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(54) Title: NEW ANTIMICROBIAL AGENTS AGAINST STAPHYLOCOCCUS AUREUS

(57) Abstract: The present invention relates to the field of antimicrobial agents active against Staphylococcus aureus bacteria. In particular, the present invention relates to polypeptides comprising the CHAP domain of LysK endolysin, the M23 endopeptidase domain of lysostaphin, the cell wall binding domain (CBD) of ALE-1, and a further peptide selected from the group consisting of an antimicrobial peptide, an amphipathic peptide, a cationic peptide, a hydrophobic peptide, a sushi peptide and a defensin. In addition, the present invention relates to nucleic acids encoding such polypeptides, vectors comprising such nucleic acids, and corresponding host cells, compositions and devices. Finally, the present invention relates to applications of the inventive polypeptides, in particular in the pharmaceutical field.



New antimicrobial agents against *Staphylococcus aureus*

The present invention relates to the field of antimicrobial agents active against *Staphylococcus aureus* bacteria. In particular, the present invention relates to polypeptides comprising the CHAP domain of LysK endolysin, the M23 endopeptidase domain of lysostaphin, the cell wall binding domain (CBD) of ALE-1, and a further peptide selected from the group consisting of an antimicrobial peptide, an amphipathic peptide, a cationic peptide, a hydrophobic peptide, a sushi peptide and a defensin. In addition, the present invention relates to nucleic acids encoding such polypeptides, vectors comprising such nucleic acids, and corresponding host cells, compositions and devices. Finally, the present invention relates to applications of the inventive polypeptides, in particular in the pharmaceutical field.

Bacterial pathogens represent a significant threat for human health. Although various types of agents having bactericidal or bacteriostatic activity are known in the art (e.g. antibiotics), microbial resistance to these, in particular to antibiotics, is steadily increasing. One of the pathogens representing a health concern are *Staphylococcus aureus* bacteria. *S. aureus* is one of the most common causes of bacteremia, infective endocarditis, as well as bone and joint infections. Furthermore, it can cause skin and soft tissue infections as well as food poisoning. *S. aureus* rapidly develops resistance to antibiotics, as illustrated by multi-drug resistant (MDR), methicillin-resistant *S. aureus* (MRSA) and the reduced susceptibility to vancomycin (vancomycin-intermediate strains). Since increasing resistance diminishes the utility of conventional antibiotics, there is a constant demand for new antimicrobial agents to control the number of *Staphylococcus aureus*, e.g. in the nosocomial (hospital) environment.

An agent highly active against *Staphylococcus aureus* bacteria is lysostaphin. Lysostaphin is a peptidoglycan hydrolase which is capable of degrading the cell wall peptidoglycan of *Staphylococcus aureus* bacteria. It is produced by *Staphylococcus simulans* biovar *staphylolyticus*. Lysostaphin exhibits two domains: a N-terminal catalytic M23 endopeptidase

domain and a C-terminal cell wall binding domain (CBD). However, *S. aureus* also rapidly develops resistance against lysostaphin.

Recently, Becker et al. (Sci Rep. 2016 Apr 28;6:25063; incorporated herewith by reference) reported the generation of fusion proteins, wherein antimicrobial activities from lysostaphin and LysK endolysin were combined. The resulting fusion protein reduced the incidence of resistant strain development significantly.

However, there is still a constant need for new antibacterial agents active against *Staphylococcus aureus* bacteria. Preferably, said agents show reduced incidence of resistant strain development while exhibiting in parallel a high antibacterial activity, as reflected by a low minimum inhibitory concentration (MIC).

The problem to be solved by the present invention was thus to provide new antibacterial agents against *Staphylococcus aureus* bacteria which show reduced incidence of resistant strain development while exhibiting in parallel a high antibacterial activity.

This object is solved by the subject matter defined in the claims and set forth below.

The term "polypeptide" as used herein refers in particular to a polymer of amino acids linked by peptide bonds in a specific sequence. The amino acid residues of a polypeptide 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, such as heme or lipid, giving rise to conjugated polypeptides which are also comprised by the term "polypeptide" as used herein. The term as used herein is intended to encompass also proteins. Thus, the term "polypeptide" also encompasses for example complexes of two or more amino acid polymer chains. The term "polypeptide" does encompass embodiments of polypeptides which exhibit optionally modifications typically used in the art, e.g. biotinylation, acetylation, pegylation, chemical changes of the amino-, SH- or carboxyl-groups (e.g. protecting groups) etc. As will become apparent from the description below, the polypeptide according to the present invention is a non-naturally occurring polypeptide. The term "polypeptide", as used herein, is not limited to a specific length of the amino acid polymer chain, but typically the polypeptide will exhibit a length of more than 250 amino acids. Usually, but not necessarily, a typical

polypeptide of the present invention will not exceed about 750 amino acids in length, preferably not exceed about 450 amino acids in length.

The term "variant sequence", as used herein, refers to an amino acid sequence which exhibits, in comparison to the respective reference sequence, one or more additions, deletions, insertions, and/or substitutions and combinations thereof. This includes for example combinations of deletions/insertions, insertions/deletions, deletions/additions, additions/deletions, insertion/ additions, additions/insertions etc. A person skilled in the art will however understand that the presence of an amino acid residue at a certain position of the variant sequence which is different from the one that is present at the respective same position in the reference sequence is not a combination of, for example, a deletion and a subsequent insertion at the same position but is a substitution as defined herein. Rather, if reference is made herein to combinations of one or more of additions, deletions, insertions, and substitutions, then combination of changes at distinct positions in the sequence are intended, e.g. an addition at the N-terminus and an intrasequential deletion. Such derived sequence will exhibit a certain level of sequence identity with the respective reference sequence, for example a given SEQ ID NO, which is preferably at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99%. Preferred variant sequences are fragments of the parent molecule, for example a given SEQ ID NO, retaining the activity of the parent molecule, i.e. exhibiting on a general level same activity as the respective parent molecule. However, said activity can be the same, higher or lower as the respective parent molecule. Also preferred variant sequences are those resulting from conservative amino acid substitutions within the parent sequence, for example a given SEQ ID NO, again retaining the activity of the parent molecule on a general level.

As used herein, the term "% sequence identity", has to be understood as follows: Two sequences to be compared are aligned to give a maximum correlation between the sequences. This may include inserting "gaps" in either one or both sequences, to enhance the degree of alignment. A % identity may then be determined over the whole length of each of the sequences being compared (so-called global alignment), that is particularly suitable for sequences of the same or similar length, or over shorter, defined lengths (so-called local alignment), that is more suitable for sequences of unequal length. In the above context, an amino acid sequence having a "sequence identity" of at least, for example, 95% to a query

amino acid sequence, is intended to mean that the sequence of the subject amino acid sequence is identical to the query sequence except that the subject amino acid sequence may include up to five amino acid alterations per each 100 amino acids of the query amino acid sequence. In other words, to obtain an amino acid sequence having a sequence of at least 95% identity to a query amino acid sequence, up to 5% (5 of 100) of the amino acid residues in the subject sequence may be inserted or substituted with another amino acid or deleted. Methods for comparing the identity and homology of two or more sequences are well known in the art. The percentage to which two sequences are identical can for example be determined by using a mathematical algorithm. A preferred, but not limiting, example of a mathematical algorithm which can be used is the algorithm of Karlin et al. (1993), PNAS USA, 90:5873-5877. Such an algorithm is integrated in the BLAST family of programs, e.g. BLAST or NBLAST program (see also Altschul et al., 1990, J. Mol. Biol. 215, 403-410 or Altschul et al. (1997), Nucleic Acids Res, 25:3389-3402), accessible through the home page of the NCBI at world wide web site ncbi.nlm.nih.gov) and FASTA (Pearson (1990), Methods Enzymol. 83, 63-98; Pearson and Lipman (1988), Proc. Natl. Acad. Sci. U. S. A 85, 2444-2448.). Sequences which are identical to other sequences to a certain extent can be identified by these programmes. Furthermore, programs available in the Wisconsin Sequence Analysis Package, version 9.1 (Devereux et al, 1984, Nucleic Acids Res., 387-395), for example the programs BESTFIT and GAP, may be used to determine the % identity between two polypeptide sequences. BESTFIT uses the "local homology" algorithm of (Smith and Waterman (1981), J. Mol. Biol. 147, 195-197.) and finds the best single region of similarity between two sequences. If herein reference is made to an amino acid sequence sharing a particular extent of sequence identity to a reference sequence, then said difference in sequence is preferably due to conservative amino acid substitutions. Preferably, such sequence retains the activity of the reference sequence, e.g. albeit maybe at a slower rate. In addition, if reference is made herein to a sequence sharing "at least" at certain percentage of sequence identity, then 100% sequence identity are preferably not encompassed.

