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Description

[0001] The present invention relates to a polypeptide variant of the polypeptide termed ply_{pitti26} comprising the sequence as depicted in SEQ ID NO:1. Furthermore, the present invention relates to nucleic acids and vectors encoding for variants of said polypeptide as well as host cells comprising these nucleic acids and/or vectors. Finally, the present invention relates to the uses of variants of said polypeptide, nucleic acid sequences, vectors and host cells, in particular for the treatment or prophylaxis of a subject infected by or exposed to *Staphylococci*.

Background of the Invention

[0002] Staphylococcal infections are a major cause of severe diseases with high mortality all over the world. The gram-positive pathogen *Staphylococcus aureus* is responsible for a variety of infections of the skin and soft tissues as well as life-threatening infections like bacteremia and endocarditis. In addition, *Staphylococcus aureus* is frequently involved in food poisoning. Due to its tolerance to low pH values and high salt conditions this pathogen grows in a variety of food products, especially of animal origin, producing a heat stable enterotoxin. Persons with a particular risk of infection are patients after surgery or during hemodialysis as well as premature infants and immunocompromised persons, or those with need for prosthetic devices. Staphylococcal infections are of particular global health concern because of their high distribution (about 25 - 30 % of the population are asymptomatic carriers) and of the increasing emergence of antibiotic resistant strains of *Staphylococcus aureus*. MRSA (methicillin resistant *Staphylococcus aureus*) is a prominent member of this group and a major cause of nosocomial infections. In addition, there are many multiresistant strains, even those which are resistant to the "drugs of the last line of defence" like vancomycin, linezolid or daptomycin. Infections with antibiotic resistant staphylococci rise enormous costs to the global health budgets, because the patients often need long-term stay in a hospital and have to be isolated from other patients.

[0003] Besides the coagulase positive *S. aureus*, pathogens from the group of the coagulase negative staphylococci are of importance. *S. haemolyticus*, for example, causes keratitis, *S. epidermidis* is frequently found in biofilms on implanted devices which are associated with serious infections (endoplasitis) and *S. saprophyticus* is responsible for urinary tract infections. Apart from infections of humans, cattle infections also play an important role. Especially, bovine mastitis, an infection of the mammary glands, is of commercial significance. Apart from *S. aureus* it is caused by some coagulase negative staphylococci like *S. epidermidis*, *S. simulans*, *S. chromogenes*, *S. hyicus*, *S. warneri* and *S. xylosus*.

[0004] Standard antibiotic therapy is becoming more and more ineffective. Hence, new strategies for treating bacterial infections are needed. They include the development of new antibiotics as well as the search for antimicrobial peptides. Uses of antibodies and putative vaccines or phage therapy are alternative approaches. However all of these methods exhibit serious disadvantages. At widespread use, novel antibiotics also will rise new resistances, antimicrobial peptides and monoclonal antibodies require a lot of additional investments until a routine use in therapy will be possible; immunization strategies against *Staphylococcus aureus* were not successful so far and phage therapy causes problems with immune response and tissue penetration as well as with a potential undesired transfer of bacterial toxins by the phages. The use of isolated peptidoglycan hydrolases, the so-called endolysins, represents an advancement of the phage therapy. Endolysins enzymatically hydrolyse the cell walls of those bacteria which are host organisms for their corresponding bacteriophages.

Description of the Relevant Art

[0005] After infection of the host bacterium, bacteriophages produce new phage particles within the host cell. At the end of the reproduction cycle the host cell must be lysed, to set free the new phage generation. Endolysins are produced as a tool for this lysis of the host cell. It was found, that endolysins also act on bacterial cell walls when they are added exogenously to non infected bacterial cells ("lysis from without"). The use of endolysins to kill contaminating bacteria in food was first disclosed by Gasson in 1991 (GB 2,255,561). First therapeutic and prophylactic applications *in vivo* using mouse model systems were described in 2001 by the group of Fischetti (Nelson & Fischetti, 2001, Proc. Natl. Acad. Sci. USA, 98, 4107-4112; Loeffler et al., 2001, Science, 94, 2170-2172). This work describes the topical application of endolysins against group A streptococci (oral application) and against pneumococci (nasopharyngeal application). Later, an application against *Bacillus anthracis* was added (Schuch et al., 2002, Nature 418, 884-889). Entenza et al. (2005, Antimicrob. Agents Chemother., 49, 4789-4792) report the use of Cpl-1 lysin against pneumococci causing endocarditis in rats. Endolysin PlyGBS was used to kill group B streptococci in the vagina and oropharynx of a mouse model (Cheng et al., 2005, Antimicrob. Agents Chemother., 49, 111-117). Fischetti (2006, BMC Oral Health, 6, 16-19) summarizes the use of phage lytic enzymes to control pathogenic bacteria.

[0006] In US 5,997,862 a multitude of methods of treatments and pharmaceutical compositions to treat and prevent bacterial infections using phage derived lysins is disclosed. Several further patents teach specific compositions and uses of phage derived lysins for treatment of e.g. dermatological infections, ocular infections, infections of mouth and

teeth, infections of the respiratory tract, various illnesses, bacterial infections in general, the parenteral use of lysin compositions, and the use of bandage compositions. US 2007-077235 describes lysin compositions to treat mastitis in animals.

[0007] Endolysins may be divided into five classes: (1) N-acetylmuramidases (lysozymes), (2) endo- β -N-acetylglucosaminidases, and (3) lytic transglycosylases, which all cleave the sugar moiety of peptidoglycan, (4) endopeptidases, which cleave the peptide moiety, and (5) N-acetylmuramoyl-L-alanine amidases, which cut the amide bond between sugar backbone and peptide linkers. Endolysins show a modular organization exhibiting a combination of different polypeptide domains showing enzymatic activity or cell binding activity, the so-called EADs (enzymatically active domains) and CBDs (cell binding domains), respectively. Mostly, EADs are located at the N-terminal part of the endolysins, and CBDs at the C-terminal parts, but there are also exceptions of this rule of thumb. It is also shown that modules can be exchanged between different cell wall lytic enzymes producing new functional enzymes, which sometimes exhibit even new functional properties (Diaz et al., 1990, Proc. Natl. Acad. Sci. USA, 87, 8125-8129; Croux et al., 1993, Molecular Microbiology, 9, 1019-1025; Donovan et al., 2006, Appl. & Environm. Microbiol., 72, 2988-2996).

[0008] Since endolysins are typically more specific than antibiotics, it is unlikely that resistance development will rapidly occur. Therefore, the use of suitable endolysins acting on *staphylococcus* bacteria is a desirable means for the fight against the respective infections. Several endolysins active against *staphylococcus* bacteria are already described in the relevant art. Protein 17 associated with phage P68 is a staphylococcal endolysin which exhibits antimicrobial activity also against clinical *S. aureus* isolates (Takac et al., 2005, Antimicrobial Agents and Chemotherapy, 49, 2934-2940). The endolysin plyTW derived from the *S. aureus* phage Twort needs only the N-terminal enzymatically active fragment for hydrolytic activity against bacterial cells, whereas the C-terminal part with homology to lysostaphin seems dispensable (Loessner et al., 1998, FEMS Microbiol. Lett, 162, 265-274). Donovan et al. (2006, Appl. & Environm. Microbiol., 72, 2988-2996) created a chimaeric endolysin between *Streptococcus agalactiae* B30 endolysin and lysostaphin of *Staphylococcus simulans* with potential use in treatment of mastitis. Several groups used the endolysin of *Staphylococcus aureus* bacteriophage phi11 in antimicrobial applications. Navarre et al (1999, J. Biol. Chem., 274, 15847-15856) identified multiple enzymatic activities in phi11 endolysin, and showed that a mutant with deletion of the amidase domain is still active. Donovan et al. (2006, FEMS Microbiol. Lett, 265, 133-139) used complete phi11 endolysin as well as C-terminally truncated versions in assays against mastitis pathogens. Different mutants of phi11 endolysin and phi12 endolysin were tested in different activity assays on *Staphylococcus aureus* cell walls, heat inactivated cells and also bacterial biofilms (Sass & Bierbaum, 2007, Appl. & Environment. Microbiol., 73, 347-352). The endolysin of the *Staphylococcus warneri* phage Φ WMY, LysWMY, although reported to be closely related to phi11 endolysin, retained full activity when the amidase as well as the cell binding domains were deleted (Yokoi et al., 2005, Gene 351, 97-108). This result indicates that the functions of and interactions between the different endolysin modules are not equivalent even in closely related endolysins.

[0009] Although different endolysins against staphylococcus bacteria are known from the art, there is still a need for efficient staphylococcal endolysins that can be produced in an efficient way and in addition show high activity against microorganisms of the genus *Staphylococcus*.

[0010] The problem is solved by the subject matter as disclosed in the claims.

Brief Description of the Drawings

[0011] To illustrate certain aspects of the invention, the following drawings are presented. They are not intended to restrict the invention in any way.

Fig. 1 provides a schematic representation of the modular organization of different *Staphylococcus* endolysins.

The endolysins are built up from CHAP (cysteine, histidine-dependent amidohydrolases/peptidases), and amidase (N-acetyl-muramyl-L-alanine amidase, ami) enzymatic domains, and SH3-modules as cell binding domains (CBDs).

Fig. 2 Agar plate showing lysis zones in an activity test on heat inactivated staphylococcus cells. Lysis plates include the respective *staphylococcus* strain to be tested in a top agar layer. Either *E. coli* strains harbouring plasmids of the endolysin constructs to be tested or preparations of isolated endolysins are stippled to the top of the plates. Lysis zones appear after incubation depending on the lysing activity on the respective host bacterium. Number 1 depicts an example, where no lysis occurred (-). Numbers 2, 3, and 4 mark weak (+), medium (++) or strong (+++) lytic activity of the respective endolysin variant. The agar plate in the figure exemplarily shows an assay, where the activity of several point mutations in EADplypiti26_CBDplyUSA were tested.

Fig. 3 Comparison of different artificial staphylococcus endolysin constructs

Artificial endolysin constructs according to the present invention are shown in this figure with respect to expression and solubility (fig. 3A), and activity in the turbidity assay (fig. 3B). Construct 3 is a synonym for

EADplypiti26_CBDplyUSA, construct 5 is a synonym for EADplypiti26_CBDplypiti20, construct 9 and 5 are further artificial endolysins. The lanes on the SDS-gel are marked "P" for unsoluble pellet fraction and "S" for soluble supernatant fraction. A molecular mass standard is indicated in the margin. The position of the full-length endolysin constructs is marked by an arrow. The expression and solubility test at 37°C is performed as described in example 2, the activity test is done like in example 5, but with a raw cell extract after expression of the proteins.

Fig. 4 Comparison of solubility after expression

Depicted are solubility tests performed after expression of endolysin constructs at 30°C (described in example 2). Fig. 4A shows an SDS-gel with the solubility test of wild-type plypiti26, fig. 4B a respective test with EADplypiti26_CBDplyUSA. "P" denotes the insoluble "pellet fraction" whereas "S" denotes the soluble protein fraction found in the supernatant. The bands of the endolysins are marked with asterisks, the arrow in fig. 4A marks an *E. coli* protein which migrates only somewhat slower than plypiti26. The molecular mass marker lane is marked by an "M".

Fig. 5 Comparison between lysis activity of EADplypiti26_CBDplyUSA and wild-type plypiti26 on untreated cells
Concentration dependent lysis profiles were recorded on *Staphylococcus aureus* cells after addition of purified plypiti26 (numbers 1, 2, 3) or EADplypiti26_CBDplyUSA (numbers 4, 5, 6). The assay was performed as described in example 5. 2 µg, 5 µg or 10 µg each of isolated endolysin proteins were added to a bacterial cell suspension (addition indicated by the arrow, increasing protein concentration with increasing numbering of the traces) and decrease of the sample turbidity recorded until complete lysis.

Fig. 6 Lysis activity in human blood serum

Fig. 6 shows lysis of *Staphylococcus aureus* cells by EADplypiti26_CBDplyUSA measured with the turbidity assay performed in human blood serum. The decrease in optical density (abs) is measured against assay time (s). EADplypiti26_CBDplyUSA at concentrations of 25 µg/ml (-----), 50 µg/ml (-----) or 100 µg/ml (-----) was applied at time point zero. The solid line represents a control without addition of endolysin. The assay is described in example 6.

Fig. 7 Stability of EADplypiti26_CBDplyUSA in comparison to wild-type piti26

The figure shows pictures of SDS-gels depicting endolysin preparations after incubation at 25°C in storage buffer (see example 6). In figure 7A, EADplypiti26_CBDplyUSA is applied onto the gel, figure 7B shows plypiti26 protein. The first lane represents a molecular standard with the time of incubation at 25°C in hours. The position of the full-length endolysin is marked by an arrow.

Fig. 8 Stability against thrombin

Figure 8A represents a picture of SDS-gels showing EADplypiti26_CBDplyUSA (lanes 3, 4) and wild-type plypiti26 (lanes 1, 2) before and after digestion with thrombin. The first lane (M) is a molecular mass standard. Lanes 1 and 3 are controls (without addition of thrombin), and lanes 2 and 4 show the protein samples after addition of thrombin. The position of the full-length endolysins is marked by an arrow. Figure 8B depicts an activity assay using turbidity measurements before and after digestion with thrombin. On the left plypiti26 is shown, on the right EADplypiti26_CBDplyUSA. Solid lines represent the lytic activity without addition of thrombin, dotted lines the residual activity after thrombin digestion. The experiment is described in example 8.

Fig. 9 Stability in human blood

Fig. 9 shows the stability of EADplypiti26_CBDplyUSA after preincubation in human blood at 37°C. The activity level achieved without preincubation is set to 100 %. (●) marks the activity after preincubation in buffer, and (▲) marks the respective preincubation in human EDTA-blood. The assays are described in example 9.

