid molecule encoding the peptide stretch Sushi I was ligated into a modified pET32 b expression vector (unmodified vector obtainable from Novagen, Darmstadt, Germany), which was also cleaved in a digest with the respective restriction enzymes Nco I and BamH I before. The modification of the pET32b expression vector refers to the deletion of the sequence encoding a S-tag and the central His-tag.

Afterwards, the nucleic acid molecule encoding the endolysin KZ144 was cleaved in a digest with the restriction enzyme BamH I and Xho I, so that the endolysin could be ligated into the pET21b expression vector (Novagen, Darmstadt, Germany) and the modified pET32 b expression vector, respectively, which were also cleaved in a digest with the respective restriction enzymes BamH I and Xho I before. The nucleic acid molecule encoding the endolysin STM0016 and the nucleic acid molecule encoding the endolysin N4gp61 were cleaved in a digest with the restriction enzyme BamH I and Xho I, so that the respective endolysin could be ligated into the pET21b expression vector (Novagen, Darmstadt, Germany).

Thus, the nucleic acid molecule encoding the peptide stretch is ligated into the respective vector at the 5'-end of the nucleic acid molecule encoding the endolysin KZ144 or STM0016. Moreover, the nucleic acid molecule encoding the endolysin KZ144 or STM0016 is ligated into the respective plasmid, so that a nucleic acid molecule encoding a His-tag consisting of six histidine residues is associated at the 3'-end of the nucleic acid molecule encoding the endolysin.

As some fusion proteins may either be toxic upon expression in bacteria, or not homogenous due to protein degradation, the strategy might be to express these fusion proteins fused or linked to other additional proteins. Example for these other additional protein is thioredoxin, which was shown to mediate expression of toxic antimicrobial peptides in *E.coli* (TrxA mediating fusion expression of antimicrobial peptide CM4 from multiple joined genes in

Escherichia coli. Zhou L, Zhao Z, Li B, Cai Y, Zhang S. Protein Expr Purif. 2009 Apr;64(2):225-230). In the case of the fusion protein consisting of the N-terminal Sushi 1 peptide and the endolysin KZ144, the Sushi 1 peptide is ligated into the modified pET32b expression vector, so that an additional thioredoxin is associated at the 5'-end of the Sushi 1 peptide. The thioredoxin could be removed from the expressed fusion protein by the use of enterokinase, therefore between the nucleic acid molecule encoding the Sushi peptide and the one encoding the thioredoxin is an enterokinase restriction site introduced.

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

Recombinant expression of the fusion proteins according to SEQ ID NO: 77 to 90 is performed in *E. coli* BL21 (DE3) pLysS and *E. coli* BL21 (DE3) cells (Novagen, Darmstadt, Germany). The cells were growing until an optical density of OD_{600nm} of 0.5-0.8 was reached. Then the expression of the fusion protein was induced with 1 mM IPTG (isopropylthiogalactoside) and the expression was performed at 37°C for a period of 4 hours.

E.coli BL21 cells were harvested by centrifugation for 20 min at 6000g and disrupted via sonication on ice. Soluble and insoluble fraction of the E.coli crude extract were separated by centrifugation (Sorvall, SS34, 30 min, 15 000 rpm). All proteins were purified by Ni²⁺ affinity chromatography (Akta FPLC, GE Healthcare) using the C-terminal 6xHis-tag, encoded by the pET21b or pET32b vectors.

As described above, some of the fusion proteins were expressed using a modified pET32b vector (S-tag and central His-tag deleted), which fuses thioredoxin on the N-terminus of the proteins of interest. The vector also contains an enterokinase cleavage site right before the protein of interest. This site allows the proteolytic cleavage between thioredoxin and the protein of interest, which can be purified via the remaining C-terminal His-tag. For antimicrobial function of the fusion protein Sushi 1-KZ144 it may be necessary to remove the thioredoxin by proteolytic cleavage. Therefore the fusion protein was cleaved with 2-4 units/mg recombinant enterokinase (Novagen, Darmstadt, Germany) to remove the thioredoxin

following the protocol provided by the manufacturer. After enterokinase cleavage the fusion protein was purified via His-tag purification as described below.

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

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

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

EXAMPLE 4: Antimicrobial activity of the endolysin KZ144 modified with various peptide stretches on the N-terminus.