The term "further peptide", as used herein refers to an amino acid subsequence within the amino acid sequence of the polypeptide of the invention. Said sequence may be the sequence of a cationic peptide, a polycationic peptide, an amphipathic peptide, a hydrophobic peptide, a sushi peptide and/or an antimicrobial peptide. The term does not refer to conventional tags like His-tags, such as His5-tags, His6-tags, His7-tags, His8-tags, His9-tags, His10-tags,

His11-tags, His12-tags, His16-tags and His20-tags, Strep-tags, Avi-tags, Myc-tags, Gst-tags, JS-tags, cystein-tags, FLAG-tags or other tags known in the art, thioredoxin or maltose binding proteins (MBP). Preferably, the sequence of the further peptide has a length of at least about 3 to at most about 50, preferably at most about 39 amino acid residues. The further peptide sequence itself does not provide any of the following enzymatic 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 (lysozyme), N-acetyl-glucosaminidase or transglycosylase. Typically, the further peptide sequence will not provide any enzymatic activity at all.

As used herein, the term "cationic peptide" refers to a peptide having positively charged amino acid residues. Preferably a cationic peptide has a pKa-value of 9.0 or greater. Typically, at least four of the amino acid residues of the cationic peptide can be positively charged, for example, lysine or arginine. "Positively charged" refers to the side chains of the amino acid residues which have a net positive charge at about physiological conditions. The term "cationic peptide" as used herein refers also to polycationic peptides, but also includes cationic peptides which comprise for example less than 20%, preferably less than 10% positively charged amino acid residues.

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

The term, "antimicrobial peptide" (AMP) as used herein refers to any naturally occurring peptide that has microbicidal and/or microbistatic activity on, for example, bacteria, viruses, fungi, yeasts, mycoplasma and protozoa. Thus, the term "antimicrobial peptide" as used

herein refers in particular to any peptide having anti-bacterial, anti-fungal, anti-mycotic, anti-parasitic, anti-protozoal, anti-viral, anti-infectious, anti-infective and/or germicidal, algicidal, amoebicidal, microbicidal, bactericidal, fungicidal, parasiticidal, protozoacidal, protozoicidal properties. Preferred are anti-bacterial peptides. The antimicrobial peptide may be a member of the RNase A super family, a defensin, cathelicidin, granulysin, histatin, psoriasin, dermicidine or hepcidin. The antimicrobial peptide may be naturally occurring in insects, fish, plants, arachnids, vertebrates or mammals. Preferably the antimicrobial peptide may be naturally occurring in radish, silk moth, wolf spider, frog, preferably in *Xenopus laevis*, *Rana* frogs, more preferably in *Rana catesbeiana*, toad, preferably Asian toad *Bufo bufo* gargarizans, fly, preferably in *Drosophila*, more preferably in *Drosophila melanogaster*, in *Aedes aegypti*, in honey bee, bumblebee, preferably in *Bombus pascuorum*, flesh fly, preferably in *Sarcophaga peregrine*, scorpion, horseshoe crab, catfish, preferably in *Parasilurus asotus*, cow, pig, sheep, porcine, bovine, monkey and human. As used herein, an "antimicrobial peptide" (AMP) may in particular be a peptide which is not a cationic peptide, polycationic peptide, amphipathic peptide, sushi peptide, defensins, and hydrophobic peptide, but nevertheless exhibits antimicrobial activity.

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

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 defence 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 "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 rest of its surface.

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

The term "hydrophobic peptide" as used herein refers to a hydrophobic peptide, which is preferably composed of mostly amino acid residues with hydrophobic groups. Such peptide is preferably composed of mostly hydrophobic amino acid residues, i.e. at least about 20, 30, 40, 50, 60, 70, 75, 80, 85, 90, 95 or at least about 100 % of the amino acid residues are hydrophobic amino acid residues. The amino acid residues being not hydrophobic are preferably neutral and preferably not hydrophilic.

As used herein, the term "tag" refers to an amino acid sequence, which is typically in the art fused to or included in another amino acid sequence for a) improving expression of the overall amino acid sequence or polypeptide, b) facilitating purification of the overall amino acid sequence or polypeptide, c) facilitating immobilisation of the overall amino acid sequence or polypeptide, and/or d) facilitating detection of the overall amino acid sequence or polypeptide. Examples for tags are His tags, such as His5-tags, His6-tags, His7-tags, His8-tags, His9-tags, His10-tags, His11-tags, His12-tags, His16-tags and His20-tags, Strep-tags, Avi-tags, Myc-tags, GST-tags, JS-tags, cystein-tags, FLAG-tags, HA-tags, thioredoxin or maltose binding proteins (MBP), CAT, GFP, YFP, etc. The person skilled in the art will know a vast number of tags suitable for different technical applications. The tag may for example

make such tagged polypeptide suitable for e.g. antibody binding in different ELISA assay formats or other technical applications.

The term "comprising" as used herein shall not be construed as being limited to the meaning "consisting of" (i.e. excluding the presence of additional other matter). Rather, "comprising" implies that optionally additional matter may be present. The term "comprising" encompasses as particularly envisioned embodiments falling within its scope "consisting of" (i.e. excluding the presence of additional other matter) and "comprising but not consisting of" (i.e. requiring the presence of additional other matter), with the former being more preferred.

The inventors of the present invention have surprisingly found new polypeptide agents which exhibit a high antibacterial activity and in parallel a reduced incidence of resistant strain development. In both aspects the polypeptides of the present invention represent an improvement over the best fusion protein of Becker et al. (Sci Rep. 2016 Apr 28;6:25063), the L-K construct.

Therefore, the present invention relates in a first aspect to a polypeptide comprising:

- i) the CHAP domain of LysK endolysin (or a variant sequence thereof exhibiting at least 80% sequence identity with the CHAP domain of LysK endolysin);
- ii) the M23 endopeptidase domain of lysostaphin (or a variant sequence thereof exhibiting at least 80% sequence identity with the M23 endopeptidase domain of lysostaphin);
- iii) the cell wall binding domain (CBD) of ALE-1 (or a variant thereof exhibiting at least 90% sequence identity with the cell wall binding domain (CBD) of ALE-1),
and
- iv) a further peptide selected from the group consisting of an antimicrobial peptide, an amphipathic peptide, a cationic peptide, a hydrophobic peptide, a sushi peptide and a defensin.