Fig. 10 A-C

Fig. 10A shows the amino acid sequence of a modified EADplypiti26_CBDplyUSA - EADplypiti26_CBDplyUSA-Add2_M5. Figs. 10B and C show nucleic acid sequences encoding the modified EADplypiti26_CBDplyUSA - EADplypiti26_CBDplyUSA-Add2_M5.

Fig. 11 Lysis activity in buffer

Fig. 11 shows a comparison of the lysis activity of different endolysin constructs against *Staphylococcus aureus* cells. Measured is the decrease in absorbance at 600 nm achieved per minute and mg protein ($\Delta A_{600}/\text{min mg}$) after addition of protein at concentrations of 1 µg/ml (left panel) or 10 µg/ml. The protein constructs used are from left to right plypiti26 (chequered), EADplypiti26_CBDplyUSA (horizontal lines), EADplypiti26_CBDplyUSA-Add2_M5

(grey), EADplypitti26_CBDplyUSA-Add2_M8 (white), EADplypitti26_CBDLS-Add2_M5 (vertical lines), EADplypitti26_CBDALE1-Add2_M5 (black), lysostaphin (chequerboard).

Fig. 12 Lysis activity in human serum

Fig. 12 shows a comparison of the lysis activity of different endolysin constructs against *Staphylococcus aureus* cells. Measured is the decrease in absorbance at 600 nm achieved per minute and mg protein ($\Delta A_{600}/\text{min mg}$) after addition of protein at concentrations of 10 $\mu\text{g/ml}$ (left panel) or 25 $\mu\text{g/ml}$. The protein constructs used are from left to right EADplypitti26_CBDplyUSA (horizontal lines), EADplypitti26_CBDplyUSA-Add2_M5 (grey), EADplypitti26_CBDplyUSA-Add2_M8 (white), EADplypitti26_CBDLS-Add2_M5 (vertical lines), EADplypitti26_CBDALE1-Add2_M5 (black).

Fig. 13 Thermal stability of *Staphylococcal* endolysins

Fig. 13 shows a representative curve measured during thermal denaturation of *Staphylococcal* endolysins. The absorption at a wavelength of 360 nm (A_{360}) is measured at increasing temperatures (T , °C). Fig. 13 shows the turbidity signal measured using EADplypitti26_CBDALE1-Add2_M5 at a concentration of 0.1 mg/ml. The arrow marks the temperature where the A_{360} signal increases significantly as aggregation of the protein begins. This temperature is defined as the aggregation temperature T_{agg} .

Fig. 14 Pharmacokinetic study for EADplypitti26_CBDplyUSA-Add2_M5

Fig. 14A shows the residual activity of the endolysin variant EADplypitti26_CBDplyUSA-Add2_M5 in rat serum measured with a turbidity assay in microliter plate format at different time points after IV injection of the protein. The activity values ($\Delta A_{620}/\text{min}$) shown represent the mean value $\pm$ standard deviation measured in samples taken from 3 rats each at the time points after injection.

Fig. 14B shows the endolysin activity of the variant EADplypitti26_CBDplyUSA-Add2_M5 in rat serum taken 5 min after IV injection of the protein (black bar) in comparison to a control using 150 $\mu\text{g/ml}$ protein in pre-immune serum (grey bar) or 150 $\mu\text{g/ml}$ protein in formulation buffer.

Detailed Description of the Invention

[0012] The term "endolysin" or "peptidoglycan hydrolase" as used herein refers to an enzyme which is suitable to hydrolyse bacterial cell walls. The enzyme comprises at least one of the following activities of which the "enzymatically active domains" (EADs) of the endolysins are constituted: endopeptidase, carboxypeptidase, N-acetyl-muramoyl-L-alanine-amidase (amidase), N-acetyl-muramidase (lysozyme or lytic transglycosylase) or N-acetyl-glucosaminidase. Either, the enzyme is phage or prophage encoded or it is derived from related enzymes coded by bacteria, the so-called "autolysins". In addition, the endolysins usually 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 endolysin refers also to lysostaphin and ALE-1 which contain an endopeptidase domain and a CBD.

[0013] The term "module" as used herein refers to a subunit of an endolysin which is ascribed a specific function. Generally, a module is a relatively small functional unit like CHAP-, ami- or SH3-modules.

[0014] The term "domain" as used herein refers to a subunit of an endolysin which is ascribed a specific function and can also coincide with structural domains. The term domain is preferentially used to describe the antagonism between EAD which can be composed of more than one module and CBD domains.

[0015] The term "CBD" as used herein refers to the cell wall binding domain or cell wall targeting domain of an endolysin, which is often found at the C-terminus of the protein. CBD domains have no enzymatic activity in terms of hydrolyzing the cell wall, but often mediate binding of the endolysin to the bacterial cell wall. CBD may contain an SH3-domain.

[0016] The term "EAD" as used herein refers to the enzymatically active domain of an endolysin which is responsible for hydrolysis of the bacterial peptidoglycan. It contains at least one of the enzymatic activities of an endolysin. The EAD can also be composed of more than one enzymatically active module.

[0017] A "CHAP" domain (cysteine, histidine-dependent amidohydrolases/peptidases) is a region between 110 and 140 amino acids that is found in proteins from bacteria, bacteriophages, archaea and eukaryotes of the Trypanosomidae family. The proteins may function mainly in peptidoglycan hydrolysis. The CHAP domain is commonly associated with bacterial type SH3 domains and with several families of amidase domains. CHAP domain containing proteins may utilize a catalytic cysteine residue in a nucleophilic-attack mechanism. The CHAP domain contains two invariant amino acid residues, a cysteine and a histidine. These residues form part of the putative active site of CHAP domain containing proteins.

[0018] The term "ami" as used herein describes an enzymatically defined module which exhibits amidase activity, i.e. it hydrolyzes the amide bond between N-acetylmuramine in the peptidoglycan backbone and the adjacent amino acid which is usually L-ala in the peptide linker. The amidase are often metal ion dependent for activity.

[0019] The term "peptidase_M23" as used herein refers to a zinc-dependent metallopeptidase domain which cleaves glycyl-glycyl peptide bonds as an endopeptidase. Peptidase_M23 domains are found for example in lysostaphin and ALE-1.

[0020] The term "SH3" domain which is sometimes also called Src homology 3 domain as used herein describes a small non-catalytic protein domain of about 60 amino acids which is characteristic for proteins which interact with other binding partners. It is identified via a proline-rich consensus motif. The SH3 domain is usually located within the CBD. SH3 domains found in peptidoglycan hydrolases are often of the SH3b or SH3_5 type.

[0021] The term "shuffling" as used herein refers to the combination of different fragments of polypeptides from different enzymes into new chimeric polypeptide constructs. In this context, the enzymes are preferentially endolysins, and the fragments are preferentially modules. Usually, the fragments are combined by molecular biological methods on nucleic acid level. Small linker sequences may be introduced between the fragments for structural or cloning reasons.

[0022] The term "wild-type" as used herein refers to a naturally occurring amino acid sequence of a protein or polypeptide or to a nucleotide sequence of a nucleic acid molecule encoding said protein or polypeptide.

[0023] The term "variant" as used herein refers to a modified form of a naturally occurring protein. Variants are generated by shuffling of polypeptides or mutations of polypeptides or addition of a tag or a marker or a combination of the different possibilities. Suitable mutations to generate a variant are deletions of amino acids, additions of amino acids or substitutions (amino acid exchanges, point mutations). The number of amino acids to be deleted, added or exchanged varies from 1 to several hundred. The respective modifications of the nucleic acids coding for the protein variants are determined by the genetic code.

[0024] The inventors isolated several lytic bacteriophages from sewage samples which were active against *Staphylococci* using standard techniques for bacteriophage isolation (Adams, 1959, In: Bacteriophages, Interscience Pub., New York, p. 447-451). Several lysogenic phages were identified using molecular biological techniques within *Staphylococcus aureus* strains isolated from epidemic strains.

[0025] One isolated phage was named pitti26. Within the phage genomes of the isolated lysogenic phages endolysin proteins were identified and isolated. One preferred endolysin was isolated from the lytic phage pitti26 and was named ply_pitti26 (SEQ ID NO:1). A further endolysin was identified within the lytic phage pitti20 and named ply_pitti20. A prophage derived endolysin, plyUSA, was identified in the genome of the prophage Φ SA2usa which was integrated into the genome of the methicillin-resistant *Staphylococcus aureus* strain USA300 (Diep et al., The Lancet, 2006, 367, 731-739; databank entry NC_007793).

[0026] Endolysins of bacteriophages specific to *Staphylococcus* bacteria are typically composed of two enzymatically active domains, namely a CHAP domain, and an amidase domain (ami), and a cell binding domain (CBD) which often is defined as an SH3-domain of the SH3b or SH3_5 type.

[0027] Lysostaphin and ALE-1, a homologue of lysostaphin, are peptidoglycan hydrolases that specifically lyse *Staphylococcus aureus* cell walls as both proteins comprise an endopeptidase domain (peptidase_M23) which cleaves the pentaglycine linkages between peptidoglycan chains, and a C-terminal SH3b domain which functions as a CBD or targeting domain. Lysostaphin is secreted by *Staphylococcus simulans* biovar *staphylolyticus*, and ALE-1 by *Staphylococcus capitis* EPK1. Lysostaphin is produced as a precursor protein which upon maturation loses tandem repeats at the N-terminus. ALE-1 comprises an N-terminal repeat domain which is not processed post-translationally. The cell wall targeting domain of ALE-1 was determined to consist of the 92 C-terminal amino acids (residues 271 to 362) (Lu et al., 2006, J. Biol. Chem., 281, 549-558). This domain is very similar to the C-terminal SH3b domain of lysostaphin (82 % identity).

[0028] The inventors of the present invention combined the CHAP and the amidase domain of the endolysin plypitti26 with the cell binding domain of the prophage endolysin plyUSA, with the cell binding domain of the endolysin plypitti20, the cell wall targeting domain of lysostaphin, and the cell wall targeting domain of ALE-1 to generate chimeric endolysins having new properties for use of the endolysin variants as a therapeutic or prophylactic agent against staphylococcal infections, as an antimicrobial agent for disinfection or sanitation against *Staphylococcus* or as a means in *Staphylococcus* diagnostics. New properties are e. g. a higher activity of the chimeric endolysins in buffer systems, in therapeutically relevant solutions like blood or serum, an increased host range of recognition within the genus *Staphylococcus* or the species *Staphylococcus aureus*, preferably the MRSA strains, a higher solubility of the chimeric endolysins preferably after expression, and a higher protein stability preferably thermostability, long term stability or stability against proteases. The isolated endolysin plypitti26 as well as the chimeric enzymes can be used to lyse bacteria of the genus *Staphylococcus*.

[0029] Described is a polypeptide termed ply_pitti26 comprising the sequence as depicted in SEQ ID NO: 1.

[0030] The present invention relates a polypeptide variant comprising the sequence as depicted in SEQ ID NO:1, wherein the polypeptide variant comprising the sequence as depicted in SEQ ID NO:1 comprises an endolysin cell binding domain (CBD) selected from the endolysin CBD of ply_USA as depicted in SEQ ID NO:3 or of ply_pitti20 as depicted in SEQ ID NO:5; or wherein the polypeptide variant comprising the sequence as depicted in SEQ ID NO: 20 or 22; the polypeptide variant further optionally comprising:

a) a polypeptide variant comprising the sequence of SEQ ID NO:1 with one or more point mutations or amino acid substitutions in said sequence,

(i) wherein the substituted residues are selected from the following amino acid residues of SEQ ID NO:1: F19, W22, W36, F42, Y44, L55, L56, F67, L74, Y78, W107, Y115, I116, Y119, W123, W128, W137, W139, W154, E163, R167, E179, E189, Y200, Y275, Y276, C282, F300 and C303, or

(ii) wherein with regard to the sequence of SEQ ID NO:1 the one or more substitutions are selected from the group consisting of: W22R, F42A, F44A F67T, Y115S, W123M, W137A, W139A, W154H, E163A, E179Q, E179A, E187Q, Y200A, Y200H, Y275A, Y275M, Y276A, C282A, F300A, C303S, W310A and W310M, or

(iii) wherein one of the following substitutions with regard to SEQ ID NO:1 are selected from the group consisting of: F67T + Y115S, F67T + W137A, F67T + W139A, F67T + W154H, Y115S + W137A, Y115S + W139A, E163Q + R169A, E163A + R169A, E163Q + R167A + E189Q, E163A + R167A + E189Q, E163Q + R167A + E179Q + E189Q, E163Q + R167A + E179A + E189Q, E163A + R167A + E179Q + E189Q, E163A + R167A + E179A + E189Q, Y200A + Y275A, Y200A + Y276A, Y200A + C282A, Y200A + F300A, Y275A + Y276A, L55H + L56T + E163A + R167A + Y200H, E163A + R167A + E179A + E189Q + Y200H, L55H + L56T + E163A + R167A + E179A + E189Q + Y200H, S237L + R354Q + A367V, and L55H + L56T + E163A + R167A + Y200H + S237L + R354Q + A367V, or

b) a polypeptide variant comprising in addition to the sequence of SEQ ID NO: 1 sequences representing marker moieties, tags or other functional polypeptide sequences, or

c) a polypeptide variant comprising one or more additional amino acid residues inserted into the amino acid sequence according to SEQ ID NO:1 selected from the group consisting of SEQ ID NO: 15, 18, and 28, or

d) a polypeptide variant comprising a polypeptide sequence representing any combination of variants a), b), and c).

[0031] As mentioned above, the present invention relates to variants of a polypeptide comprising the sequence of SEQ ID NO: 1. Variants of said polypeptide are considered to be the polypeptides according to the present invention. All of them share the common feature to function as an endolysin capable to lyse *Staphylococcus* bacteria. Said specificity for *Staphylococci* can be tested by a plurality of methods which are known in the art or described as set forth below, e.g. by adding the recombinant endolysin variant to a sample comprising one or more of said *Staphylococcus* species and determining the change in turbidity after addition of the (recombinant) endolysin.