The fusion protein consisting of KZ144 and the peptide stretch $\alpha 4$ helix was constructed as described in example 1. The other fusion proteins consisting of KZ144 and the respective peptide stretches were constructed as described in example 3.

E. coli DSMZ 11753, *Acinetobacter baumannii* DSMZ 30007 and *Pseudomonas aeruginosa* PAO1p cells (Burn wound isolate, Queen Astrid Hospital, Brussels; Pirnay JP et al. (2003), *J*

Clin Microbiol., 41(3):1192-1202) were used as test strains. Overnight cultures were diluted 10-fold in fresh LB medium and grown to OD₆₀₀=0.6. The culture was spun down and diluted 10-fold in dilution buffer (10 mM HEPES, 0.5 mM EDTA; pH 7.4). Bacteria were incubated at room temperature with each 10 µg undialyzed fusion protein at a final concentration of 100 µg/ml in buffer (20 mM NaH₂PO₄-NaOH pH 7.4; 0.5 M NaCl; 0.5 M imidazole). After 1 hour cell dilution series were made in PBS and plated on LB. Additionally, a negative control was plated using buffer (20 mM NaH₂PO₄-NaOH pH 7.4; 0.5 M NaCl; 0.5 M imidazole). The residual colonies were counted after an overnight incubation at 37°C. Based on the counted cell numbers the antibacterial activity as logarithmic units ($=\log_{10}N_0/N_i$ with N_0 = number of untreated cells and N_i = number of treated cells) was calculated (Table 11). All samples were replicated at least in four fold.

The antimicrobial activity of these fusion proteins is given in the following table.

Table 11: Antimicrobial activity of KZ144 modified with various peptide stretches against gram-negative bacteria

Fusion protein	Enzyme part	Peptide stretch (N-terminal unless otherwise indicated)	Activity against <i>Pseudomonas</i> <i>aeruginosa</i>	Activity against <i>E.</i> <i>coli</i> DSMZ 11753	Activity against <i>Acinetobacter</i> <i>baumannii</i> DSMZ 30007
SEQ ID NO: 77	KZ144 (SEQ ID NO:25)	Pseudin 1 (SEQ ID NO:29)	+	n.d.	n.d.
SEQ ID NO: 78	KZ144 (SEQ ID NO:25)	Ranalexin (SEQ ID NO:30)	+	n.d.	n.d.
SEQ ID NO: 79	KZ144 (SEQ ID NO:25)	Sushi 1 (SEQ ID NO:32)	+	n.d.	++
SEQ ID NO: 80	KZ144 (SEQ ID NO:25)	WLBU2-Variant (SEQ ID NO:33)	n.d.	+	n.d.
SEQ ID NO: 81	KZ144 (SEQ ID NO:25)	Melittin (SEQ ID NO:31)	+	n.d.	n.d.
SEQ ID NO: 82	KZ144 (SEQ ID NO:25)	SMAP-29 (SEQ ID NO:11)	+++	+++	n.d.
SEQ ID NO: 83	KZ144 (SEQ ID NO:25)	Cecropin A (A. aegypti) (SEQ ID NO:14)	++	+	++
SEQ ID NO: 84	KZ144 (SEQ ID NO:25)	Pleurocidin (SEQ ID NO: 6)	+	n.d.	n.d.
SEQ ID NO: 85	KZ144	Cecropin A (A.	+	n.d.	n.d.

	(SEQ ID NO:25)	melanogaster) (SEQ ID NO:15)			
SEQ ID NO: 86	KZ144 (SEQ ID NO:25)	Buforin II (SEQ ID NO:8)	+	n.d.	n.d.
SEQ ID NO: 87	KZ144 (SEQ ID NO:25)	Sarcotoxin IA (SEQ ID NO:16)	++	++	++
SEQ ID NO: 93	KZ144 (SEQ ID NO:25)	α 4 helix (SEQ ID NO:92)	$\pm$	n.d.	n.d.

Abbreviations: $\pm$ < 1 log; +: 1 log; ++: 2-3 log; +++: 4 or more logs; n.d. means that this strain was not tested with the respective fusion protein.

5

Example 5: Antimicrobial activity of the endolysin STM0016 modified with various peptide stretches on the N-terminus

The fusion proteins consisting of STM0016 and the peptide stretch Sarcotoxin IA or SMAP-29 was constructed as described in example 3.