The inventive polypeptide comprises at least four sequence elements. The first element is the CHAP domain (cysteine-histidine dependent amido-hydrolase/peptidase domain) of LysK endolysin (see SEQ ID NO:1) or a variant sequence thereof. The polypeptide of the invention may comprise aside of the CHAP domain of LysK also corresponding longer sequence

elements of LysK and respective variant sequences. For example, the polypeptide may comprise the sequence of SEQ ID NO:2, which reflects the N-terminal part of LysK endolysin (including the CHAP domain) but for the N-terminal methionine. The second element is the M23 endopeptidase domain of lysostaphin (see SEQ ID NO:3) or a variant sequence thereof. This domain is the catalytic domain of the bacteriocin lysostaphin. The polypeptide of the invention may comprise aside of the M23 endopeptidase domain of lysostaphin also longer sequence elements (and respective variant sequences), such as the sequence of SEQ ID NO:4, which reflects the N-terminal part of lysostaphin (aa 4-152; including the M23 endopeptidase domain). The third element is the cell wall binding domain (CBD) of ALE-1 endolysin (see SEQ ID NO:5) or a variant sequence thereof. This is a non-catalytic domain. The polypeptide of the invention may comprise aside of the CBD of ALE-1 endolysin also longer sequence elements of ALE-1 endolysin (and respective variant sequences), such as the sequence of SEQ ID NO:6, which reflects the C-terminal part of ALE-1 endolysin (aa 234-327; including the CBD domain). The two catalytic domains (CHAP, M23) and the CBD domain (of ALE-1) may in principle occur in any order. However, preferably the different elements (irrespective whether they are the natural occurring or variant sequences) are arranged as follows (from N- to C-terminus): CHAP domain - M23-endopeptidase - CBD of ALE-1. There may also be other, e.g. intervening, sequence elements present. Preferably, the intervening sequences are short linker sequences not exceeding in each case more than 10, more preferably not more than 5 amino acids in length. Most preferably, they are only one or two amino acids in length. Linker sequences are preferably flexible sequences, comprising one or more glycine residues. An example for such linker is a glycine-serine linker or the sequence GGGGS (SEQ ID NO: 7). Preferably, the fourth element of the inventive polypeptide, the further peptide (see below) is positioned N- or C-terminal of the unit formed by the two catalytic domains (CHAP, M23) and the CBD domain (of ALE-1), with the C-terminal position being more preferred. If the the two catalytic domains (CHAP, M23) and the CBD domain form a unit (i.e. the peptide is C- or N-terminal of said unit), then said unit is preferably less than 500 amino acids in length, preferably less than 450 amino acids in length. It is preferred if a polypeptide according to the first aspect of the invention comprises at least the amino acid sequence of SEQ ID NO:3 (M23), and/or of SEQ ID NO:5 (ALE-1). Most preferably, the polypeptide according to the present invention comprises in any event the amino acid sequence according to SEQ ID NO:5. A preferred arrangement of these last two elements (M23, ALE-1) is reflected in SEQ ID NO:8.

Regarding the CHAP domain the present invention provides a number of possible variant sequence, i.e. there is absolutely no obligation to specifically use the amino acid sequence of SEQ ID NO:1 or SEQ ID NO:2. Possible mutations (identified vis-à-vis SEQ ID NO:2) are for example K17E, K28N, H51Q, T86A, G154S or G154C, E172G, T173N, or A174R. Most preferred are variant sequences of SEQ ID NO:2, which comprise the mutations K17E and H51Q. Most preferably, said variant sequences comprise the mutations K17E, K28N, H51Q, T86A and G154S. These mutations have been shown to improve antibacterial activity of the polypeptides according to the present invention and to increase thermal stability. If the naturally occurring sequence of SEQ ID NO:2 is employed, then a preferred arrangement of the first three elements of the present invention is reflected in SEQ ID NO:9 (w/o N-terminal methionine) and in SEQ ID NO:10. Contemplated for use in the present invention are also variant sequences thereof, e.g. variant sequences comprising at least 80% sequence identity with SEQ ID NO:8, SEQ ID NO:9 or SEQ ID NO:10.

The inventive polypeptide comprises - aside of the first three elements (CHAP domain - M23-endopeptidase - CBD of ALE-1) – a fourth element, namely a peptide selected from the group consisting of an antimicrobial peptide, an amphipathic peptide, a cationic peptide, a hydrophobic peptide, a sushi peptide and a defensin. Preferably, the further peptide sequence is heterologous to the CHAP domain of LysK endolysin, the M23 endopeptidase domain of lysostaphin and the cell wall binding domain (CBD) of ALE-1, i.e. the further peptide sequence does not occur in the LysK endolysin sequence, the lysostaphin sequence and/or the ALE-1 endolysin sequence. Preferably, the fourth element of the inventive polypeptide is positioned N- or C-terminal of the unit formed by the two catalytic domains (CHAP, M23) and the CBD domain (of ALE-1), with the C-terminal position being more preferred. Suitable arrangements are thus: a) CHAP domain - M23-endopeptidase - CBD of ALE-1 - peptide, and b) Peptide - CHAP domain - M23-endopeptidase - CBD of ALE-1. The peptide may be linked to the enzyme unit (CHAP domain - M23-endopeptidase - CBD of ALE-1) directly or via intervening sequences, e.g. linker sequences.

Examples for cationic/ polycationic amino acid sequences, which may be used as further peptide, are listed in the following table.

Table 1:

Amino acid sequence	Length	SEQ ID NO:
KRKKRK	6	11
KRXKR	5	12
KRSKR	5	13
KRGSG	5	14
KRKKRKKRK	9	15
RRRRRRRRR	9	16
KKKKKKKK	8	17
KRKKRKKRKK	10	18
KRKKRKKRKKRK	12	19
KRKKRKKRKKRKKR	14	20
KKKKKKKKKKKKKKKK	16	21
KRKKRKKRKKRKKRKKR	18	22
KRKKRKKRKKRKKRKKRKK	19	23
RRRRRRRRRRRRRRRRRR	19	24
KKKKKKKKKKKKKKKKKK	19	25
KRKKRKKRKRKRKKRKKR	20	26
KRKKRKKRKRKRKRKKRKK	21	27
KRKKRKKRKKRKKRKKRKKR	21	28
KRKKRKKRKRKRGSGKRKKRKKR	22	29
KRKKRKKRKRKRGSGGKRKKRKKR	24	30
KRKKRKKRKKRKKRKKRKKRKKR	25	31
KRKKRKKRKRKRKRKKRKRKRKRKR	31	32
KRKKRKKRKRKRGSGGKRKKRKKRKRKRGSGGKRKKRKKR	38	33
KRKKRKKRKKRKKRKKRKKRKKRKKRKKRKKRKKRKKR	39	34
KRKKRKKRKRKRKRKKRKRKRKRKRKRKRKRKRKRKRKR	42	35
KNA	3	36
GGSKNA	6	37
KNK	3	38
GGSKNK	6	39

Examples for antimicrobial amino acid sequences which may be used in carrying out the present invention are listed in the following table.

Table 2:

Peptide	Sequence	SEQ ID NO
LL-37	LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLRVPR TES	40
SMAP-29	RGLRRLGRKIAHGPKYKGYPTVLRIRIAG	41
Indolicidin	IIPWKWPWWPWR	42