[0032] Also described is the variant of ply_pitti26 which is a polypeptide wherein the CBD domain of the ply_pitti26 has been replaced by the CBD of another endolysin. In principle there is no restraint with regard to choosing other CBD domains from anti-staphylococcal endolysins as long as the resulting (recombinant) endolysin retains a specificity for *Staphylococcus* bacteria, in particular for *S. aureus*, *S. aureus* (MRSA), *S. epidermidis*, *S. haemolyticus*, *S. simulans*, *S. saprophyticus*, *S. chromogenes*, *S. hyicus*, *S. warneri* and/or *S. xylosus*. Also described is a recombinant endolysin which comprises an endolysin cell binding domain of the SH3 type. Preferably, the CBD domains are selected from the endolysin CBD domains of ply_USA or ply_pitti20 or lysostaphin or ALE-1. As CBD of ply_USA in particular the sequence as denoted in SEQ ID NO: 3 is preferred. As CBD of ply_pitti20 in particular the sequence as denoted in SEQ ID NO: 5 is preferred. Examples for such recombinant endolysin variants of ply_pitti26 with exchanged CBD are given in SEQ ID NOs: 7 and 11.

[0033] In a further preferred embodiment the variant of ply_pitti26 comprises the sequence of ply_pitti 26 except for a certain number of N-terminal residues. The inventors of the present invention discovered, that if more than 28 N-terminal amino acid residues are removed from SEQ ID NO:1 then the endolysin activity is lost. Therefore, suitable variants of ply_pitti26 comprise in principle the sequence of SEQ ID NO:1 but lack 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27 or 28 N-terminal amino acid residues of SEQ ID NO:1. Particularly preferred are variants which lack 4, 9 or 28 of the N-terminal amino acid residues of ply_pitti26.

[0034] In a preferred embodiment, the variants of the polypeptide termed ply_pitti26 exhibit single or multiple substitutions with regard to SEQ ID NO: 1. In particular, the sites to be substituted for other amino acid residues are F19, W22, W36, F42, Y44, L55, L56, F67, L74, Y78, W107, Y115, I116, Y119, W123, W128, W137, W139, W154, E163, R167, E179, E189, Y200, Y275, Y276, C282, F300, C303 and/or W310. All positions are indicated with regard to SEQ ID NO: 1.

[0035] In an even more preferred embodiment hydrophobic amino acid residues such as F, W, Y, I, and L, especially the ones supposed to lie on the surface of the protein are exchanged against less hydrophobic amino acids such as R, D, E, N, K, Q, H, S, T, M, G, A, wherein A is particularly preferred. As hydrophobic proteins tend to aggregate said substitutions increase the solubility of the protein. The potential surface exposure of amino acid residues can be predicted from a high resolution structure of the protein or from a model, if the structure of homologous proteins or moduls is known. Charged amino acids such as E, D and R, K are preferentially exchanged against uncharged amino acids (e.g. Q or A for E, N or A for D, A for R or K) as charged amino acids are often recognition sites for proteases. Cysteines are preferentially exchanged against A or S as cyteines tend to build disulfide bridges under oxidising conditions

which is potentially deleterious for enzyme structure and function.

[0036] Particularly preferred are variations in the sequence of SEQ ID NO: 1 selected from the following group of mutations. All substitutions are given with regard to the position in SEQ ID NO:1: W22R, F42A, Y44A L55H, L56T, F67T, Y115S, W123M, W137A, W139A, W154H, E163Q, E163A, R167A, E179Q, E179A, E187Q, E189Q, Y200A, Y200H, Y275A, Y275M, Y276A, C282A, F300A, C303S, W310A and/or W310M. Said substitutions may be single mutants of SEQ ID NO: 1 or may be a combination of two or more of said substitutions. Preferred multiple mutants of SEQ ID NO:1 are selected from the group comprising the following multiple mutants: L55H + L56T, F67T + Y115S, F67T + W137A, F67T + W139A, F67T + W154H, Y115S + W137A, Y115S + W139A, E163Q + R167A, E163A + R167A, E163Q + R169A, E163A + R169A, E163Q + R167A + E189Q, E163A + R167A + E189Q, E163Q + R167A + Y200H, E163A + R167A + Y200H, E163Q + R167A + E179Q + E189Q, E163Q + R167A + E179A + E189Q, E163A + R167A + E179Q + E189Q, E163A + R167A + E179A + E189Q, Y200A + Y275A, Y200A + Y276A, Y200A + C282A, Y200A + F300A, Y275A + Y276A, Y275A + F300A, C282A + F300A, Y200A + Y275A + Y276A, Y275A + Y276A + F300A, L55H + L56T + E163Q + R167A + Y200H, L55H + L56T + E163A + R167A + Y200H, E163Q + R167A + E179A + E189Q + Y200H, E163A + R167A + E179A + E189Q + Y200H, L55H + L56T + E163Q + R167A + E179A + E189Q + Y200H, L55H + L56T + E163A + R167A + E179A + E189Q + Y200H, and S237L + R354Q + A367V, L55H + L56T + E163A + R167A + Y200H + S237L + R354Q + A367V and L55H + L56T + E163Q + R167A + Y200H + S237L + R354Q + A367V.

[0037] In a further preferred embodiment the variant of ply_pitti26 comprises additional marker moieties such as biotin or streptavidin or tags such as HA-tag (amino acid sequence EQKLISEEDL), His-tag (Nieba et al., 1997, Anal. Biochem., 252, 217-228), Strep-tag (Voss & Skerra, 1997, Protein Eng., 10, 975-982), Myc-tag (Evan et al., Mol&Cell Biol, 5, 3610-3616), GST-tag (Peng et al. 1993, Protein Expr. Purif., 412, 95-100), JS-tag (WO 2008/077397), cysteine tag (EP1399551) or other tags known in the art. Preferred other functional polypeptide sequences are for example protease cleavage sites. In some embodiments the above mentioned variants of ply_pitti26 may serve for facilitating the biotechnological production of ply_pitti26 or said variants.

[0038] In a further preferred embodiment the variant of ply_pitti26 comprises one or more additional amino acid residues. Preferably, the polypeptide according to the present invention comprises one, two, three or four additional amino acid residues, wherein the residues may be contiguous residues or they may be inserted into independent positions. Preferred is the addition of one or two, more preferred of one amino acid residue. Most preferred is the addition of one amino acid residue after position one, preferably is the addition of the amino acid residue A. If one or more amino acid residues have been inserted into ply_pitti26 the position set forth above being preferred positions for amino acid substitutions have to be adjusted to the number and position of inserted amino acid residues. Exemplary, if an amino acid residue is inserted in position two the position F19 will be F20, W22 will be W23 and so on. If more than one amino acid residue is inserted the new positions have to be adjusted accordingly.

[0039] It has to be understood that the above illustrated variants of ply_pitti26 must not be considered as strictly separate embodiments but instead may be combined. For example, a variant of ply_pitti26 according to the present invention may comprise one or more of the variants selected from the group of a N-terminal truncation of SEQ ID NO:1, one or more substitutions as indicated above, the CBD domain of another endolysin, one or more amino acid additions as well as for example a His-tag with the proviso that such recombinant endolysin is still specific for staphylococci. The person skilled in the art will readily realize which of said variants is suitable for his purposes and can always test such variants for activity against staphylococci by routine methods in the art, for example by assaying the effect of lysis activity on the optical density of a staphylococci containing solution. Illustrative examples for such combinatorial variants are given in SEQ ID NO: 9 (ply_pitti26 with CBD of plyUSA and additional C-terminal His-tag) and SEQ ID NO: 13 (ply_pitti26 with CBD of ply_pitti20 and additional C-terminal His-tag).

[0040] An illustrative example of a ply_pitti26 variant having a replaced CBD and further one amino acid addition at position two is given in SEQ ID NO:28 (ply_pitti26 with CBD of plyUSA and additional amino acid residue A at position two) designated EADplypitti26_CBDplyUSA-Add2.

[0041] Further illustrative examples of ply_pitti26 variants having a replaced CBD, one or more amino acid substitutions and further an additional amino acid residue at position two are described below. Particularly preferred is the polypeptide having an amino acid sequence according to SEQ ID NO:15. Said polypeptide is derived from ply_pitti26 with the CBD of plyUSA as depicted in SEQ ID NO:7 having the following five single amino acid substitutions L56H, L57T, E164A, R168A and Y201H and one additional A2. The variant is named EADplypitti26_CBDplyUSA-Add2_M5. The annotation "...M5" stands for every construct comprising the particularly preferred combination of the five single amino acid substitutions L56H, L57T, E164A, R168A and Y201H within the EAD of ply_pitti26. The annotation "Add2" stands for every construct comprising the additional single amino acid residue at position two of the EAD of ply_pitti26. Further particularly preferred is a variant combining the EAD of ply_pitti26 with the CBD of lysostaphin named EADplypitti26_CBDSL-Add2_M5 and having the amino acid sequence as depicted in SEQ ID NO.20 or the variant combining the EAD of ply_pitti26 with the CBD of ALE-1 lysin named EADplypitti26_CBDALE1-Add2_M5 and having the amino acid sequence as depicted in SEQ ID NO.22.

[0042] Particularly preferred is in addition the polypeptide having the amino acid sequence according to SEQ ID NO:18.

Said polypeptide is derived from ply_pitti26 with the CBD of plyUSA as depicted in SEQ ID NO:7 having the following eight single amino acid substitutions L56H, L57T, E164A, R168A, Y201H, S238L, R355Q, and A368V and one additional A2. The variant is named EADplypitti26_CBDplyUSA-Add2_M8.

[0043] In another aspect the present invention relates to a nucleic acid molecule encoding one of the polypeptides of the invention. It is apparent to a person skilled in the art that there may be many ways of constructing nucleic acid molecules encoding for one of the polypeptides according to the present invention, for example in view of the degenerate genetic code. A person skilled in the art with knowledge about the amino acid sequence of the polypeptide of the present invention is capable of choosing an adequate nucleotide sequence which suits his purposes most, for example in which the codon usage has been adapted to the codon usage of his desired expression host. A nucleic acid molecule comprising a sequence encoding for a polypeptide of the invention is considered to be the nucleic acid or nucleotide sequence, respectively, of the present invention.

[0044] In a preferred embodiment said nucleic acid molecule encodes for a variant of the endolysin comprising the sequence of SEQ ID NO: 1. Also described is a nucleic acid which comprises the nucleotide sequence as depicted in SEQ ID NO: 2.

[0045] In another preferred embodiment the polypeptide to be encoded is a variant of ply_pitti26, in particular a variant wherein the CBD domain is replaced by a CBD of another staphylococcal bacteriophage endolysin. Illustrative examples for nucleotide sequences encoding the CBD of plyUSA or ply_pitti20 or lysostaphin or ALE-1 are given in SEQ ID NOs: 4 and 6, and 25 and 27 respectively. Further example illustrating nucleotide sequences of the present invention are given in SEQ ID NOs: 8 (encoding for ply_pitti26 with CBD of ply_USA), 10 (encoding for ply_pitti26 with CBD of ply_USA plus additional C-terminal His-tag), 12 (encoding for ply_pitti26 with CBD of ply_pitti20) and 14 (encoding for ply_pitti26 with CBD of ply_pitti20 plus additional C-terminal His-tag), respectively.

[0046] In a preferred embodiment the nucleic acid molecule encodes for the polypeptide comprising the sequence of SEQ ID NO:15, namely EADplypitti26_CBDplyUSA-Add2_M5. Preferably, said nucleotide comprises the nucleotide sequence as depicted in SEQ ID NO:16 and 17. SEQ ID NO:16 is the nucleotide sequence isolated from the phage pitfi26 and prophage Φ SA2usa, respectively, with modifications of those nucleotide codons which encode the amino acid residues which have been substituted, i.e. L56H, L57T, E164A, R168A and Y201H and with an inserted codon encoding the additional amino acid residue A2. SEQ ID NO: 17 is derived from SEQ ID NO:16 but has been codon optimized for expression in E.coli K12. Further preferred embodiments are the nucleotide sequences SEQ ID NO: 19 which is codon optimized also, SEQ ID NO:21, and SEQ ID NO:23, encoding for the polypeptides according to the invention EADplypitti26_CBDplyUSA-Add2_M8, EADplypitti26_CBDLS-Add2_M5, and EADplypitti26_CBDALE1-Add2_M5, respectively.

[0047] In a further aspect the present invention relates to a vector comprising a nucleic acid sequence of the invention. Preferably, said vector provides for the expression of said polypeptide of the invention in a suitable host cell. Said host cell may be selected due to mere biotechnological reasons, e.g. yield, solubility, costs, etc. but may be also selected from a medical point of view, e.g. a non-pathological bacteria or yeast, human cells, if said cells are to be administered to a subject. Said vector may provide for the constitutive or inducible expression of said polypeptides according to the present invention.

[0048] In a further aspect of the present invention the above mentioned polypeptides and/or cells are employed in a method for the treatment or prophylaxis of *Staphylococcus* infections in a subject, in particular for the treatment or prophylaxis of infections by *S. aureus*, *S. aureus* (MRSA), *S. epidermidis*, *S. haemolyticus*, *S. simulans*, *S. saprophyticus*, *S. chromogenes*, *S. hyicus*, *S. warneri* and/or *S. xylosus*. Said subject may be a human subject or an animal, in particular animals used in livestock farming and/or dairy farming such as cattle. Said method of treatment encompasses the application of said polypeptide of the present invention to the site of infection or site to be prophylactically treated against infection in a sufficient amount.

[0049] In particular said method of treatment may be for the treatment or prophylaxis of infections, in particular by *Staphylococcus aureus*, of the skin, of soft tissues, of bacteremia and/or endocarditis.

[0050] In a further preferred embodiment a polypeptide according to the present invention is employed in a method for the treatment of keratitis, in particular of keratitis caused by *S. haemolyticus*.

[0051] In a further preferred embodiment a polypeptide according to the present invention is used to treat or prevent endoplatitis, in particular endoplatitis caused by *S. epidermidis*.