E. coli DSMZ 11753, *Salmonella typhimurium* DSMZ 17058 and *Pseudomonas aeruginosa* PAO1p cells (Burn wound isolate, Queen Astrid Hospital, Brussels; Pirnay JP et al. (2003), *J Clin Microbiol.*, 41(3):1192-1202) were used as test strains. The antimicrobial activity of the fusion proteins consisting of the endolysin STM0016 and the peptide Sarcotoxin IA or SMAP-29 was examined as described in example 4. The antimicrobial activity of these fusion proteins is given in the following table.

Table 12:

Fusion protein	Enzyme part	Peptide stretch (N-terminal unless otherwise indicated)	Activity against <i>Pseudomonas</i> <i>aeruginosa</i>	Activity against <i>E.</i> <i>coli</i> DSMZ 11753	Activity against <i>Salmonella</i> <i>typhimurium</i> DSMZ 17058
SEQ ID NO: 88	STM0016 (SEQ ID NO: 22)	Sarcotoxin IA (SEQ ID NO:16)	+	n.d.	+
SEQ ID NO: 89	STM0016 (SEQ ID NO: 22)	SMAP-29 (SEQ ID NO: 11)	+	+	+

Abbreviations: +: 1 log; n.d. means that this strain was not tested with the respective fusion protein.

20

Example 6: Antimicrobial activity of the endolysin N4gp61 modified with a peptide stretch on the N-terminus

The fusion protein consisting of N4gp61 and the peptide stretch SMAP-29 was constructed as described in example 3.

E. coli DSMZ 11753, *Salmonella typhimurium* DSMZ 17058 and *Pseudomonas aeruginosa* PAO1p cells (Burn wound isolate, Queen Astrid Hospital, Brussels; Pirnay JP et al. (2003), *J Clin Microbiol.*, 41(3):1192-1202) were used as test strains. The antimicrobial activity of the fusion protein consisting of the endolysin N4gp61 and the peptide SMAP-29 was examined as described in example 4. The antimicrobial activity of this fusion protein is given in the following table.

Table 13:

Fusion protein	Enzyme part	Peptide stretch (N-terminal unless otherwise indicated)	Activity against <i>Pseudomonas aeruginosa</i>	Activity against <i>E. coli</i> DSMZ 11753	Activity against <i>Salmonella typhimurium</i> DSMZ 17058
SEQ ID NO: 90	N4-gp61 (SEQ ID NO:23)	SMAP-29 (SEQ ID NO: 11)	+	+	+

Abbreviations: +: 1 log; n.d. means that this strain was not tested with the respective fusion protein.

Example 7: Antimicrobial activity of the endolysin gp188 modified with a peptide stretch on the N-terminus

The fusion proteins consisting of the endolysin gp188 and the peptide stretches α 4 helix, SMAP-29 or Sarcotoxin IA were constructed as described in example 1. *E. coli* DSMZ 11753, *Acinetobacter baumannii* DSMZ 30007 and *Pseudomonas aeruginosa* PAO1p cells (Burn wound isolate, Queen Astrid Hospital, Brussels; Pirnay JP et al. (2003), *J Clin Microbiol.*, 41(3):1192-1202) were used as test strains. The antimicrobial activity of the

fusion proteins consisting of the endolysin gp188 and the respective peptide stretches was examined as described in example 4. The antimicrobial activity of these fusion proteins is given in the following table.

5 Table 14:

Fusion protein	Enzyme part	Peptide stretch (N-terminal unless otherwise indicated)	Activity against <i>Pseudomonas</i> <i>aeruginosa</i>	Activity against <i>E.</i> <i>coli</i> DSMZ 11753	Activity against <i>Acinetobacter</i> <i>baumannii</i> DSMZ 30007
SEQ ID NO: 94	gp188 (SEQ ID NO:2)	α 4 helix (SEQ ID NO: 92)	$\pm$	n.d.	n.d.
SEQ ID NO: 95	gp188 (SEQ ID NO:2)	SMAP-29 (SEQ ID NO: 11)	++	++	++
SEQ ID NO: 96	gp188 (SEQ ID NO:2)	Sarcotoxin IA (SEQ ID NO: 16)	+	+	+

Abbreviations: $\pm$ < 1 log; +: 1 log; ++: 2-3 log; n.d. means that this strain was not tested with the respective fusion protein.