Peptide	Sequence	SEQ ID NO
Protegrin	RGGRLCYCRRRFCVCVGR	43
Cecropin P1	SWLSKTAKKLENSAKKRRISEGIAIAIQGGPR	44
Magainin	GIGKFLHSAKKFGKAFVGEIMNS	45
Pleurocidin	GWGSFFKKAHVGHVGHVKAALHLYL	46
Cecropin A (<i>A. aegypti</i>)	GGLKKGKLEGGAGKRVFNAAEKALPVVAGAKAL RK	47
Cecropin A (<i>D. melanogaster</i>)	GWLKKGKGIKIERVGHVGHVGHVKAALHLYL AATARG	48
Buforin II	TRSSRAGLQFPVGRVHRLLRK	49
Sarcotoxin IA	GWLKKGKGIKIERVGHVGHVGHVKAALHLYL AATAR	50
Apidaecin	ANRPVYIPPPRPPHRL	51
Ascaphine 5	GIKDWIKGAAKLIKTVASHIANQ	52
Nigrocine 2	GLLSKVLGVGKVKVLCVSGLVG	53
Pseudin 1	GLNTLKKVFGQLHEAIKLINNHVQ	54
Ranalexin	FLGGLIVPAMICAVTKKC	55
Melittin	GIGAVLKVLTGGLPALISWIKRKRQQ	56
Lycotoxin 1	IWLTALKFLGKHAACKLAKQQLSKL	57
Parasin 1	KGRGKQGGKVRKAKTRSS	58
Buforin I	AGRKQGGKVRKAKTRSSRAGLQFPVGRVHRLR RKGNY	59
Dermaseptin 1	ALWKTMLKKGTMALHAGKAALGAAADTISQGTQ	60
Bactenecin 1	RLCRIVVIRVCR	61
Thanatin	GSKKPVPIIYCNRRRTGKCQRM	62
Brevinin 1T	VNPIILGVLPKVLITKKC	63
Ranateurin 1	SMLSVLKNLGKVLGFGVACKINIKQC	64
Esculentin 1	GIFSKLGRKKIKNLLISGLKNVGVGMDVVRTG IKIAGCKIKGEC	65
Tachyplesin	RWCFRVCYRGICYRKCR	66
Androctonin	RSVCRQIKICRRRGGCYKCTNRPY	67
alpha- defensin	DCYCRIPACIAGERRYGTCTIYQGRWLWAFCC	68
beta- defensin	NPVSCVRNKGICVPIRCPGSMKQIGTCVGRAVKC CRKK	69
theta- defensin	GFCRCLCRRGVCRICTR	70
defensin (sapecin A)	ATCDLLSGTGINHSACAAHCLLRGNRGGYCNGKA VCVCRN	71
Thionin (crambin)	TTCCPSIVARSNFNVCRIPTPEAICATYTGCI I IPGATCPGDYAN	72
defensin from radish	QKLCQRPSTWVSGVCGNNAACKNQICIRLEKARHG SCNYVFPAPHCICYFPC	73
Drosomycin	DCLSGRYKGPCAVWDNETCRRVCKEEGRSSGHCS PSLKCWCEGC	74
Hepcidin	DTHFPICIFCCGCCHRSKCGMCKT	75

Peptide	Sequence	SEQ ID NO
Bac 5	RFRPPIRRPPIRPPFYPPFRPPIRPPIFPPIRPP FRPPLGRPFP	76
PR-39	RRRPRPPYLP RPRPPFFFPRLPPRIPP GFPPRF PPRFP	77
Pyrrhocoricin	VDKGSYLPRPTPPRPIYNRN	78
Histatin 5	DSHAKRHHGYKRKFHEKHHSRHY	79
ECP19	RPPQFTRAQWF AIQHISLN	80
MSI-594	GIGKFLKKAKKGIGAVLKVLTG	81
TL-ColM	METLTVHAPSPSTNLP SYNGAFSLSAPHVPGAG P	82
SBO	KLKKIAQKIKNFFAKLVA	83
Macedocin	GKNGVFKTISHECHLNTWAF LATCCS	84
Macedocin (Trunc)	GKNGVFKTISHECHLNTWAF LA	85
D16	ACKLKSLLKTL SKAKKKLKTLLKALSK	86
CPF-C1	GFGSLLGKALRLGANVL	87
TL-ColM(-Met)	ETLTVHAPSPSTNLP SYNGAFSLSAPHVPGAGP	88
TM-174E	LISKGWPYLLVVVLGATIYFWGNSNG	89
ECP45	RPPQFTRAQWF AIQHISLNPPRCTIAMRAINNYR WRCKNQNTFLR	90
ColicinE3_1- 51 (S37F)	SGGDGRGHNTGAHSTSGNINGGPTGLGVGGGASD GFGWSSENNPWGGGSG	91
ColicinE3_1- 69 (S37F)	SGGDGRGHNTGAHSTSGNINGGPTGLGVGGGASD GFGWSSENNPWGGGSGSGIHWGGGSGHGNGGGNG	92
ColicinD_1-53	SDYEGSGPTEGIDYGHSMVWPSTGLISGGDVKP GGSSGIAPSMPPGWDYS	93
Cathepsin G (77-83)	HPQYNQR	94

The further may be 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: 95. Other preferred sushi peptides are sushi peptides S1 and S3 and multiples thereof (Tan et al, FASEB J. 2000 Sep;14(12):1801-13).

Preferred hydrophobic peptides are Walmagh1 having the amino acid sequence according to SEQ ID NO: 96 and the hydrophobic peptide having the amino acid sequence Phe-Phe-Val-Ala-Pro (SEQ ID NO: 97).

Preferred amphipathic peptides are α 4-helix of T4 lysozyme according to SEQ ID NO: 98 and WLBU2-Variant having the amino acid sequence according to SEQ ID NO: 99 and Walmagh 2 according to SEQ ID NO: 100.

More preferably, the sequence of the further peptide is selected from the group consisting of SEQ ID NO: 36, SEQ ID NO: 37, SEQ ID NO: 38, SEQ ID NO: 39 and SEQ ID NO: 94. Most preferably, the sequence of the further peptide is selected from the group consisting of SEQ ID NO: 38 and SEQ ID NO: 94.

In particular in cases where the inventive polypeptide is to be recombinantly expressed by a host cell, it is preferred if the inventive polypeptide comprises a methionine residue at the N-terminus.

The inventive polypeptide may comprise additionally one or more tag sequences. Such tag sequence may for example be located at the N- or C-terminus of the inventive polypeptide, or between the peptide sequence and the enzyme unit (CHAP domain - M23-endopeptidase - CBD of ALE-1). In a preferred embodiment, the one or more tag sequence is located on the C-terminal side of the enzyme unit (CHAP domain - M23-endopeptidase - CBD of ALE-1). The one or more tag sequences may be linked for example directly or via a short linker to the enzyme unit (see above). Numerous examples for tags are known in the art, some of which have already been mentioned above. In the context of the present invention a particularly preferred tag sequence is a His-tag, preferably a His tag according to SEQ ID NO: 101. Preferred sequences comprising the enzyme unit (CHAP domain - M23-endopeptidase - CBD of ALE-1) and a tag are SEQ ID NO: 102 and SEQ ID NO: 103 (both with an optional methionine at the N-terminus). The peptide sequence is preferably located C-terminal (e.g. of SEQ ID NO: 102 or SEQ ID NO: 103), either directly linked thereto or via linker sequences.

Particularly preferred examples of polypeptides according to the present invention are polypeptides comprising the sequence of SEQ ID NO:104 (SEQ ID NO:105 with N-terminal methionine) or SEQ ID NO: 106 (SEQ ID NO: 107 with N-terminal methionine). Other examples are SEQ ID NO: 108 (SEQ ID NO:109 with N-terminal methionine) and SEQ ID NO: 110 (SEQ ID NO:111 with N-terminal methionine). The present invention also contemplates to utilize polypeptides comprising variant sequences of any of these eight

sequences, in particular variant sequences exhibiting at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% sequence identity to any one of these eight sequences. Preferably, said polypeptides are more heat stable and/or exhibit higher activity than the respective reference sequence. Preferably, said variant sequences still comprise the sequence of SEQ ID NO: 38 or SEQ ID NO: 94, and/or still comprise the sequence of SEQ ID NO:5 or SEQ ID NO:6. Sites for variation are in particular those for which the present invention identified suitable mutations. The variant sequences may also lack the N-terminal methionine.

In further aspects the present invention relates to polypeptides comprising an improved CHAP domain of LysK. The inventors have identified mutations which improve the antibacterial activity and/or the thermal stability. As protein domains are capable of folding independently of other domains, these properties will be retained and can be exploited when shuffling this domain with other domains, e.g. of other endolysins.

Therefore, the present invention relates in a second aspect of the present invention to a further polypeptide, said polypeptide comprising a variant sequence of SEQ ID NO:1, wherein said variant sequence comprises at least 80% sequence identity with the amino acid sequence of SEQ ID NO:1, and wherein said variant sequence exhibits mutation H8N and/or T43A compared to the amino acid sequence of SEQ ID NO:1. As said polypeptide needs to exhibit at least one of these two mutations, said polypeptide does not comprises 100% sequence identity with the amino acid sequence of SEQ ID NO:1. Preferably, the polypeptide of the second aspect of the invention exhibits at least the H8N mutation and exhibits most preferably both mutations.