[0052] In a further preferred embodiment a polypeptide according to the present invention is used to treat or prevent urinary tract infections, in particular endoplatitis caused by *S. saprophyticus*.

[0053] In a further preferred embodiment a polypeptide according to the present invention is used in a method of treatment (or prophylaxis) of staphylococcal infections in animals, in particular in livestock and dairy cattle. In particular a polypeptide of the present application is suitable for use in methods of treatment (or prophylaxis) of bovine mastitis, in particular of bovine mastitis caused by *S. aureus*, *S. epidermidis*, *S. simulans*, *S. chromogenes*, *S. hyicus*, *S. warneri* and *S. xylosus*.

[0054] Furthermore, a polypeptide of the present invention may be used prophylactically as sanitizing agent, in particular

before or after surgery, or for example during hemodialysis. Similarly, premature infants and immunocompromised persons, or those subjects with need for prosthetic devices can be treated with a polypeptide of the present invention, either prophylactically or during acute infection. In the same context, nosocomial infections by *Staphylococcus*, in particular by *S. aureus* or *S. aureus* (MRSA), may be treated prophylactically or during acute phase with a polypeptide of the present invention. In this embodiment, a polypeptide of 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.

[0055] In a particularly preferred embodiment a polypeptide of the present invention is used for medical treatment, if the infection to be treated (or prevented) is caused by multiresistant *Staphylococcus* strains, in particular by strains resistant against vancomycin, linezolid or daptomycin. Furthermore, a polypeptide of the present invention can be used in methods of treatment by administering them in combination with conventional antibacterial agents, such as antibiotics, lanthibiotics, bacteriocins other endolysins, etc.

[0056] 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 in particular embodiments oral, topical, nasopharyngeal, parenteral, intravenous, rectal or any other route of administration.

[0057] For application of a polypeptide of the present invention to a site of infection (or site endangered to be infected) a polypeptide of the present invention may be formulated in such manner that the endolysin is protected from environmental influences such as proteases, oxidation, immune response etc., until it reaches the site of infection.

[0058] Therefore, a polypeptide of the present invention may be formulated as capsule, dragee, pill, suppository, injectable solution or any other medical reasonable galenic formulation. In some embodiments these galenic formulation may comprise suitable carriers, stabilizers, flavourings, buffers or other suitable reagents.

[0059] For example, for topical application a polypeptide of the present invention may be administered by way of a lotion or plaster.

[0060] For nasopharyngeal application a polypeptide according to the present invention may be formulated in saline in order to be applied via a spray to the nose.

[0061] For treatment of the intestine, for example in bovine mastitis, suppository formulation can be envisioned. Alternatively, oral administration may be considered. In this case, the polypeptide of the present invention has to be protected from the harsh digestive environment until the site of infection is reached. This can be accomplished for example by using bacteria as carrier, which survive the initial steps of digestion in the stomach and which secret later on a polypeptide of the present invention into the intestinal environment.

[0062] All medical applications rely on the effect of the polypeptides of the present invention to lyse specifically and immediately staphylococcal bacteria when encountered. This has an immediate impact on the health status of the treated subject by providing a reduction in pathogenic bacteria and bacterial load and simultaneously relieves the immune system. Thus, the major task a person skilled in the art faces is to formulate the polypeptides of the present invention accurately for the respective disease to be treated. For this purpose usually the same galenic formulation as employed for conventional medicaments for these applications can be used.

[0063] In a further aspect of the present invention the above mentioned polypeptides and/or cells are a component of a pharmaceutical composition, which optionally comprises a carrier substance.

[0064] In an even further aspect the polypeptides and/or cells are part of a cosmetics composition. As mentioned above, several staphylococcal 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 staphylococcal pathogens, special cosmetic preparations may be employed, which comprise sufficient amounts of polypeptides of the present invention in order to lyse already existing or freshly settling *Staphylococci*.

[0065] In a further aspect the present invention relates to the use of said polypeptides according to the present invention in foodstuff, on food processing equipment, in food processing plants, on surfaces coming into contact with foodstuff such as shelves and food deposit areas and in all other situations, where staphylococcal bacteria can potentially infest food material.

[0066] A further aspect of the present invention relates to the use of said polypeptides according to the present invention in diagnostics of *Staphylococcus*. In this aspect the polypeptides according to the invention are used as a means to specifically lyse *Staphylococcus* bacteria. The lysis of the bacterial cells by the polypeptides according to the present invention may 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 lysis is needed as an initial step for subsequent specific detection of *Staphylococcus* 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 *Staphylococcus* bacteria.

[0067] A further aspect of the present invention relates to a diagnostic kit comprising the polypeptide according to the

present invention, detergents like Triton X-100 or other additives which weaken the bacterial cell envelope like polymyxin B. The kit may contain furthermore means and substances for the detection like PCR means, means for 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 *Staphylococcus* bacteria.

Examples

[0068] All cloning procedures were performed using standard techniques according to Sambrook et al. (Molecular cloning, A laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press 1989). Mutations and deletions were also introduced using standard techniques.

Example 1: Cloning of the endolysins according to the invention

[0069] For EADplypitti26_CBDplyUSA, the nucleotide sequence of the CHAP-Ami2 domain was amplified from the lytic phage pitti26 (own isolate) and the CBD sequence was amplified from plyUSA. For EADplypitti26_CBDplypitti20, the CBD was derived from plypitti20 (own isolate). The fragments were combined by ligation, and cloned into the expression vector pET14b via the restriction sites NcoI and BamHI. In order to add a C-terminal His-tag to the sequence, the new construct was cloned into the expression vector pQE60 via the restriction sites NcoI and BamHI. This construct contains an additional sequence of Ser-Arg-Ser-(His6) at the C-terminus.

Example 2: Expression and solubility testing of the endolysins according to the invention

[0070] For expression of the cloned constructs *E. coli* HMS174(DE3) (pET14b construct) and *E. coli* M15 (pQE construct) were used, respectively. Cells were grown in LB medium containing ampicillin and rifampicin (HMS174(DE3)) or ampicillin (M15) at 30°C or 37 °C with shaking and induced with 1 mM IPTG at OD_{600 nm} 0.4-0.6 (mid log phase). After shaking for further 3-4 hours the cells were harvested by centrifugation and frozen at -20°C.

For solubility testing, harvested cells were resuspended in lysis buffer (20 mM Tris/HCl pH 8.0, 5 mM EDTA), sonicated (2 x 30 sec) and centrifuged. The pellet was solved in exactly the same volume of buffer as the supernatant. Identical sample volumes of pellet and supernatant fractions were analyzed with 12 % SDS-PAGE.

[0071] Whereas upon expression at 37 °C the solubility of recombinantly produced endolysins was generally poor, the solubility of EADplypitti26_CBDplyUSA was significantly higher than that of wild-type plypitti26 at 30 °C. Whereas almost all protein was found in the insoluble pellet fraction with plypitti26, a soluble fraction of around 30 % to 40 % was found upon expression of EADplypitti26_CBDplyUSA. This holds similar for EADplypitti26_CBDplypitti20.

Example 3: Purification of the endolysins according to the invention

[0072] For the purification of the soluble His-tagged versions of the endolysin constructs according to the invention a Ni-NTA-Sepharose column (Amersham) was used according to the manufacturer's instructions. The cell pellet was resuspended in equilibration buffer (25 mM Tris/HCl pH 8.0, 500 mM NaCl, 20 mM imidazole, 0.1 % Tween 20, 10 % glycerol), centrifuged and added to the column equilibrated with the same buffer. Elution of the protein was carried out in elution buffer (25 mM Tris/HCl pH 8.0, 500 mM imidazole, 0.1 % Tween 20, 10 % glycerol), and the eluted fractions were analyzed with SDS-PAGE. The combined fractions containing purified endolysins were dialyzed against storage buffer (20 mM Tris/HCl pH 7.5, 10 mM DTE, 0.1 mM ZnSO₄) and stored at -20°C.

[0073] The purification of the soluble version of the endolysins according to the invention without His-tag followed standard purification procedures with anion exchange chromatography, size exclusion chromatography and hydrophobic chromatography using for example Streamline HST, Superdex, and HiTrap Capto MMC columns (GE Healthcare).

[0074] The amount of active endolysin can be increased if insoluble endolysin material deposited in inclusion bodies (pellet fraction of example 2) is solubilized under denaturing conditions and subsequently refolded. The amount of protein deposited in inclusion bodies can be increased by increasing expression temperature to 37 °C, for example. Suitable conditions for solubilization and refolding are described for example in Navarre et al., 1999, J. Biol. Chem. 274, 15847-15856.

Example 4: Agar plate activity assay

[0075] An overnight culture of a *staphylococcus* strain which was growing for about 18 hours at 37°C in Brain Heart Infusion medium (Oxoid), was collected by centrifugation, the cell pellet was resuspended in 1x DPBS (Merck) thereby reducing the original volume by a factor of 100 and heat inactivated at 80°C for 20 minutes. The cells were sonicated

and solved in LB topagar including ampicillin and IPTG (0.7 % agar (Bacto); 100 µg/ml ampicillin (Sigma); 1 mM IPTG (Roth)), and used for the preparation of "lysis plates". These plates can be overlaid with protein solutions or solutions of *E. coli* harbouring plasmids which code for the desired proteins. A lysis zone on the plate is visible if the lytic protein is active respectively is expressed at least partly in soluble and active form.

[0076] The agar plate assay was used to test the lysis activity of EADplypitti26_CBDplyUSA in comparison with the wild-type form of pitti26 using different coagulase positive (*Staphylococcus aureus*) and coagulase negative (e. g. *S. epidermidis*, *S. haemolyticus*, *S. saprophyticus*, *S. simulans*) *Staphylococcus* strains. This was done in an agar plate lysis assay. Concentrated heat inactivated (e. g. 20 min at 80 °C) *Staphylococcus* cells are fixed in a top agar layer in a way that a dense bacterial lawn is achieved. Either isolated endolysin solution (5 µl to 10 µl) or *E. coli* transformants harbouring the respective endolysin containing plasmids are stippled onto the surface of the top agar layer. The plates are incubated at 30 °C for several hours (1 h to 12 h), and then checked for lysis zones around the endolysin spots. Normally, an increase in the lysis zone diameter also coincides with a clearing of the lysis zone indicating more efficient cell lysis. The results are depicted in table 1.

Table 1: Host range for lysis by EADplypitti26_CBDplyUSA and plypitti26

PROFOS culture collection Nr.	Origin	Staphylococcus species	plypitti26	EADplypitti26_CBDplyUSA
S462	Clinical isolate	<i>S. aureus</i>	+++	+++
S460	Clinical isolate	<i>S. aureus</i>	++	+++
S1516	Clinical isolate	<i>S. aureus</i>	+++	+++
S459	Clinical isolate	<i>S. aureus</i>	+++	+++
S457	Clinical isolate	<i>S. aureus</i>	+++	+++
S456	Clinical isolate	<i>S. aureus</i>	+++	+++
S1519	Clinical isolate	<i>S. aureus</i>	+++	+++
S463	Clinical isolate	<i>S. aureus</i>	+++	+++
S1551	DSMZ346	<i>S. aureus</i>	++	+++
S1517	Clinical isolate	<i>S. aureus</i>	+++	+++
S458	Clinical isolate	<i>S. aureus</i>	+++	+++
S1550	DSMZ20231	<i>S. aureus</i>	++	++
S467	Clinical isolate	<i>S. aureus</i> , MRSA	+	+++
S469	Clinical isolate	<i>S. aureus</i> , MRSA	+++	+++
S468	Clinical isolate	<i>S. aureus</i> , MRSA	+++	+++
S1573	Own isolate, patient	<i>S. epidermidis</i>	-	+++
S1508	Clinical isolate	<i>S. epidermidis</i>	+++	+++
S1510	Clinical isolate	<i>S. epidermidis</i>	+++	+++
S1546	DSMZ20044	<i>S. epidermidis</i>	+	+++
S27	Own isolate	<i>S. haemolyticus</i>	+	+++
S1548	DSMZ20228	<i>S. haemolyticus</i>	+	+++
S1509	Clinical isolate	<i>S. haemolyticus</i>	-	+++
S1549	DSMZ20263	<i>S. haemolyticus</i>	+	+++
S1429	Own isolate, food	<i>S. saprophyticus</i>	-	+++
S1512	Clinical isolate	<i>S. simulans</i>	++	+++

[0077] Table 1 depicts the host range for the EADplypitti26_CBDplyUSA compared to the wild-type endolysin Pitti26. Lysis activity on different staphylococcus strains was tested with the agar plate activity assay. Strong (+++), medium (++) , weak (+) and no (-) lysis are defined as described in the legend of Fig. 2. The first column depicts the strain number

corresponding to the PROFOS culture collection. The second column mentions the origin of the respective strain. "Clinical isolate" means a strain isolated from a patient with diagnosed staphylococcal infection. DSMZ numbers represent strains that can be ordered from the "Deutsche Sammlung für Mikroorganismen und Zellkulturen" (Braunschweig) the remaining being own isolates from our laboratory. The lysis assay demonstrated that the activity of EADplypitti26_CBDplyUSA is often better than the activity of the wild-type enzyme plypitti26, especially on the coagulase negative non *S. aureus* strains. This means also that EADplypitti26_CBDplyUSA has a broader host range compared to plypitti26. Coagulase negative staphylococci (species other than *Staphylococcus aureus*) are involved in infections in weakened and immune compromised persons, cause problems in biofilm formation in persons wearing indwelling devices or other implants, and are involved in mastitis. *S. saprophyticus* causes special problems in urinary tract infections.