10 Example 8: Antimicrobial activity of the *Salmonella* endolysin modified with the peptide stretch SMAP-29 on the N-terminus

The fusion proteins consisting of the *Salmonella* endolysin having an amino acid sequence according to SEQ ID NO: 3 and the peptide stretch SMAP-29 were constructed analogous to
 15 example 3. *E. coli* DSMZ 11753 and *Salmonella typhimurium* DSMZ 17058 were used as test strains. The antimicrobial activity of the fusion protein was examined as described in example 4. The antimicrobial activity of this fusion protein is given in the following table.

Table 15:

Fusion protein	Enzyme part	Peptide stretch (N-terminal unless otherwise indicated)	Activity against <i>E. coli</i> DSMZ 11753	Activity against <i>Salmonella</i> <i>typhimurium</i> DSMZ 17058
SEQ ID NO: 97	Salmonella endolysin (SEQ ID NO:3)	SMAP-29 (SEQ ID NO: 11)	+	+

Abbreviations: +: 1 log;

5 Example 9: Antimicrobial activity of the *Acinetobacter baumannii* endolysin modified with various peptide stretches on the N-terminus

The fusion proteins consisting of the *Acinetobacter baumannii* endolysin having an amino acid sequence according to SEQ ID NO: 5 and the peptide stretches SMAP-29, Pseudin 1 and Sushi 1 were constructed analogous to example 3. *Acinetobacter baumannii* DSMZ 30007 and *Pseudomonas aeruginosa* PAO1p cells (Burn wound isolate, Queen Astrid Hospital, Brussels; Pirnay JP et al. (2003), *J Clin Microbiol.*, 41(3):1192-1202) were used as test strains. The antimicrobial activity of the fusion proteins was examined as described in example 4. The antimicrobial activity of these fusion proteins is given in the following table.

15

Table 16:

Fusion protein	Enzyme part	Peptide stretch (N-terminal unless otherwise indicated)	Activity against <i>Pseudomonas</i> <i>aeruginosa</i>	Activity against <i>Acinetobacter</i> <i>baumannii</i> DSMZ 30007
SEQ ID NO: 98	<i>Acinetobacter</i>	Pseudin 1	±	n.d.

	<i>baumannii</i> endolysin (SEQ ID NO:5)	(SEQ ID NO: 29)		
SEQ ID NO: 99	<i>Acinetobacter</i> <i>baumannii</i> endolysin (SEQ ID NO:5)	SMAP-29 (SEQ ID NO: 11)	++	++
SEQ ID NO: 100	<i>Acinetobacter</i> <i>baumannii</i> endolysin (SEQ ID NO:5)	Sushi 1 (SEQ ID NO: 32)	+	+

Abbreviations: $\pm < 1$ log; +: 1 log; ++: 2-3 log; n.d. means that this strain was not tested with the respective fusion protein.

- 5 The fusion proteins in Table 11 to 16 without any tag and linker were also tested with the activity assays described above. They all showed antimicrobial activity against the used bacterial strains (data not shown).

CLAIMS

1. A fusion protein composed of an endolysin having the activity of degrading the cell wall of Gram-negative bacteria and a peptide stretch fused to the enzyme at the N- or C-terminus or at both termini, wherein the peptide stretch is an amphipatic peptide, sushi peptide, defensin, hydrophobic peptide or an antimicrobial peptide, wherein the antimicrobial peptide exhibits an amino acid sequence according to SEQ ID NO: 6 to 16 or 26 to 31.
2. The fusion protein according to claim 1, wherein said fusion protein exhibits an amino acid sequence according to SEQ ID NO: 63 to 90.
3. The fusion protein according to claim 1 or 2, wherein said fusion protein exhibits an additional amino acid residue on the N-terminus.
4. The fusion protein according to any of the preceding claims, wherein said fusion protein comprises a tag or additional protein on the C- and/or N-terminus.
5. The fusion protein according to claim 4, wherein said tag or additional protein is linked to the fusion protein by one or more additional amino acid residues.
6. The fusion protein according to any of the preceding claims, wherein the peptide stretch is linked to the fusion protein by one or more additional amino acid residues.
7. The fusion protein according to claim 6, wherein the enzyme exhibits an amino acid sequence according to SEQ ID NO: 22-25.
8. The fusion protein according to claim 1, wherein the sushi peptide exhibits an amino acid sequence according to SEQ ID NO: 32.
9. The fusion protein according to claim 1, wherein the hydrophobic peptide exhibits an amino acid sequence according to SEQ ID NO: 28, 33 or 35.