In a third the present invention relates to a further polypeptide comprising a variant sequence of SEQ ID NO:2, wherein said variant sequence comprises at least 80% sequence identity with the amino acid sequence of SEQ ID NO:2, and wherein said variant sequence exhibits one or more mutations selected from the group consisting of K16E, K27N, H50Q, T85A, G154C, G154S compared to the amino acid sequence of SEQ ID NO:2. Preferably, the polypeptide according to the third aspect of the invention comprises a variant sequence exhibiting at least mutations K16E and H50Q compared to the amino acid sequence of SEQ ID NO:2. Even more preferably, the polypeptide according to the third aspect of the invention

comprises a variant sequence exhibiting at least mutations K16E, K27N, H50Q, T85A, and G154S compared to the amino acid sequence of SEQ ID NO:2.

The polypeptide of the second or third aspect of the invention may comprise further sequence elements, in particular further domains, such as domains providing the fusion protein with antibacterial activity (for instance against Staphylococci, such as *S. aureus*). For example, a polypeptide of the second or third aspect of the invention may comprise at least one (e.g. one, two, or more than two) catalytic domains of a peptidoglycan hydrolyse. If the polypeptide of the second or third aspect of the invention comprises more than one additional domain, then said domains may derive from different sources, i.e. may be heterologous to each other.

An example for a polypeptide according to the second or third aspect of the invention may be a polypeptide wherein said variant sequence exhibits at least 95% sequence identity with the amino acid sequence of SEQ ID NO:105, SEQ ID NO: 107, SEQ ID NO:109, or SEQ ID NO:111, preferably at least 96%, at least 97%, at least 98% or at least 99% sequence identity with the amino acid sequence of SEQ ID NO:105, SEQ ID NO: 107, SEQ ID NO:109, or SEQ ID NO:111.

The length of a polypeptide according to any aspect of the present invention is in principle not limited, but preferably the length will not be excessively large. Preferably, a polypeptide according to the present invention has an overall length not exceeding about 600 amino acids, preferably not exceeding about 500 amino acids.

A polypeptide according to the present invention is preferably characterized by the ability to degrade the peptidoglycan of *S. aureus* bacteria. If the enzyme is active, degradation of the peptidoglycan layer will lead to a drop of turbidity, which can be measured photometrically (see for example Briers et al., J. Biochem. Biophys Methods 70: 531-533, (2007)).

The present invention does also relate to nucleic acids encoding one or more inventive polypeptides of the present invention. The inventive nucleic acid may take all forms conceivable for a nucleic acid. In particular the nucleic acids according to the present invention may be RNA, DNA or hybrids thereof. They may be single-stranded or double-stranded. They may have the size of small transcripts or of entire genomes, such as a

bacteriophage genome. As used herein, a nucleic acid encoding one or more inventive polypeptides of the present invention may be a nucleic acid reflecting the sense strand. Likewise, the antisense strand is also encompassed. The nucleic acid may encompass a heterologous promotor for expression of the inventive polypeptide.

In a further aspect, the present invention relates to a vector, which comprises a nucleic acid according to the present invention. Such vector may for example be an expression vector allowing for expression of an inventive polypeptide. Said expression may be constitutive or inducible. The vector may also be a cloning vector comprising the nucleic acid sequence of an inventive polypeptide for cloning purposes.

In a further aspect, the present invention relates to a host cell comprising a polypeptide according to the present invention, a nucleic acid according to the present invention, and/or a vector according to the present invention. The host cells may be selected in particular from the group consisting of bacterial cells and yeast cells. Particularly preferred host cells are *E. coli* cells.

In a further aspect, the present invention relates to composition comprising a polypeptide according to the present invention, a nucleic acid according to the present invention, a vector according to the present invention, and/or a host cell according to the present invention and further a suitable diluent, excipient or carrier. Preferred compositions comprise the polypeptide according to the present invention. Preferably, a composition according to the present invention comprises a pharmaceutically acceptable diluent, excipient or carrier. Such composition may be a pharmaceutical composition. Furthermore, a composition according to the present invention, comprising a polypeptide according to the present invention, a nucleic acid according to the present invention, a vector according to the present invention, and/or a host cell according to the present invention, may be an aqueous solution (preferably a buffer or a physiological solution), a powder, a suppository, an emulsion, a suspension, a gel, a lotion, a cream, salve, ointment, injectable solution, syrup, spray, inhalant or any other medical reasonable galenic composition or formulation, a coating composition, preferably an implant coating composition, a stent coating composition, or a catheter coating composition, a biomaterial, preferably bone cement.

In a further aspect the present invention relates to a device, in particular a medical device comprising a polypeptide according to the present invention, a nucleic acid according to the present invention, a vector according to the present invention, a host cell according to the present invention, and/or a composition according the present invention. Such device (or at least a portion thereof) may for example be coated or impregnated with a polypeptide according to the present invention, a nucleic acid according to the present invention, a vector according to the present invention, a host cell according to the present invention, and/or a composition according the present invention. Particularly preferred are coating or impregnation with a polypeptide according to the present invention or a composition according the present invention. Such device may for example be an implant, stent or catheter. These devices may find for example application in cardiac or orthopedic surgery. Other suitable devices of the invention may be plasters, compresses or dressings comprising a polypeptide according to the present invention (or a nucleic acid according to the present invention, a vector according to the present invention, a host cell according to the present invention, and/or a composition according the present invention). These may find for instance application in wound care.

In a further aspect the present invention relates to a polypeptide according to the present invention, a nucleic acid according to the present invention, a vector according to the present invention, a host cell according to the present invention, and/or a composition according the present invention for use in a method for treatment of the human or animal body by surgery or therapy or in diagnostic methods practiced on the human or animal body.

Diagnostic methods practiced on the human or animal body may involve the taking of samples from a subject, establishing bacterial cultures and the analysis whether addition of, e.g., a polypeptide of the present invention to the culture inhibits bacterial growth. If so, presence of *S. aureus* is likely.

If the nucleic acids of the invention or the vector of the present invention is used in this context, then preferably said nucleic acid or vector provides for secretion of an inventive polypeptide from a cell. If a host cell of the invention is used, then it is preferred that the respective host cell secretes an inventive polypeptide.

The present invention also relates to a polypeptide according to the present invention (and likewise a nucleic acid according to the present invention, a vector according to the present invention, a host cell according to the present invention, and/or a composition according to the present invention) for use in a method of treatment or prevention of infections, in particular for use in a method of treatment or prevention of infections involving *S. aureus* bacteria. In this respect the present invention relates also to a polypeptide according to the present invention (and likewise a nucleic acid according to the present invention, a vector according to the present invention, a host cell according to the present invention, and/or a composition according to the present invention) for use in a method of treatment, amelioration or prevention of dermatitis (such as atopic dermatitis) or otitis. *S. aureus* bacteria are frequently involved in these disease states, e.g. as secondary infection.

The present invention also relates to a polypeptide according to the present invention (and likewise a nucleic acid according to the present invention, a vector according to the present invention, a host cell according to the present invention, and/or a composition according to the present invention) for use in a method of treatment of wounds of a subject, in particular acute wounds such as iatrogenic wounds, or chronic wounds. The subject may be, for example, an animal or a human being.

The present invention also relates to a method of treatment or prevention of infections caused by *S. aureus* bacteria in a subject, the method comprising contacting said subject with a polypeptide according to the present invention (or likewise a nucleic acid according to the present invention, a vector according to the present invention, a host cell according to the present invention, and/or a composition according to the present invention). In particular, the present invention relates to a method of treatment or prevention of infections involving *S. aureus* bacteria in a subject, the method comprising contacting said subject with a polypeptide according to the present invention.

In a further aspect the present invention relates to the use of a polypeptide according to the present invention (or a nucleic acid according to the present invention, a vector according to the present invention, a host cell according to the present invention, and/or a composition according to the present invention) for disinfecting inanimate surfaces, compositions and/or objects, in particular in the nosocomial environment or in a doctor's office.