Example 5: Turbidity assay to control lysis activity

[0078] The second activity test we use for endolysin function is a turbidity test, where lysis of bacterial cells is measured "online" in a photometer. The absorption at 600 nm is a measure for the density of the cell culture and decreases upon cell lysis when the sample also becomes visibly clear. In contrast to the agar plate assay described above, this assay uses non-heat-treated bacterial cells, and is therefore more stringent and closer to reality in medical applications. Lysis of bacteria by the phage endolysins results in a drop of optical density (OD) which is measured by the turbidity assay. Target bacteria were grown in Brain Heart Infusion (Oxoid) until an OD₆₀₀ of around 0.8 is reached (exponential phase). The cells were harvested and resuspended in TBST buffer (20 mM Tris/HCl pH 7.5, 60 mM NaCl, 0.1 % Tween) plus 2 mM CaCl₂ to an OD₆₀₀ of 1. Small volumes of concentrated endolysin solutions are added to final protein concentrations as indicated in the figures. Changes in OD₆₀₀ were followed at 30 °C in a sample volume of 1 ml in a photometer (Jasco) until a stable baseline is reached. The addition of the test proteins occurred without interruption of the measurement.

[0079] Different concentrations of isolated plypitti26 and EADplypitti26_CBDplyUSA endolysin were tested on *Staphylococcus aureus* cells. EADplypitti26_CBDplyUSA generally showed higher activity at the same protein concentration than plypitti26, again indicating superior properties compared to the naturally occurring endolysin. With respect to the relatively low protein concentrations used in the assay, complete lysis (indicated by no residual absorption) is achieved in a very short time span of several minutes with EADplypitti26_CBDplyUSA. The test was also performed with protein concentrations as low as 1 µg, 0.5 µg and even 0.2 µg, resulting also in complete lysis over a longer period of time. The resulting specific activity, defined as ΔOD₆₀₀ per mg protein and minute, was calculated for EADplypitti26_CBDplyUSA as a value of ca. 50 units. Comparing this specific activity with state of the art full-length and truncated versions of phi11 endolysin, which are between 0.8 and 1.5 units (Donovan et al., 2006, FEMS Microbiol. Lett, 265, 133-139), EADplypitti26_CBDplyUSA turns out to be an extremely efficient staphylococcal endolysin. Even plypitti26 is significantly better than the phi11 endolysin.

Example 6: Activity assay in human blood serum

[0080] The activity of EADplypitti26_CBDplyUSA in blood serum was tested with the photometric turbidity assay according to example 5. Log phase *S. aureus* cells were resuspended in blood serum and subsequently lysis assays with different concentrations of EADplypitti26_CBDplyUSA were performed. This assay allows a direct measuring of lysis of *S. aureus* cells in human blood serum. For efficient lysis in serum around 10fold the amount of protein is required in comparison to lysis in osmotically optimized lysis buffer. In addition, the kinetics of lysis are slower in blood serum than in buffer.

[0081] The activity of EADplypitti26_CBDplyUSA was tested in human blood serum. It could be demonstrated that EADplypitti26_CBDplyUSA is also an efficient lysin on *S. aureus* cells under conditions existent in human plasma, albeit a somewhat higher protein concentration is needed than under optimized standard assay conditions, and the lysis is somewhat slower.

Example 7: Stability during long term incubation at room temperature

[0082] The stability of EADplypitti26_CBDplyUSA in comparison to plypitti26 was tested after incubation in storage buffer (20 mM Tris/HCl pH 7.5, 10 mM DTE, 0.1 mM ZnSO₄) at 25 °C for up to one week. Degradation of the proteins was monitored by SDS-PAGE and in turbidity assays. Whereas EADplypitti26_CBDplyUSA was stable over the whole time period tested as no changes were observed, plypitti26 was obviously degraded at incubation times longer than 9 hours, and no full-length protein was visible after 120 h of incubation, but protein bands corresponding to smaller fragments became visible.

[0083] Remaining activity of the tested proteins was recorded using the turbidity assay under standard conditions (see example 5). In accordance with the stability data obtained by SDS-PAGE, EADplypitti26_CBDplyUSA shows a slower decrease in activity in comparison to plypitti26. EADplypitti26_CBDplyUSA remains fully active for a long time, whereas

the activity of the pypitt26 drops significantly already after 4 hours.

[0084] This correlated with the decrease in activity, suggesting that only full-length endolysins were enzymatically active under these conditions. The experiment demonstrated, that especially EADpypitt26_CBDplyUSA is stable for reasonable periods of time even at room temperature. The stability extends a lot when the proteins are stored at lower temperatures, e.g. at 4 °C in a refrigerator or at temperatures of - 20 °C or - 80 °C in a deep freezer.

Example 8: Protease stability against thrombin and V8 protease

[0085] For the protease stability assay 50 µg thrombin were incubated over night at 25 °C with the respective proteins at a final concentration of 1.1 mg/ml. The incubation buffer was 20 mM Tris/HCl pH 7.5, 10 mM DTE, 0.1 mM ZnSO₄. On the next day, the protein samples were analyzed on SDS-gels and in the turbidity assay. Whereas only a small amount of EADpypitt26_CBDplyUSA is degraded during the time of the assay, no full-length protein of pypitt26 is left under the same conditions. This means that EADpypitt26_CBDplyUSA is still highly active after incubation with thrombin whereas pypitt26 loses its activity completely. EADpypitt26_CBDplyUSA exhibits thrombin resistance as a property which renders it helpful for application in wounds or intravenous.

[0086] Protease stability against V8 protease was tested incubating endolysin (concentration 0.2 mg/ml) and V8 protease (1 µg/ml) in the following buffer: 20 mM Tris/HCl, 100 mM NaCl, pH 8.0 at 25 °C over night. On the next day, the protein samples were analyzed on SDS-gels and in the turbidity assay.

Example 9: Stability in human blood

[0087] Activity was measured with the turbidity assay (example 5) after preincubation of EADpypitt26_CBDplyUSA at 37 °C for the times indicated in either storage buffer (20 mM Tris/HCl pH 7.5, 10 mM DTE, 0.1 mM ZnSO₄) or human EDTA-blood. Human blood samples are centrifuged to sediment red blood cells at the times indicated, and 100 µl of protein solution from the supernatant are added to start the turbidity assay. Up to 2 h of incubation time, there is almost identical activity measured after preincubation in buffer and in blood. EADpypitt26_CBDplyUSA is inactivated almost completely after 4 h incubation in blood at 37 °C, whereas there is 80 % residual activity measured with the control incubation.

Example 10: Modified endolysins

[0088] We have generated several modified chimeric endolysins starting from the sequences of EADpypitt26_CBDplyUSA, EADpypitt26_CBDpypitt20, EADpypitt26_CBDALE1, and EADpypitt26_CBDLS.

N-terminal truncations

[0089] Several N-terminally truncated forms of EADpypitt26_CBDplyUSA were constructed and tested for activity in the agar plate assay. Two constructs that were N-terminally shortened by 4 and 9 amino acids, respectively, exhibited lytic activity in the plating assay, whereas a construct that was shortened by 29 amino acids did not exhibit activity any more.

Site directed mutagenesis

[0090] In order to further stabilize and solubilize pypitt26, EADpypitt26_CBDplyUSA, EADpypitt26_CBDpypitt20, EADpypitt26_CBDALE1, and EADpypitt26_CBDLS, site directed mutations at selected positions within the amino acid sequence were performed using specific primers for single amino acid substitutions, and the activity of the respective mutants was tested using the agar plate activity assay. We substituted hydrophobic amino acids (F, W, Y, I, L) against the less hydrophobic amino acids R, D, E, N, K, Q, H, S, T, M, G, A by standard site directed mutagenesis methods. The charged amino acids E and R were preferentially exchanged against uncharged amino acids (Q or A for E, A for R). The C were preferentially exchanged against A or S. The substitutions as listed in table 2 turned out to maintain the activity or to improve the properties of the endolysin as a therapeutic, diagnostic and prophylactic agent against staphylococcal infections.

Table 2: Modified endolysins exhibiting single amino acid mutations in pypitti26, EADpypitti26_CBDplyUSA, EADpypitti26_CBDpypitti20, EADpypitti26_CBDALE1, and EADpypitti26_CBDLS showing lytic activity against staphylococci. It has to be understood that the numbers of the single amino acid mutations change if they are present in a pypitti26 variant with an amino acid addition, e.g. in a variant with an additional A at position 2 like the preferred embodiments EADpypitti26_CBDLS-Add2_M5, EADpypitti26_CBDplyUSA-Add2_M5, EADpypitti26_CBDALE1-Add2_M5 or EADpypitti26_CBDplyUSA-Add2_M8.

Single amino acid mutations	
mutations	activity
W22R	Yes
F42A	Yes
Y44A	Yes
L55H	Yes
L56T	Yes
F67T	Yes
Y115S	Yes
W123M	Yes
W137A	Yes
W139A	Yes
W154H	Yes
E163Q	Yes
R167A	Yes
E179Q	Yes
E179A	Yes
E187Q	Yes
E189Q	Yes
Y200A	Yes
Y200H	Yes
Y275A	Yes
Y275M	Yes
Y276A	Yes
C282A	Yes
F300A	Yes
C303S	Yes
W310A	Yes
W310M	Yes
Mutation of 2 to 7 residues	
Mutations	activity
L55H + L56T	yes
F67T + Y115S	yes
F67T + W137A	yes

(continued)

Mutation of 2 to 7 residues	
Mutations	activity
F67T + W139A	yes
F67T + W154H	yes
Y115S + W137A	yes
Y115S + W139A	yes
E163Q + R167A	yes
E163Q + R169A	yes
E163A + R169A	yes
E179A + E189Q	yes
E179Q + E189Q	yes
E163Q + R167A + E189Q	yes
E163A + R167A + E189Q	yes
E163Q + R167A + Y200H	yes
E163Q + R167A + E179Q + E189Q	yes
E163Q + R167A + E179A + E189Q	yes
E163A + R167A + E179Q + E189Q	yes
E163A + R167A + E179A + E189Q	yes
Y200A + Y275A	yes
Y200A + Y276A	yes
Y200A + C282A	yes
Y200A + F300A	yes
Y275A + Y276A	yes
Y275A + F300A	yes
C282A + F300A	yes
Y200A + Y275A + Y276A	yes
Y275A + Y276A + F300A	yes
L55H + L56T + E163A + R167A + Y200H	yes
E163A + R167A + E179A + E189Q + Y200H	yes
L55H + L56T + E163A + R167A + E179A + E189Q + Y200H	yes
S237L + R354Q + A367V	yes

Example 11: Comparison of lysis activity in buffer of different lysis proteins against *Staphylococcus aureus* cells

[0091] The assay was performed according to the protocol "turbidity assay to control lysis activity" described in example 5. The specific activity ($\Delta A_{600} / \text{min mg}$) was calculated from the initial slopes of the lysis curves where the decrease in absorbance was almost linear. The assay was performed using lysis protein concentrations of 1 $\mu\text{g/ml}$ and 10 $\mu\text{g/ml}$. It is seen that the specific activities are generally lower at a concentration of 10 $\mu\text{g/ml}$ protein as the decrease in absorbance ($\Delta A_{600} / \text{min}$) measured has no linear correlation with the protein concentration. The following order of lysis activity in buffer resulted from the experiment: EADplypitti26_CBDLS-Add2_M5 better than EADplypitti 26_CBDplyUSA-Add2_M8 better than EADplypitti26_CBDALE1-Add2_M5 better than EADplypitti26_CBDplyUSA-Add2_M5 better than

EADplypitti26_CBDplyUSA better than plypitti26 better than lysostaphin. Lysostaphin has the weakest performance in this assay, and plypitti26 the second weakest. Surprisingly however, a combination of the EAD of plypitti26 and the CBD of lysostaphin - EADplypitti26_CBDLS-Add2_M5 - shows the second best performance. This is also reflected in the variant EADplypitti26_CBDALE1-Add2_M5, as ALE1 lysin is very similar to lysostaphin. The plypitti26 variants EADplypitti26_CBDplyUSA and EADplypitti26_CBDplyUSA-Add2_M5 show better lysis activity than plypitti26.

Example 12: Comparison of lysis activity in human blood serum of different lysis proteins against *Staphylococcus aureus* cells

[0092] The assay was performed according to the protocol "activity assay in human blood serum" described in example 6. The specific activity ($\Delta A_{600} / \text{min mg}$) was calculated from the initial slopes of the lysis curves where the decrease in absorbance was almost linear. The assay was performed using lysis protein concentrations of 10 $\mu\text{g/ml}$ and 25 $\mu\text{g/ml}$. It is seen that the specific activities are somewhat lower at a concentration of 25 $\mu\text{g/ml}$ protein as the decrease in absorbance ($\Delta A_{600} / \text{min}$) measured has no linear correlation with the protein concentration. The following order of lysis activity in human blood serum resulted from the experiment using 10 $\mu\text{g/ml}$ lysis protein: EADplypitti26_CBDLS-Add2_M5 better than EADplypitti26_CBDplyUSA-Add2_M5 better than EADplypitti26_CBDALE1-Add2_M5 better than EADplypitti26_CBDplyUSA better than EADplypitti26_CBDplyUSA-Add2_M8. It turned out, that lysis efficiency of the different endolysin constructs in human blood serum is different from that measured in buffer. Endolysin plypitti26 exhibited no lytic activity on *Staphylococcus aureus* cells in human blood serum, but seems to be inhibited by an unknown factor. The modified variants of plypitti26 exhibiting the CBD of plyUSA, namely EADplypitti26_CBDplyUSA and EADplypitti26_CBDplyUSA-Add2_M5 and EADplypitti26_CBDplyUSA-Add2_M8 show specific lysis activities in human blood serum better than plypitti26. The construct EADplypitti26_CBDLS-Add2_M5 using the CBD of lysostaphin shows the best specific lysis activity in human blood serum although the lysis activity in buffer of lysostaphin was very poor. The construct EADplypitti26_CBDALE1-Add2_M5 using the CBD of ALE-1 lysin shows the third best specific lysis activity in human blood serum.