10. The fusion protein according to any of the preceding claims, wherein the Gram-negative bacteria are selected from the group consisting of
- Enterobacteriaceae,
- in particular Escherichia, Salmonella, Shigella, Citrobacter, Edwardsiella,
- 5 Enterobacter, Hafnia, Klebsiella, Morganella, Proteus, Providencia, Serratia, and Yersinia,
- Pseudomonadaceae,
- in particular Pseudomonas, Burkholderia, Stenotrophomonas, Shewanella, Sphingomonas and Comamonas,
- 10 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,
- 15 Bacteroidaceae,
- in particular Bacteroides, Fusobacterium, Prevotella and Porphyromonas, and Acinetobacter,
- in particular A. baumannii.
- 20 11. The fusion protein according to claim 1, wherein the amphipatic peptide comprises at least one positively charged amino acid residue selected out of the group consisting of lysine, arginine and histidine residues, combined to at least one hydrophobic amino acid residue selected out of the group consisting of valine, isoleucine, leucine, methionine, phenylalanine, tryptophan, cysteine, alanine, tyrosine, histidine, threonin, serine, proline
- 25 and glycine residues.
12. The fusion protein according to claim 1, wherein at least about 70% of the said amino acid residues in said amphipatic peptide are either arginine or lysine residues and at least about 30% of the said amino acid residues in said amphipatic peptide are valine,
- 30 isoleucine, leucine, methionine, phenylalanine, tryptophan, cysteine, alanine, tyrosine, histidine, threonin, serine, proline or glycine residues.

3. The fusion protein according to any of the preceding claims, wherein the peptide stretch comprises about 5 to about 100 amino acid residues, in particular about 5 to 50 amino acid residues, in particular about 5 to 30 amino acid residues.
- 5 14. An isolated nucleic acid molecule encoding a fusion protein according to any of claims 1 to 13.
15. A vector comprising the nucleic acid molecule according to claim 14.
- 10 16. A host cell comprising the nucleic acid molecule according to claim 14 or the vector according to claim 15.
17. A host cell according to claim 16, wherein the cell is a bacterial cell or a yeast cell.
- 15 18. The fusion protein according to any one of claims 1 to 13 for use as a medicament, diagnostic means or cosmetic substance.
19. The fusion protein according to any one of claims 1 to 13 for use as a medicament for treatment or prevention of Gram-negative bacterial infections.
- 20 20. The fusion protein according to any one of claims 1 to 13 for use as a disinfectant.
21. The use of the fusion protein according to any one of claims 1 to 13 for the treatment or prevention of Gram-negative bacterial contamination of foodstuff, of food processing
25 equipment, of food processing plants, of surfaces coming into contact with foodstuff, of medical devices, of surfaces in hospitals and surgeries.
22. The use of the fusion protein according to any one of claims 1 to 3 as a diagnostic means in medicinal, food or feed or environmental diagnostics.
- 30 23. Pharmaceutical composition comprising a fusion protein according to any one of claims 1 to 13.

Antimicrobial Agents**ABSTRACT**

5 The present invention relates to antimicrobial agents against Gram-negative bacteria, in particular to fusion proteins composed of an enzyme having the activity of degrading the cell wall of Gram-negative bacteria and a peptide stretch fused to the enzyme at the N- or C-terminus. Moreover, the present invention relates to nucleic acid molecules encoding said fusion protein, vectors comprising said nucleic acid molecules and host cells comprising either said nucleic acid molecules or said vectors. In addition, the present invention relates to 10 said fusion protein for use as a medicament, in particular for the treatment or prevention of Gram-negative bacterial infections, as diagnostic means or as cosmetic substance. The present invention also relates to the treatment or prevention of Gram-negative bacterial contamination of foodstuff, of food processing equipment, of food processing plants, of surfaces coming into contact with foodstuff, of medical devices, of surfaces in hospitals and surgeries. 15 Furthermore, the present invention relates to a pharmaceutical composition comprising said fusion protein.