In a further aspect, the present invention relates to the use of a polypeptide according to the present invention (or a nucleic acid according to the present invention, a vector according to the present invention, a host cell according to the present invention, and/or a composition according to the present invention) for preventing contamination of inanimate surfaces, compositions and/or objects with bacteria, in particular for preventing contamination with *S. aureus* bacteria.

In a further aspect, the present invention relates to a method for disinfecting inanimate surfaces, compositions and/or objects, in particular in the nosocomial environment or in a doctor's office, wherein the method comprises contacting the inanimate surfaces, compositions and/or objects with a polypeptide according to the present invention (or a nucleic acid according to the present invention, a vector according to the present invention, a host cell according to the present invention, and/or a composition according to the present invention).

In a further aspect, the present invention relates to a method for preventing contamination of inanimate surfaces, compositions and/or objects with bacteria, in particular for preventing contamination with *S. aureus* bacteria, wherein the method comprises contacting the inanimate surfaces, compositions and/or objects with a polypeptide according to the present invention (or a nucleic acid according to the present invention, a vector according to the present invention, a host cell according to the present invention, and/or a composition according to the present invention).

Examples

In the following a specific example illustrating embodiments and aspects of the invention is presented. However, the present invention shall not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become readily apparent to those skilled in the art from the foregoing description and the examples below. All such modifications fall within the scope of the appended claims.

Example 1: Antibacterial activity on *S. aureus* of two fusion proteins of the invention

Becker et al. (Sci Rep. 2016 Apr 28;6:25063) reported construction of fusion protein L-K with reduced incidence of resistant *S. aureus* strain development. In an attempt to provide other improved fusion proteins against *S. aureus*, the inventors generated two fusion proteins, each comprising the CHAP domain of LysK endolysin, the M23 endopeptidase domain of lysostaphin; and the cell wall binding domain (CBD) of ALE-1. In addition, one of the fusion proteins did comprise the cathepsin G (77-83) peptide (SEQ ID NO:94), the other the cationic peptide KNK (SEQ ID NO:38). The resulting fusion proteins (SEQ ID NO:105 and SEQ ID NO: 107) were assayed for antibacterial activity and resistance development. For this purpose MIC (minimal inhibitory concentration) assays (see below) were carried out for several culture cycles.

MIC assay

S. aureus Sp10 was grown in (Luria-Bertani) medium and diluted 1:10 in Mueller-Hinton medium. At an optical density OD₆₀₀ of about 0.6, bacteria were diluted in the same medium 1:10 followed by a 1:500 dilution. Protein buffer (20 mM HEPES, 500 mM NaCl, pH 7.4) and proteins or gentamycin were pipetted into a 96 well plate, using different concentrations of proteins/ gentamycin and an end volume of 20 µl. The proteins used were the fusion proteins according to SEQ ID NO:105 and SEQ ID NO: 107. 180 µl of bacterial cells or a medium (Mueller-Hinton) control were given to the 96 well plate and mixed. The plate was incubated for 18-22 hours at 37°C and the bacterial growth was determined measuring the OD₆₀₀ values of the wells. The well with the lowest concentration of protein/gentamycin showing the same OD₆₀₀ value as the medium control was taken as MIC. For the next cycle,

the bacterial solution from the sub-MIC well was used. The sub-MIC well is the well in which the next lower concentration to the MIC concentration was tested, i.e. the well with the highest concentration of protein/gentamycin but still OD₆₀₀ above the medium control. For further cycles of the resistance assay, the bacteria from this sub-MIC well of the previous cycle were taken for the next over-night culture.

The results are given in tables 3a and b below.

Table 3a:

Cycle	Minimal inhibitory concentration (MIC; µg/ml)		
	SEQ ID NO:105	SEQ ID NO: 107	Gentamycin
1	4	4	0.8
6	4	4	4
12	6	6	6

Table 3b below illustrates said results of table 3a as fold change over the initial MIC (cycle 1).

Table 3b:

Cycle	Fold change of MIC		
	SEQ ID NO:105	SEQ ID NO: 107	Gentamycin
1	1	1	1
6	1	1	5
12	1.5	1.5	7.5

As evident from table 3b above, both fusion proteins of the invention are less prone to resistant strain development over time than gentamycin.

Moreover, both fusion proteins of the invention showed in comparison to the results reported for the fusion protein L-K of the prior art (see Becker et al. (Sci Rep. 2016 Apr 28;6:25063) surprisingly reduced incidence of resistant strain development after more cycles (1.5 fold change MIC for both fusions proteins of the present invention after 12 cycles vs. 2 fold change MIC for L-K fusion protein of Becker et al. after 10 cycles (see Becker et al., Fig. 1B), while exhibiting in parallel a higher antibacterial activity (initial MIC of 4 µg/ml for the fusion proteins of the present invention vs. 7.8 µg/ml for the L-K fusion protein of Becker et al.).

Example 2: Variants of polypeptides according to the present invention

The inventors of the present invention also created variants of the above mentioned polypeptides. For this purpose, mutations were introduced in the sequence of SEQ ID NO: SEQ ID NO:105.

Table 4:

Clone	Mut1	Mut2	Mut3	Mut4
1	K17E	H51Q		
2	T127I			
3	K28N	T86A	G154C	
4	N126S	A16I		
5	G85D		G119S	
6	A16I	F36L	A46V	I80T
7	G166S	E172R	T173N	A174R
8	E172G	T173N	A174R	
9	V26A	Q114P		
10	N138D	G154S	K171I	

Table 5 below illustrates the results of these mutations on the activity for the fusion protein of SEQ ID NO:105. As used herein, strain DSM 346 refers to *Staphylococcus aureus* strain

DSM 346 (Leibniz-Institut DSMZ - Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany). S64 and S69 are *Staphylococcus aureus* strains S64 and S69 obtained from Prof. Rob Lavigne (Katholieke Universiteit Leuven, Belgium).

Table 5:

Clone	Strain	MIC [$\mu\text{g/mL}$]
1	DSM 346	4
	S64	6
	S69	6
2	DSM 346	2
	S64	4
	S69	4
3	DSM 346	4
	S64	8
	S69	8
4	DSM 346	2
	S64	4
	S69	4
5	DSM 346	4
	S64	4
	S69	4
6	DSM 346	6
	S64	20
	S69	12
7	DSM 346	6
	S64	10
	S69	8
8	DSM 346	4
	S64	6
	S69	6
9	DSM 346	4
	S64	4
	S69	4
10	DSM 346	4
	S64	6
	S69	8

All mutants retained antibacterial activity on *S. aureus*.

Some of the above mentioned mutations were also verified for the sequence of SEQ ID NO: 107. In addition, some of the above mutations were combined. In addition, mutation G154S was used instead of G154C. Table 6 below illustrates the mutations tested for the fusion protein of SEQ ID NO: 107.

Table 6:

Clone	Mut1	Mut2	Mut3	Mut4	Mut 5	Mut 6
11	K17E	H51Q				
12	K17E	H51Q	E172G	T173N	A174R	
13	K28N	T86A	G154S			
14	K28N	T86A	G154S	E172G	T173N	A174R
15	K17E	H51Q	K28N	T86A	G154S	

Table 7 below illustrates the results of these mutations on the activity for the fusion protein of SEQ ID NO: 107.

Table 7:

Clone	Strain	MIC [$\mu\text{g/mL}$]
11	DSM 346	4
	S64	4
	S69	12
12	DSM 346	8
	S64	10
	S69	10
13	DSM 346	4
	S64	4
	S69	4
14	DSM 346	10
	S64	8
	S69	8
15	DSM 346	2
	S64	4
	S69	4

All mutants retained antibacterial activity on *S. aureus*.

In addition, thermal stability was assessed for two of said mutants in comparison to the unmodified fusion protein. The thermal stability assay was carried out with the strain *Staphylococcus aureus* DSM 346. The proteins were diluted to a concentration of 0,3 mg/ml followed by an incubation for 20 minutes at different temperatures (see table 8 below). A standard MIC assay was carried out after this incubation time. The higher the temperature after which the protein still shows (a high) activity, the better is the thermal stability of a protein.