Example 13: Thermal stability assay

[0093] The thermal stability of the *Staphylococcus* endolysins was measured in a thermostability assay measuring aggregation of the protein at a wavelength of 360 nm in a photometer. The proteins (concentration 0.1 mg/ml) were dissolved in a buffer containing 20 mM Hepes, 10 mM CaCl_2 , 50 mM arginin, pH 7.5. A cuvette containing the buffered protein solution was put in a photometer and heated in steps of 1 °C per minute. The absorbance at 360 nm (A_{360}) was measured between room temperature and around 65 °C. Without protein denaturation, the A_{360} signal shows only a slight slope, but beginning from a specific temperature, the aggregation temperature T_{aggr} , the absorption line begins to rise and describes kind of a peak. This signal change coincides with aggregation and concomitant inactivation of the protein. Thus, the aggregation temperature T_{aggr} observed is a measure for the thermal stability of the protein. A comparison of the T_{aggr} for different *Staphylococcus* endolysin variant is given in table 3.

Table 3:

Protein	T_{aggr} °C
ply_pitti26	50
EADplypitti26_CBDplyUSA	55
EADplypitti26_CBDplyUSA-Add2_M5	55
EADplypitti26_CBDplyUSA-Add2_M8	55
EADplypitti26_CBDLS-Add2_M5	56
EADplypitti26_CBDALE1-Add2_M5	51

[0094] It turned out that ply_pitti26 is the least stable enzyme measured in this experiment. The exchange of the CBD of ply_pitti26 for the CBD from plyUSA or from lysostaphin increases the stability by 5 °C. EADplypitti26_CBDLS-Add2_M5 is the most stable endolysin with a T_{aggr} of 56 °C, followed by EADplypitti26_CBDplyUSA-Add2_M5, EADplypitti26_CBDplyUSA, and EADplypitti26_CBDplyUSA-Add2_M8 exhibiting T_{aggr} of 55 °C, each. The exchange of the CBD of ply_pitti26 for the CBD from ALE-1 lysin results only in a marginal stabilisation of 1 °C.

Example 14: Characterisation of plypitti26 and variants thereof with respect to advantageous properties for use as a therapeutic, diagnostic and prophylactic agent against staphylococcal infections.

[0095] Different chimaeric endolysins derived from plypitti26, and modified proteins exhibiting up to 7 single amino acid mutations were compared with respect to different properties concerning activity and stability of the endolysins. Protease resistance was tested according to the protocol described in example 8, lysis activity in buffer and serum according to examples 5 and 6, respectively, the host range was determined in the agar plate assay (example 4), and the stability during long term incubation with the assay described in example 7. The results are summarised in a semi-quantitative manner in table 4.

Table 4: Comparison of properties of different protein variants of *Staphylococcus endolysin* ply...piti26 for a use according to the invention

Endolysin construct	plypiti26	EADpiti26... CBDUSA	EADpiti26... CBDUSA- Add2	EADpiti26... CBDpiti20	EADpiti26... CBDUSA- Add2_M5	EADpiti26... CBDUSA-Add2	EADpiti26... CBDUSA- Add2	EADpiti26... CBDUSA- Add2	EADpiti26... CBDUSA- Add2
Amino acid substitutions		E163A, R167A, Y200H	E164A, R168A, E180A, E190Q, Y201H		L56H, L57T, E164A, R168A, Y201H	L56H, L57T, E164A, R168A, E180A, E190Q, Y201H	L56H, L57T, E164A, R168A		
	property	assay							
	thrombin resistance	SDS- PAGE	+	+	+++	+++	+++	+++	+
	thrombin resistance	turbidity	-	-	-	+	-	-	+
V8 protease resistance	SDS- PAGE		-	-	+	-	-	-	-
activity in buffer	turbidity	+	+++	+	+++	+++	++	+	+++
activity in serum	turbidity	n.d.	n.d.	n.d.	+++	++	n.d.	n.d.	+
host range <i>Staphylococ- cus</i>	agar plate	++	+++	+	+++	+++	+++	+++	+++
host range <i>S. aureus</i>	agar plate	++	+++	+++	+++	+++	+++	+++	+++
host range <i>S. aureus</i> MRSA	agar plate	++	+++	+	+++	+++	+++	+++	+++

(continued)

Endolysin construct	plypiti26	EADpiti26... CBDUSA	EADpiti26... CBDUSA- Add2	EADpiti26... CBDpiti20	EADpiti26... CBDUSA- Add2_M5	EADpiti26... CBDUSA-Add2	EADpiti26... CBDUSA- Add2	EADpiti26... CBDUSA- Add2	EADpiti26... CBDUSA- Add2
Amino acid substitutions		E163A, R167A, Y200H	E164A, R168A, E180A, E190Q, Y201H		L56H, L57T, E164A, R168A, Y201H	L56H, L57T, E164A, R168A, E180A, E190Q, Y201H	L56H, L57T, E164A, R168A		
property	assay								
storage stability									
turbidity	+	n.d.	n.d.	n.d.	+++	++	+++	++	+++
+++: property measured in the respective assay very good compared with the other endolysin constructs measured ++: property measured in the respective assay good compared with the other endolysin constructs measured +: property measured in the respective assay less pronounced compared with the other endolysin constructs measured -: property not measurable under the conditions used in the respective assay compared with the other endolysin constructs measured n.d.: property not determined with the respective endolysin construct									

[0096] All of the variants of pypitt26 constructed as described in this invention show advantages over the naturally occurring endolysin in stability or activity or both. The variants exhibiting the mutations E164A (or E163A in EADpitt26_CBDUSA) and R168A (or R167A in EADpitt26_CBDUSA), but also the chimaeric constructs EADpitt26_CBDUSA, EADpitt26_CBDUSA-Add2 and EADpitt26_CBDpitt20 show better resistance to thrombin than pypitt26. In the variants EADpitt26_CBDUSA-Add2_M5 (L56H, L57T, E164A, R168A, Y201H) and EADpitt26_CBDUSA-Add2 (L56H, L57T, E164A, R168A, E180A, E190Q, Y201H) and to a lower degree in the variants EADpitt26_CBDUSA, EADpitt26_CBDUSA-Add2 and EADpitt26_CBDpitt20 this is also pronounced when testing the residual endolysin activity after thrombin digestion. This shows that two strategies lead to an improved resistance against proteases. First, the mutations of residues involved in the substrate recognition of the proteases, (in this case single amino acid substitutions like R168Q) and second, a change in the overall conformation of the protein in the way that the accessibility of the digestion site for the protease is probably reduced (in the case the construction of chimaeric constructs by the exchange of CBDs). The lysis activity against *Staphylococcus* cells was superior to pypitt26 in the variant EADpitt26_CBDUSA-Add2 (L56H, L57T) and especially in the variants EADpitt26_CBDUSA-Add2, EADpitt26_CBDUSA-Add2 (E164A, R168A, E180A, E190Q, Y201H), EADpitt26_CBDUSA-Add2 (L56H, L57T, E164A, R168A, Y201H) and EADpitt26_CBDUSA-Add2 (L56H, L57T, E164A, R168A, E180A, E190Q, Y201H). The variants EADpitt26_CBDUSA-Add2 (L56H, L57T, E164A, R168A, Y201H) and EADpitt26_CBDUSA-Add2 (L56H, L57T, E164A, R168A, E180A, E190Q, Y201H) also show a very good to good lysis activity in human blood serum. It turned out that especially the mutations L56H, L57T, and Y201H are beneficial for the lysis activity of the protein. The host range of the pypitt26 variants was tested using 67 strains of the genus *Staphylococcus*, 32 strains of *Staphylococcus aureus*, and 12 *Staphylococcus aureus* MRSA strains. A very good sensitivity with respect to host range (+++) means the lysis of more than 80 % of the *Staphylococcus* strains, more than 90 % of the *Staphylococcus aureus* strains, and more than 75 % of the *Staphylococcus aureus* MRSA strains. Almost all variants show a very good host range sensitivity towards *Staphylococcus* cells. Pypitt26 host range sensitivity is slightly reduced, and the sensitivity of the chimaeric construct EADpitt26_CBDpitt20 is weaker towards cells from the genus *Staphylococcus*, and MRSA strains. Long term stability at room temperature was improved in all the variants tested compared to pypitt26. Especially preferred with respect to storage stability are the variants EADpitt26_CBDUSA, EADpitt26_CBDUSA-Add2 (L56H, L57T) and EADpitt26_CBDUSA-Add2 (L56H, L57T, E164A, R168A, E180A, E190Q, Y201H). The mutations L56H and L57T have a positive effect on stability and solubility of pypitt26. In summary, all the variants presented show advantages over the naturally existing endolysin pypitt26. Especially preferred are the variants EADpitt26_CBDUSA-Add2 (L56H, L57T, E164A, R168A, Y201H) and EADpitt26_CBDUSA-Add2 (L56H, L57T, E164A, R168A, E180A, E190Q, Y201H) which show the best performance over all properties tested.

Example 15: Immunogenicity study

[0097] For a use of the endolysins as a therapeutic or prophylactic agent against staphylococcal infections, the immunogenicity of the proteins has to be tested in an animal model in order to get information about the immune response of the organisms elicited by the foreign proteins. 10 female Balb/c mice per group were used as an animal model for the immunogenicity study for IV-therapy. The pypitt26 endolysin variant EADpypitt26_CBDplyUSA-Add2_M5 was applied with a slow, low pressure IV bolus injection in formulation buffer (25 mM Tris pH 7.5, 10 mM citrate, 10 mM CaCl₂, 300 mM arginine) at t=0 h, 24 h and 48 h for the three groups at a dosis of 15 mg/kg body weight. Endpoint serum withdrawal was after 2 or 4 or 8 weeks for the three groups, respectively. 3 mice in each group achieved medication with buffer without endolysin as a control. 3 female Balb/c mice per group were used as an animal model for the control immunogenicity study for IP-injection. Here, the pypitt26 endolysin variant EADpypitt26_CBDplyUSA-Add2_M5 was applied with IP injection with adjuvans water/oil emulsion at t=0 h, 14 days and 28 days for the three groups at a dosis of 15 mg/kg body weight. Endpoint serum withdrawal was after 10 or 21 or 35 days for the three groups, respectively. The degree of immunogenicity was tested in an ELISA assay using EADpypitt26_CBDplyUSA-Add2_M5 coupled to microtiter plates and colorimetric signal detection using a secondary antibody against the diverse groups of antibodies resulting in the immune response. The results for the IV and IP injection are given in tables 5 and 6. Depicted is the lowest antibody titer which still gives a signal significantly below the background signal which was derived from the controls.

Table 5: Antibody titers after IV injection of EADpypitt26_CBDplyUSA-Add2_M5

time (weeks)	antibody titer (1/x)				
	IgA	IgM	IgG1	IgG2a	IgE
0	0	0	0	0	0
2	100	100	400	400	100

EP 2 185 699 B1

(continued)

time (weeks)	antibody titer (1/x)				
	IgA	IgM	IgG1	IgG2a	IgE
4	6400	1600	6400	6400	400
8	6400	6400	25000	6400	6400

Table 6: Antibody titers after IP injection of EADplypitti26_CBDplyUSA-Add2_M5

time (days)	antibody titer (1/x)				
	IgA	IgM	IgG1	IgG2a	IgE
0	0	0	0	0	0
10	1600	6400	100000	25000	6400
21	100000	400000	400000	1600000	100000
35	1600000	1600000	1600000	1600000	1600000

[0098] It is seen that antibody titers were low after IV injection of EADplypitti26_CBDplyUSA-Add2_M5 compared to IP injection which was used as a control for a strong immune response.

Example 16: Neutralisation of the activity of EADplypitti26_CBDplyUSA-Add2_M5 by mouse antisera

[0099] In order to test whether the antibodies built during the immune response after IV injection against EADplypitti26_CBDplyUSA-Add2_M5 are able to neutralize the activity of the endolysin, the lysis activity against a staphylococcal cell wall preparation was tested in a microtiter plate turbidity assay. EADplypitti26_CBDplyUSA-Add2_M5 (0.1 mg/ml) in buffer 20 mM Hepes, 150 mM NaCl, pH 7.4 was incubated for 1 h at 30 °C with serial dilutions (dilution factors of 0, 0.5, 0.2, 0.1, 0.05, 0.1, 0.05, 0.01, 0.001, 0.0001) of mice antisera generated 4 and 8 weeks after IV injection according to the protocol described in example 15. 10 µl of the incubated solutions of the different serial dilutions were mixed with 190 µl staphylococcal cell wall preparation, and the decrease in absorption at 620 nm measured over time starting from an A_{620} of ca. 2.0. The time needed for a decrease in absorption of $\Delta A_{620} = 0.1$ is a measure for the activity of the endolysin. The activities measured for samples after addition of preimmune sera at the respective dilutions served as controls for activity. The activity values presented in table 7 represent mean values derived from the sera of the 7 mice each IV-injected with EADplypitti26_CBDplyUSA-Add2_M5 as described in example 15.

Table 7: Endolysin activity against staphylococcal cell wall preparations after neutralisation with sera derived from IV injection with EADplypitti26_CBDplyUSA-Add2_M5

serum dilution	activity, %		
	preimmune serum	serum 4 weeks	serum 8 weeks
0	100.0	94.9	99.4
0.5	100.0	102.3	115.0
0.2	100.0	109.0	114.8
0.1	100.0	110.8	108.0
0.05	100.0	114.4	102.5
0.01	100.0	103.5	100.2
0.001	100.0	110.4	83.2
0.0001	100.0	109.1	100.0

[0100] The activity assay shows that the staphylococcal endolysin variant according to the invention EADplypitti26_CBDplyUSA-Add2_M5 is not inactivated by the antibodies generated after IV injection to a significant

level. On the contrary, a slight activation of the endolysin activity is observed after addition of antisera in most of the samples. Thus, the antibodies generated during the immune response after IV injection are not able to neutralise the endolysin activity of EADplypiti26_CBDplyUSA-Add2_M5 to a significant degree.