Table 8:

	MIC [$\mu\text{g}/\text{mL}$]							
	RT	44,6°C	47,1°C	49,7°C	52,3°C	54,9°C	57,4°C	60°C
SEQ ID NO: 107	6	20	25	30	30	>30	>30	>30
Clone 11	4	6	10	10	16	>30	>30	>30
Clone 15	4	4	4	4	4	4	6	8

Clones 11 and 15 thus showed increased thermal stability in comparison to the unmodified fusion protein.

Claims

1. Polypeptide comprising:
 - i) the CHAP domain of LysK endolysin or a variant thereof exhibiting at least 80% sequence identity with the CHAP domain of LysK endolysin;
 - ii) the M23 endopeptidase domain of lysostaphin or a variant thereof exhibiting at least 80% sequence identity with the M23 endopeptidase domain of lysostaphin;
 - iii) the cell wall binding domain (CBD) of ALE-1 or a variant thereof exhibiting at least 90% sequence identity with the cell wall binding domain (CBD) of ALE-1, and
 - iv) a further peptide selected from the group consisting of an antimicrobial peptide, an amphipathic peptide, a cationic peptide, a hydrophobic peptide, a sushi peptide and a defensin.

2. The polypeptide according to claim 1, wherein the polypeptide comprises:
 - i) the amino acid sequence of SEQ ID NO:1, or of a variant thereof exhibiting at least 80% sequence identity with the amino acid sequence of SEQ ID NO:1,
 - ii) the amino acid sequence of SEQ ID NO:3, or of a variant thereof exhibiting at least 80% sequence identity with the amino acid sequence of SEQ ID NO:3, and/or
 - iii) the amino acid sequence of SEQ ID NO:5, or of a variant thereof exhibiting at least 90% sequence identity with the amino acid sequence of SEQ ID NO:5.

3. The polypeptide according to claim 2, wherein the polypeptide comprises the amino acid sequence of SEQ ID NO:1, SEQ ID NO:3 and/or of SEQ ID NO:5, preferably wherein the polypeptide comprises the amino acid sequence of SEQ ID NO:3, and/or SEQ ID NO:5 even more preferably wherein the polypeptide comprises the amino acid sequence of SEQ ID NO:5.

4. The polypeptide according to claim 1, wherein the polypeptide comprises:
 - i) the amino acid sequence of SEQ ID NO:2, or of a variant thereof exhibiting at least 80% sequence identity with the amino acid sequence of SEQ ID NO:2,
 - ii) the amino acid sequence of SEQ ID NO:4, or of a variant thereof exhibiting at least 80% sequence identity with the amino acid sequence of SEQ ID NO:4, and/or

- iii) the amino acid sequence of SEQ ID NO:6, or of a variant thereof exhibiting at least 90% sequence identity with the amino acid sequence of SEQ ID NO:6.
5. The polypeptide according to claim 4, wherein the variant sequence of SEQ ID NO:2 exhibits one or more mutations selected from the group consisting of K16E, K27N, H50Q, T85A, G154C, G154S compared to the amino acid sequence of SEQ ID NO:2, preferably wherein said variant sequence exhibits at least mutations K16E and H50Q compared to the amino acid sequence of SEQ ID NO:2, even more preferably wherein said variant sequence exhibits at least mutations K16E, K27N, H50Q, T85A, and G154S compared to the amino acid sequence of SEQ ID NO:2.
 6. The polypeptide according to any one of the preceding claims, wherein the polypeptide comprises one or more sequences selected from the group consisting of SEQ ID NO:2, SEQ ID NO:4 and SEQ ID NO:6, preferably wherein the polypeptide comprises the amino acid sequence of SEQ ID NO:4, and/or SEQ ID NO:6 even more preferably wherein the polypeptide comprises the amino acid sequence of SEQ ID NO:6.
 7. The polypeptide according to any one of the preceding claims, wherein the polypeptide comprises the amino acid sequence of SEQ ID NO:2, SEQ ID NO:4 and SEQ ID NO:6, preferably wherein the polypeptide comprises the amino acid sequence of SEQ ID NO:8 or SEQ ID NO:9, preferably wherein the polypeptide comprises the amino acid sequence of SEQ ID NO:9.
 8. The polypeptide according to any one of the preceding claims, wherein the order of elements or of their respective variants is from the N- to the C-terminus:
 - a) CHAP domain - M23-endopeptidase - CBD of ALE-1 - peptide, or
 - b) Peptide - CHAP domain - M23-endopeptidase - CBD of ALE-1.
 9. The polypeptide according to any one of the preceding claims, wherein the polypeptide comprises a tag, preferably a His₆-tag.
 10. The polypeptide according to anyone of the preceding claims, wherein the further peptide is:

- i) an antimicrobial peptide selected from the group consisting of the SEQ ID Nos. from SEQ ID NO: 40 to 94,
 - ii) an amphipathic peptide selected from the group consisting of SEQ ID NO: 98, SEQ ID NO: 99 and SEQ ID NO: 100,
 - iii) a cationic peptide selected from the group consisting of the SEQ ID Nos. from SEQ ID NO: 11 to 39,
 - iv) a sushi peptide according to SEQ ID NO: 95, or
 - iii) a hydrophobic peptide selected from the group consisting of SEQ ID NO: 96 and SEQ ID NO: 97.
11. The polypeptide according to claim 10, wherein the further peptide is selected from the group consisting of SEQ ID NO: 38 and SEQ ID NO: 94.
 12. The polypeptide according to anyone of the preceding claims, wherein the polypeptide comprises a sequence selected from the group consisting of SEQ ID NO:105, SEQ ID NO: 107, SEQ ID NO:109 and SEQ ID NO:111 and variant sequences at least 90% identical to any of these four sequences.
 13. The polypeptide according to anyone of the preceding claims, wherein the polypeptide does not exceed 500 amino acids in length.
 14. The polypeptide according to any one of the preceding claims, wherein said polypeptide is capable of degrading the cell wall of *Staphylococcus aureus* bacteria.
 15. Polypeptide comprising a variant sequence of SEQ ID NO:1, wherein said variant sequence comprises at least 80% sequence identity with the amino acid sequence of SEQ ID NO:1, and wherein said variant sequence exhibits mutation H8N and/or T43A compared to the amino acid sequence of SEQ ID NO:1.
 16. Polypeptide comprising a variant sequence of SEQ ID NO:2, wherein said variant sequence comprises at least 80% sequence identity with the amino acid sequence of SEQ ID NO:2, and wherein said variant sequence exhibits one or more mutations selected

- from the group consisting of K16E, K27N, H50Q, T85A, G154C, G154S compared to the amino acid sequence of SEQ ID NO:2.
17. The polypeptide according to claim 16, wherein said variant sequence exhibits at least mutations K16E and H50Q compared to the amino acid sequence of SEQ ID NO:2.
 18. The polypeptide according to claim 16, wherein said variant sequence exhibits at least mutations K16E, K27N, H50Q, T85A, and G154S compared to the amino acid sequence of SEQ ID NO:2.
 19. The polypeptide according to any one of claims 16 to 18, wherein said polypeptide further comprises the sequence of at least one catalytic domain of a peptidoglycan hydrolase and/or exhibits antibacterial activity, in particular antibacterial activity against *S. aureus*.
 20. The polypeptide according to claim 19, wherein said variant sequence exhibits at least 95% sequence identity with the amino acid sequence of SEQ ID NO:109 or SEQ ID NO:111, preferably at least 96%, at least 97%, at least 98% or at least 99% sequence identity with the amino acid sequence of SEQ ID NO:109 or SEQ ID NO:111.
 21. The polypeptide according to claim 19, wherein said polypeptide comprises the sequence of SEQ ID NO: 108 or SEQ ID NO: 110.
 22. A nucleic acid encoding a polypeptide according to any one of claims 1 to 21.
 23. A vector comprising a nucleic acid according claim 22.
 24. A host cell comprising a polypeptide according to any one of claims 1 to 21, a nucleic acid according claim 22, or a vector according claim 23.
 25. A composition comprising a polypeptide according to any one of claims 1 to 21, a nucleic acid according claim 22, a vector according claim 23, and/or a host cell according claim 24, and further comprising a carrier, diluent or excipient.