Example 17: High dose toxicity of the staphylococcal endolysin variant EADplypiti26_CBDplyUSA-Add2_M5 in mice

[0101] In order to test the potential toxicity of EADplypiti26_CBDplyUSA-Add2_M5, 10 healthy female CFW1 mice were IV bolus injected with a dosis of 100 mg endolysin per kg body weight. Injection was repeated after time periods of 1 h, 6 h, and 22 h. The injection volume was 0.2 ml each in buffer 20 mM Tris, 10 mM CaCl₂, 10 mM citrate, 300 mM arginine, pH 7.5. The mice were controlled for clinical signs, body weight, and lethality daily during the next 5 days. In none of the mice tested, there was a loss of body weight or other clinical signs like fever or tremor during the next five days indicating that even a high dose of the staphylococcal endolysin variant EADplypiti26_CBDplyUSA-Add2_M5 was not toxic for healthy mice after repeated IV injection. From the animal model, the staphylococcal endolysin variant EADplypiti26_CBDplyUSA-Add2_M5 appears to be applicable for therapy or prevention of staphylococcal infections without toxic effects.

Example 18: Pharmacokinetic study using the staphylococcal endolysin variant EADplypiti26_CBDplyUSA-Add2_M5

[0102] A pharmacokinetic study in rats was performed in order to determine the half-life of EADplypiti26_CBDplyUSA-Add2_M5 in a living organism. 24 male sprague dawley (CD) rats were inoculated IV into the leg vein with a dosis of 12 mg endolysin per kg body weight. EADplypiti26_CBDplyUSA_M5 was dissolved in buffer 20 mM Tris, 10 mM CaCl₂, 10 mM citrate, 300 mM arginine, pH 7.5. 3 animals each were killed 5 min, 15 min, 30 min, 60 min, 120 min, 240 min or 480 min after IV injection. 3 animals of the control group received only buffer for injection. After killing, blood samples were taken immediately, serum prepared, and freezed in liquid nitrogen. In addition, the organs heart, kidney, liver, spleen and lung were prepared and also freezed in liquid nitrogen. The residual activity of the endolysin variant EADplypiti26_CBDplyUSA-Add2_M5 was determined in a turbidity assay performed in a microtiter plate format. 20 µl serum was mixed with 200 µl staphylococcal cell wall preparation which had an absorption at 620 nm of A₆₂₀ = 2.0. The time needed for the decrease of A₆₂₀ of 0.1 (ΔA₆₂₀ = 0.1) was taken as a measure for the activity of the endolysin. As controls, the activity of respective concentrations (150 µg/ml) of EADplypiti26_CBDplyUSA-Add2_M5 endolysin in buffer and pre-immune serum were measured. A second assay for determination of the half-life of EADplypiti26_CBDplyUSA-Add2_M5 endolysin was a Western-blot analysis of the protein using polyclonal anti EADplypiti26_CBDplyUSA-Add2_M5 endolysin rabbit antiserum in a dilution of 1: 10⁵ and an alkaline phosphatase goat anti-rabbit IgG conjugate as secondary antibody. This Western-blot analysis was also used for the organ extracts, which were prepared by homogenisation of the thawed organs using glass beads and spatula. Organ extracts, blood and serum samples were applied to SDS-polyacrylamide gels, and the separated polypeptide bands blotted to PVDF membranes and developed using the antibodies described.

[0103] From the activity assay described the half-life of the variant EADplypiti26_CBDplyUSA-Add2_M5 after IV injection in rat was about 60 min. 2 h after application there was almost no residual activity measurable. A comparison of the first sample taken after 5 minutes to the activity of the protein in pre-immune serum and formulation buffer showed that there is almost no loss of activity directly after application, which could be due to adsorption of the protein to blood cells or epithelial cells of the blood vessels. From the Western blot assay which was also performed in samples taken from the different organs, it became evident that the disappearance of the protein band for EADplypiti26_CBDplyUSA-Add2_M5 coincided with the loss of activity. Very similar pharmacokinetics were observed in blood, serum and the organs examined suggesting that clearance of the protein is similar in different parts of the body. The only difference observed was that the endolysin was visible as the full-length form in blood, serum, heart, lung, liver, and spleen samples, whereas fragments of the protein were detected at all time points in the kidney sample. The endolysin variant EADplypiti26_CBDplyUSA-Add2_M5 seems to be digested in rat kidney by proteases present in that organ. The pharmacokinetic study identified the staphylococcal endolysin variant EADplypiti26_CBDplyUSA-Add2_M5 as a potential pharmaceutical agent suitable for use in therapy and prevention of staphylococcal infections.

Example 19: Efficacy study of endolysin EADpiti26_CBDUSA-Add2_M5 after systemic infection of mice with *Staphylococcus aureus* cells

[0104] In order to test the efficacy of endolysin EADpiti26_CBDUSA-Add2_M5 as a therapeutic agent against *Staphylococcus* infections, mice were IV infected with *Staphylococcus aureus* cells, and the IV treatment with endolysin EADpiti26_CBDUSA-Add2_M5 was compared with other treatments with respect to survival of the test animals. 48 female healthy CFW1 mice were IV inoculated with 2 x 10⁸ *Staphylococcus aureus* cells of the strain DSMZ 11823 per mouse. The mice were divided into 7 groups á 6 animals. The first three groups of mice were treated with high (100 mg

EP 2 185 699 B1

protein per kg body weight), medium (25 mg protein per kg body weight), or low (5 mg protein per kg body weight) dosages of endolysin EADpiti26_CBDUSA-Add2_M5 which were given in three IV injections 1 h, 6 h, and 22 h post infection. One group of mice received the same injections of formulation buffer (20 mM Tris, 10 mM CaCl₂, 10 mM citrate, 300 mM arginine, pH 7.5). Three groups of mice were treated with high (20 mg protein per kg body weight), medium (5 mg protein per kg body weight), or low (1.25 mg protein per kg body weight) dosages of the antibiotic vancomycin. One group of mice did not receive any treatment, and served as a control for the virulence of the *Staphylococcus aureus* cells. The survival of the mice was observed for the next 5 days after infection. The results are summarized in table 8.

Table 8: Survival of mice after *Staphylococcus aureus* infection achieving different treatments

treatment	concentration (mg/kg)	survival (number of animals)					
		day 0	day 1	day 2	day 3	day 4	day 5
EADpiti26_CBDUSA-Add2_M5 high dosage	100	6	6	6	6	6	6
EADpiti26_CBDUSA-Add2_M5 medium dosage	25	6	6	6	6	6	6
EADpiti26_CBDUSA-Add2_M5 low dosage	5	6	6	6	6	6	6
formulation buffer	-	6	4	0	0	0	0
vancomycin high dosage	20	6	6	6	6	6	6
vancomycin medium dosage	5	6	6	6	6	6	6
vancomycin low dosage	1,25	4	4	4	4	4	2
control	-	6	6	2	0	0	0

[0105] It turned out that IV application of endolysin EADpiti26_CBDUSA-Add2_M5 after IV inoculation of mice with *Staphylococcus aureus* cells at a concentration of 2×10^8 cells guaranteed survival of all mice within the observation period at all three dosages tested. Without treatment, all the animals from the control group had died on day 3. The formulation buffer for the endolysin itself seemed to have a somewhat negative effect, as in this group all the animals had died already on day 2. After treatment with the antibiotic vancomycin all the animals survived in the groups treated with high and medium dosages, but some animals died in the group treated with the low dosage of antibiotic. From the efficacy study, endolysin EADpiti26_CBDUSA-Add2_M5 appears a good tool for treatment of *Staphylococcus aureus* infections, and an alternative to treatment with antibiotics were often resistances occur.

SEQUENCE LISTING

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EP 2 185 699 B1

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EP 2 185 699 B1

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EP 2 185 699 B1

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EP 2 185 699 B1

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EP 2 185 699 B1

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 Arg Gln His Ala Tyr Asp Phe Pro Met Trp Phe Ile Arg Pro Asn Phe
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 Lys Ser Glu Thr Ala Pro Arg Ser Ile Gln Ser Pro Thr Gln Ala Ser
 165 170 175
 Lys Lys Glu Thr Ala Lys Pro Gln Pro Lys Ala Val Glu Leu Lys Ile

EP 2 185 699 B1

180 185 190
 Ile Lys Asp Val Val Lys Gly Tyr Asp Leu Pro Lys Arg Gly Gly Asn
 195 200 205
 5 Pro Lys Gly Ile Val Ile His Asn Asp Ala Gly Ser Lys Gly Ala Thr
 210 215 220
 Ala Glu Ala Tyr Arg Asn Gly Leu Val Asn Ala Pro Ser Ser Arg Leu
 225 230 235 240
 Glu Ala Gly Ile Ala His Ser Tyr Val Ser Gly Asn Thr Val Trp Gln
 245 250 255
 10 Ala Leu Asp Glu Ser Gln Val Gly Trp His Thr Ala Asn Gln Leu Gly
 260 265 270
 Asn Lys Tyr Tyr Tyr Gly Ile Glu Val Cys Gln Ser Met Gly Ala Asp
 275 280 285
 Asn Ala Thr Phe Leu Lys Asn Glu Gln Ala Thr Phe Gln Glu Cys Ala
 290 295 300
 15 Arg Leu Leu Lys Lys Trp Gly Leu Pro Ala Asn Arg Asn Thr Ile Arg
 305 310 315 320
 Leu His Asn Glu Phe Thr Ser Thr Ser Cys Pro His Arg Ser Ser Val
 325 330 335
 20 Leu His Thr Gly Phe Asp Pro Val Thr Arg Gly Leu Leu Pro Glu Asp
 340 345 350
 Lys Arg Leu Gln Leu Lys Asp Tyr Phe Ile Lys Gln Ile Arg Ala Tyr
 355 360 365
 Met Asp Gly Lys Ile Pro Val Ala Thr Val Ser Asn Glu Ser Ser Ala
 370 375 380
 25 Ser Ser Asn Thr Val Lys Pro Val Ala Glu Leu Met Pro Pro Val Pro
 385 390 395 400
 Ala Gly Tyr Thr Leu Asp Lys Asn Asn Val Pro Tyr Lys Lys Glu Gln
 405 410 415
 30 Gly Asn Tyr Thr Val Ala Asn Val Lys Gly Asn Asn Val Arg Asp Gly
 420 425 430
 Tyr Ser Thr Asn Ser Arg Ile Thr Gly Val Leu Pro Asn Asn Thr Thr
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 Ile Thr Tyr Asp Gly Ala Tyr Cys Ile Asn Gly Tyr Arg Trp Ile Thr
 450 455 460
 35 Tyr Ile Ala Asn Ser Gly Gln Arg Arg Tyr Ile Ala Thr Gly Glu Val
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<212> DNA

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<220>

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EP 2 185 699 B1

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 ggtgcaaaaag atataccatt tgcaaacaaat ttcgatggac tagctactgt ataccaaaat 240
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 25 agatggatta cttatattgc taatagtgga caacgtcgct atattgcgac cggagaggta 1440
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35 <220>
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<400> 11

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EP 2 185 699 B1

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 20 25 30
 Val Asp Leu Trp Tyr Gly Phe Gln Cys Phe Asp Tyr Ala Asn Ala Gly
 35 40 45
 Trp Lys Val Leu Phe Gly Leu Leu Lys Gly Leu Gly Ala Lys Asp
 50 55 60
 Ile Pro Phe Ala Asn Asn Phe Asp Gly Leu Ala Thr Val Tyr Gln Asn
 65 70 75 80
 Thr Pro Asp Phe Leu Ala Gln Pro Gly Asp Met Val Val Phe Gly Ser
 85 90 95
 Asn Tyr Gly Ala Gly Tyr Gly His Val Ala Trp Val Ile Glu Ala Thr
 100 105 110
 Leu Asp Tyr Ile Ile Val Tyr Glu Gln Asn Trp Leu Gly Gly Gly Trp
 115 120 125
 Thr Asp Arg Ile Glu Gln Pro Gly Trp Gly Trp Glu Lys Val Thr Arg
 130 135 140
 Arg Gln His Ala Tyr Asp Phe Pro Met Trp Phe Ile Arg Pro Asn Phe
 145 150 155 160
 Lys Ser Glu Thr Ala Pro Arg Ser Ile Gln Ser Pro Thr Gln Ala Ser
 165 170 175
 Lys Lys Glu Thr Ala Lys Pro Gln Pro Lys Ala Val Glu Leu Lys Ile
 180 185 190
 Ile Lys Asp Val Val Lys Gly Tyr Asp Leu Pro Lys Arg Gly Gly Asn
 195 200 205
 Pro Lys Gly Ile Val Ile His Asn Asp Ala Gly Ser Lys Gly Ala Thr
 210 215 220
 Ala Glu Ala Tyr Arg Asn Gly Leu Val Asn Ala Pro Ser Ser Arg Leu
 225 230 235 240
 Glu Ala Gly Ile Ala His Ser Tyr Val Ser Gly Asn Thr Val Trp Gln
 245 250 255
 Ala Leu Asp Glu Ser Gln Val Gly Trp His Thr Ala Asn Gln Leu Gly

EP 2 185 699 B1

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	Arg	Leu	Leu	Lys	Lys	Trp	Gly	Leu	Pro	Ala	Asn	Arg	Asn	Thr	Ile	Arg
	305					310					315					320
	Leu	His	Asn	Glu	Phe	Thr	Ser	Thr	Ser	Cys	Pro	His	Arg	Ser	Ser	Val
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	385					390					395					400
	Pro	Lys	Gly	Trp	Ser	Glu	Asn	Pro	Tyr	Gly	Thr	Tyr	Tyr	Lys	Lys	Val
				405						410						415
20	Asp	Lys	Thr	Phe	Ile	Val	Gly	Ser	Glu	Lys	Ile	Glu	Thr	Arg	Ile	Gly
				420					425					430		
	Ser	Pro	Phe	Leu	Ser	Ala	Pro	Ser	Gly	Gly	His	Val	Thr	Pro	Asn	Gln
			435					440					445			
	Lys	Met	Thr	Phe	Asp	Tyr	Leu	Ala	Gln	Gln	Asp	Gly	Tyr	Glu	Trp	Gly
		450					455					460				
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<400> 12