26. The composition according to claim 25, wherein the carrier, diluent or excipient is pharmaceutically acceptable.
27. The composition according to claim 25, wherein the composition is an aqueous solution (preferably a buffer or a physiological solution), a powder, a suppository, an emulsion, a suspension, a gel, a lotion, a cream, salve, ointment, injectable solution, syrup, spray, inhalant, a coating composition, preferably an implant coating composition, a stent coating composition, or a catheter coating composition, or a biomaterial, preferably bone cement.
28. A device comprising a polypeptide according to any one of claims 1 to 21, a nucleic acid according claim 22, a vector according claim 23, a host cell according claim 24 and/or a composition according to any one of claims 25 to 27.
29. The device of claim 28, wherein the device is a medical device, in particular wherein the device is an implant, a stent, a catheter, a plaster, a compress or a dressing.
30. A polypeptide according to any one of claims 1 to 21, a nucleic acid according claim 22, a vector according claim 23, a host cell according claim 24 and/or a composition according to any one of claims 25 to 27 for use in a method for the treatment of the human or animal body by surgery or therapy or in diagnostic methods practiced on the human or animal body.
31. The polypeptide, nucleic acid, vector, host cell or composition for use according to claim 30, wherein the polypeptide, nucleic acid, vector, host cell or composition is used for the treatment or prevention of bacterial infections in a subject, in particular for the treatment or prevention of bacterial infections with *Staphylococcus aureus* bacteria.
32. The polypeptide, nucleic acid, vector, host cell or composition for use according to claim 30, wherein the polypeptide, nucleic acid, vector, host cell or composition is used for the treatment of wounds of a subject, in particular acute wounds such as iatrogenic wounds, or chronic wounds, or for the treatment of dermatitis or otitis.

33. Use of a polypeptide according to any one of claims 1 to 21, a nucleic acid according claim 22, a vector according claim 23, a host cell according claim 24 and/or a composition according to any one of claims 25 to 27 for disinfecting inanimate surfaces, compositions and/or objects, in particular in the nosocomial environment or in a doctor's office.

34. Use of a polypeptide according to any one of claims 1 to 21, a nucleic acid according claim 22, a vector according claim 23, a host cell according claim 24 and/or a composition according to any one of claims 25 to 27 for preventing contamination of inanimate surfaces, compositions and/or objects with bacteria, in particular for preventing contamination with *Staphylococcus aureus* bacteria.

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2017/079769

A. CLASSIFICATION OF SUBJECT MATTER					
INV.	A61K38/16	A61K38/47	A61K38/48	A61P31/04	C07K7/00
	C07K7/06	C07K7/08	C07K14/47	C12N9/36	C12N9/52
	A61L27/00	A61F2/07	A61F13/00	A61M25/10	A61L15/44
According to International Patent Classification (IPC) or to both national classification and IPC					

B. FIELDS SEARCHED
Minimum documentation searched (classification system followed by classification symbols) A61K A61P C07K C12N A61L A61F A61M

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

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	STEPHEN C. BECKER ET AL: "Triple-acting Lytic Enzyme Treatment of Drug-Resistant and Intracellular Staphylococcus aureus", SCIENTIFIC REPORTS, vol. 6, no. 1, 28 April 2016 (2016-04-28), XP055442167, DOI: 10.1038/srep25063 cited in the application the whole document figure 1	1-14, 22-34
Y	WO 2016/142445 A2 (MICREOS HUMAN HEALTH B V [NL]) 15 September 2016 (2016-09-15) the whole document claims 1-17 pages 29-30; tables 2, 3	1-14, 22-34
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Further documents are listed in the continuation of Box C.

See patent family annex.

* Special categories of cited documents :	
"A" document defining the general state of the art which is not considered to be of particular relevance	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"E" earlier application or patent but published on or after the international filing date	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O" document referring to an oral disclosure, use, exhibition or other means	"&" document member of the same patent family
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search	Date of mailing of the international search report
30 April 2018	07/05/2018

Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Authorized officer Fayos, Cécile
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INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2017/079769

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	<p>BRIERS YVES ET AL: "Breaking barriers: expansion of the use of endolysins as novel antibacterials against Gram-negative bacteria", FUTURE MICROBIO, LONDON : FUTURE MEDICINE LTD, 2006, GB, vol. 10, no. 3, 1 January 2015 (2015-01-01), pages 377-390, XP008180525, ISSN: 1746-0921, DOI: 10.2217/FMB.15.8 the whole document page 383, right-hand column, last paragraph - page 388, left-hand column, last paragraph</p> <p style="text-align: center;">-----</p>	1-14, 22-34
X	<p>US 2016/097044 A1 (DONOVAN DAVID M [US]) 7 April 2016 (2016-04-07) claims 1-35 the whole document page 5 - paragraph [0045] page 7 - paragraph [0058] examples 2-11</p> <p style="text-align: center;">-----</p>	1-14, 22-34
Y	<p>WO 2015/121443 A1 (LYSANDO AG [LI]) 20 August 2015 (2015-08-20) the whole document pages 26-27; table 4 page 24 - page 25; table 3</p> <p style="text-align: center;">-----</p>	1-14, 22-34
Y	<p>EP 2 338 916 A1 (HYGLOS INVEST GMBH [DE]) 29 June 2011 (2011-06-29) the whole document claims 1-30</p> <p style="text-align: center;">-----</p>	1-14, 22-34
Y	<p>EP 2 468 856 A1 (LYSANDO AG [LI]; UNIV LEUVEN KATH [BE]) 27 June 2012 (2012-06-27) the whole document</p> <p style="text-align: center;">-----</p>	1-14, 22-34

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2017/079769

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

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

2. Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.

3. As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-14(completely); 22-34(partially)

Remark on Protest

- The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

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

1. claims: 1-14(completely); 22-34(partially)

A polypeptide comprising:

i) the CHAP domain of LysK endolysin or a variant thereof exhibiting at least 80% sequence identity with the CHAP domain of LysK endolysin;

ii) the M23 endopeptidase domain of lysostaphin or a variant thereof exhibiting at least 80% sequence identity with the M23 endopeptidase domain of lysostaphin;

iii) the cell wall binding domain (CBD) of ALE-1 or a variant thereof exhibiting at least 90% sequence identity with the cell wall binding domain (CBD) of ALE-1, and

iv) a further peptide selected from the group consisting of an antimicrobial peptide, an amphipathic peptide, a cationic peptide, a hydrophobic peptide, a sushi peptide and a defensin; the corresponding nucleic acid, vector, host cell and composition, the corresponding therapeutic or diagnostic use, methods for disinfecting inanimate surfaces and a device comprising the above defined polypeptides/compositions.

2. claims: 15(completely); 22-34(partially)

A polypeptide comprising a variant sequence of SEQ ID NO:1, wherein said variant sequence comprises at least 80% sequence identity with the amino acid sequence of SEQ ID NO:1, and wherein said variant sequence exhibits mutation H8N and/or T43A compared to the amino acid sequence of SEQ ID NO: 1; the corresponding nucleic acid, vector, host cell and composition, the corresponding therapeutic or diagnostic use, methods for disinfecting inanimate surfaces and a device comprising the above defined polypeptides/compositions.

3. claims: 16-21(completely); 22-34(partially)

A polypeptide comprising a variant sequence of SEQ ID NO:2, wherein said variant sequence comprises at least 80% sequence identity with the amino acid sequence of SEQ ID NO:2, and wherein said variant sequence exhibits one or more mutations selected from the group consisting of K16E, K27N, H50Q, T85A, G154C, G154S compared to the amino acid sequence of SEQ ID NO:2; the corresponding nucleic acid, vector, host cell and composition, the corresponding therapeutic or diagnostic use, methods for disinfecting inanimate surfaces and a device comprising the above defined polypeptides/compositions.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2017/079769

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