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EP 2 185 699 B1

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	acaccggact	ttttggcaca	acccggcgac	atgggtgtat	tcggtagcaa	ttacggtgca	300
	ggatacggac	acgtagcatg	ggtaattgaa	gcaactttag	attatatcat	tgtatatgag	360
	cagaattggc	taggcgggtg	ctggactgac	agaatcgaac	aaccgggctg	gggttgggaa	420
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	cctaaagggt	ggagtgaaaa	tccttatggt	acttactata	aaaaagttga	taaaacattt	1260
25	attgtagggt	cagaaaaaat	tgaaacacga	attgggttcac	ctttcttata	tgcaccaagt	1320
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	tatgaatggg	gacaacttga	aaataataga	ggacaacaag	aatttgtacc	aattagacca	1440
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<210> 13
 <211> 501
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<220>
 <223> pitti26-pitti20

<400> 13

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EP 2 185 699 B1

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 Lys Glu Phe Ile Glu Trp Leu Lys Thr Ser Glu Gly Lys Gln Phe Asn
 20 25 30
 Val Asp Leu Trp Tyr Gly Phe Gln Cys Phe Asp Tyr Ala Asn Ala Gly
 35 40 45
 Trp Lys Val Leu Phe Gly Leu Leu Lys Gly Leu Gly Ala Lys Asp
 50 55 60
 Ile Pro Phe Ala Asn Asn Phe Asp Gly Leu Ala Thr Val Tyr Gln Asn
 65 70 75 80
 Thr Pro Asp Phe Leu Ala Gln Pro Gly Asp Met Val Val Phe Gly Ser
 85 90 95
 Asn Tyr Gly Ala Gly Tyr Gly His Val Ala Trp Val Ile Glu Ala Thr
 100 105 110
 Leu Asp Tyr Ile Ile Val Tyr Glu Gln Asn Trp Leu Gly Gly Gly Trp
 115 120 125
 Thr Asp Arg Ile Glu Gln Pro Gly Trp Gly Trp Glu Lys Val Thr Arg
 130 135 140
 Arg Gln His Ala Tyr Asp Phe Pro Met Trp Phe Ile Arg Pro Asn Phe
 145 150 155 160
 Lys Ser Glu Thr Ala Pro Arg Ser Ile Gln Ser Pro Thr Gln Ala Ser
 165 170 175
 Lys Lys Glu Thr Ala Lys Pro Gln Pro Lys Ala Val Glu Leu Lys Ile
 180 185 190
 Ile Lys Asp Val Val Lys Gly Tyr Asp Leu Pro Lys Arg Gly Gly Asn
 195 200 205
 Pro Lys Gly Ile Val Ile His Asn Asp Ala Gly Ser Lys Gly Ala Thr
 210 215 220
 Ala Glu Ala Tyr Arg Asn Gly Leu Val Asn Ala Pro Ser Ser Arg Leu
 225 230 235 240
 Glu Ala Gly Ile Ala His Ser Tyr Val Ser Gly Asn Thr Val Trp Gln
 245 250 255
 Ala Leu Asp Glu Ser Gln Val Gly Trp His Thr Ala Asn Gln Leu Gly
 260 265 270
 Asn Lys Tyr Tyr Tyr Gly Ile Glu Val Cys Gln Ser Met Gly Ala Asp
 275 280 285
 Asn Ala Thr Phe Leu Lys Asn Glu Gln Ala Thr Phe Gln Glu Cys Ala
 290 295 300
 Arg Leu Leu Lys Lys Trp Gly Leu Pro Ala Asn Arg Asn Thr Ile Arg
 305 310 315 320
 Leu His Asn Glu Phe Thr Ser Thr Ser Cys Pro His Arg Ser Ser Val
 325 330 335
 Leu His Thr Gly Phe Asp Pro Val Thr Arg Gly Leu Leu Pro Glu Asp
 340 345 350
 Lys Arg Leu Gln Leu Lys Asp Tyr Phe Ile Lys Gln Ile Arg Ala Tyr
 355 360 365

EP 2 185 699 B1

Met Asp Gly Lys Ile Pro Val Ala Thr Val Ser Asn Glu Ser Ser Ala
 370 375 380
 Ser Ser Asn Thr Val Lys Pro Val Ala Glu Leu Met Lys Arg Lys Lys
 385 390 395 400
 Pro Lys Gly Trp Ser Glu Asn Pro Tyr Gly Thr Tyr Tyr Lys Lys Val
 405 410 415
 Asp Lys Thr Phe Ile Val Gly Ser Glu Lys Ile Glu Thr Arg Ile Gly
 420 425 430
 Ser Pro Phe Leu Ser Ala Pro Ser Gly Gly His Val Thr Pro Asn Gln
 435 440 445
 Lys Met Thr Phe Asp Tyr Leu Ala Gln Gln Asp Gly Tyr Glu Trp Gly
 450 455 460
 Gln Leu Glu Asn Asn Arg Gly Gln Gln Glu Phe Val Pro Ile Arg Pro
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 gaagcgggta ttgcgcatag ttatgtatca ggtaacacag tgtggcaagc tttagatgaa 780
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 <212> PRT

EP 2 185 699 B1

<213> artificial sequence

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<223> pitti26__prophage SA2 USA

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<400> 15

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EP 2 185 699 B1

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 1 5 10 15
 Lys Lys Glu Phe Ile Glu Trp Leu Lys Thr Ser Glu Gly Lys Gln Phe
 20 25 30
 5 Asn Val Asp Leu Trp Tyr Gly Phe Gln Cys Phe Asp Tyr Ala Asn Ala
 35 40 45
 Gly Trp Lys Val Leu Phe Gly His Thr Leu Lys Gly Leu Gly Ala Lys
 50 55 60
 10 Asp Ile Pro Phe Ala Asn Asn Phe Asp Gly Leu Ala Thr Val Tyr Gln
 65 70 75 80
 Asn Thr Pro Asp Phe Leu Ala Gln Pro Gly Asp Met Val Val Phe Gly
 85 90 95
 Ser Asn Tyr Gly Ala Gly Tyr Gly His Val Ala Trp Val Ile Glu Ala
 100 105 110
 15 Thr Leu Asp Tyr Ile Ile Val Tyr Glu Gln Asn Trp Leu Gly Gly Gly
 115 120 125
 Trp Thr Asp Arg Ile Glu Gln Pro Gly Trp Gly Trp Glu Lys Val Thr
 130 135 140
 Arg Arg Gln His Ala Tyr Asp Phe Pro Met Trp Phe Ile Arg Pro Asn
 145 150 155 160
 20 Phe Lys Ser Ala Thr Ala Pro Ala Ser Ile Gln Ser Pro Thr Gln Ala
 165 170 175
 Ser Lys Lys Glu Thr Ala Lys Pro Gln Pro Lys Ala Val Glu Leu Lys
 180 185 190
 25 Ile Ile Lys Asp Val Val Lys Gly His Asp Leu Pro Lys Arg Gly Gly
 195 200 205
 Asn Pro Lys Gly Ile Val Ile His Asn Asp Ala Gly Ser Lys Gly Ala
 210 215 220
 Thr Ala Glu Ala Tyr Arg Asn Gly Leu Val Asn Ala Pro Ser Ser Arg
 225 230 235 240
 30 Leu Glu Ala Gly Ile Ala His Ser Tyr Val Ser Gly Asn Thr Val Trp
 245 250 255
 Gln Ala Leu Asp Glu Ser Gln Val Gly Trp His Thr Ala Asn Gln Leu
 260 265 270
 Gly Asn Lys Tyr Tyr Tyr Gly Ile Glu Val Cys Gln Ser Met Gly Ala
 275 280 285
 35 Asp Asn Ala Thr Phe Leu Lys Asn Glu Gln Ala Thr Phe Gln Glu Cys
 290 295 300
 Ala Arg Leu Leu Lys Lys Trp Gly Leu Pro Ala Asn Arg Asn Thr Ile
 305 310 315 320
 40 Arg Leu His Asn Glu Phe Thr Ser Thr Ser Cys Pro His Arg Ser Ser
 325 330 335
 Val Leu His Thr Gly Phe Asp Pro Val Thr Arg Gly Leu Leu Pro Glu
 340 345 350
 Asp Lys Arg Leu Gln Leu Lys Asp Tyr Phe Ile Lys Gln Ile Arg Ala
 355 360 365
 45 Tyr Met Asp Gly Lys Ile Pro Val Ala Thr Val Ser Asn Glu Ser Ser
 370 375 380
 Ala Ser Ser Asn Thr Val Lys Pro Val Ala Glu Leu Met Pro Pro Val
 385 390 395 400
 50 Pro Ala Gly Tyr Thr Leu Asp Lys Asn Asn Val Pro Tyr Lys Lys Glu
 405 410 415
 Gln Gly Asn Tyr Thr Val Ala Asn Val Lys Gly Asn Asn Val Arg Asp
 420 425 430
 Gly Tyr Ser Thr Asn Ser Arg Ile Thr Gly Val Leu Pro Asn Asn Thr
 435 440 445

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EP 2 185 699 B1

Thr Ile Thr Tyr Asp Gly Ala Tyr Cys Ile Asn Gly Tyr Arg Trp Ile
 450 455 460
 Thr Tyr Ile Ala Asn Ser Gly Gln Arg Arg Tyr Ile Ala Thr Gly Glu
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 <212> DNA
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<220>
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<220>
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<400> 17

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	ctgggcgcgga	aagatattcc	gtttgcgaat	aactttgatg	gcctggccac	cgtgtatcag	240
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	gcggttatg	gccatgtggc	gtgggtgatt	gaagcgaccc	tggattatat	catcgtgtac	360
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10	gaaaaagtga	cccgtcgtca	gcatgcgtac	gattttccga	tgtggtttat	tgcgccgaac	480
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	ccggccggtt	ataccctgga	taaaaacaac	gtgccgtata	aaaaagaaca	gggcaactat	1260
	accgtggcga	acgtgaaagg	caacaacgtg	cgtgatggct	atagcaccac	cagccgtatt	1320
	accggcgtgc	tgccgaacaa	caccaccatt	acctatgatg	gcgcgtattg	cattaacggc	1380
25	tatcgtcgga	ttacctatat	cgcgaacagc	ggccagcgtc	gttatattgc	gaccggcgaa	1440
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<213> artificial sequence

<220>

<223> pitti26_prophage SA2 USA

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<400> 18

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EP 2 185 699 B1

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 Lys Lys Glu Phe Ile Glu Trp Leu Lys Thr Ser Glu Gly Lys Gln Phe
 20 25 30
 5 Asn Val Asp Leu Trp Tyr Gly Phe Gln Cys Phe Asp Tyr Ala Asn Ala
 35 40 45
 Gly Trp Lys Val Leu Phe Gly His Thr Leu Lys Gly Leu Gly Ala Lys
 50 55 60
 10 Asp Ile Pro Phe Ala Asn Asn Phe Asp Gly Leu Ala Thr Val Tyr Gln
 65 70 75 80
 Asn Thr Pro Asp Phe Leu Ala Gln Pro Gly Asp Met Val Val Phe Gly
 85 90 95
 Ser Asn Tyr Gly Ala Gly Tyr Gly His Val Ala Trp Val Ile Glu Ala
 100 105 110
 15 Thr Leu Asp Tyr Ile Ile Val Tyr Glu Gln Asn Trp Leu Gly Gly Gly
 115 120 125
 Trp Thr Asp Arg Ile Glu Gln Pro Gly Trp Gly Trp Glu Lys Val Thr
 130 135 140
 Arg Arg Gln His Ala Tyr Asp Phe Pro Met Trp Phe Ile Arg Pro Asn
 145 150 155 160
 20 Phe Lys Ser Ala Thr Ala Pro Ala Ser Ile Gln Ser Pro Thr Gln Ala
 165 170 175
 Ser Lys Lys Glu Thr Ala Lys Pro Gln Pro Lys Ala Val Glu Leu Lys
 180 185 190
 25 Ile Ile Lys Asp Val Val Lys Gly His Asp Leu Pro Lys Arg Gly Gly
 195 200 205
 Asn Pro Lys Gly Ile Val Ile His Asn Asp Ala Gly Ser Lys Gly Ala
 210 215 220
 Thr Ala Glu Ala Tyr Arg Asn Gly Leu Val Asn Ala Pro Leu Ser Arg
 225 230 235 240
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EP 2 185 699 B1

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<211> 1497

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<213> artificial sequence

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<223> pitt26 prophage SA2 USA

<400> 19

EP 2 185 699 B1

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	aacaccccg	attttctggc	ccagccgggc	gatatgggtg	tgtttggcag	caactatggc	300
	gcgggttatg	gccatgtggc	gtgggtgatt	gaagcgaccc	tggattatat	catcgtgtac	360
	gaacagaaact	ggctgggcgg	tggctggacc	gatcgtattg	aacagccggg	ctggggctgg	420
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	ccggccgggtt	ataccctgga	taaaaacaac	gtgccgtata	aaaaagaaca	gggcaactat	1260
	accgtggcga	acgtgaaagg	caacaacgtg	cgtgatggct	atagcaccaa	cagccgtatt	1320
	accggcgtgc	tgccgaacaa	caccaccatt	acctatgatg	gcgcgtattg	cattaacggc	1380
25	tatcgctgga	ttacctatat	cgcgaaacagc	ggccagcgtc	gttatattgc	gaccggcgaa	1440
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30 <212> PRT

<213> artificial sequence

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<223> pitti26 lysostaphin

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<400> 20

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EP 2 185 699 B1

Met Ala Ser Ile Ile Met Glu Val Ala Thr Met Gln Ala Lys Leu Thr
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 Lys Lys Glu Phe Ile Glu Trp Leu Lys Thr Ser Glu Gly Lys Gln Phe
 20 25 30
 Asn Val Asp Leu Trp Tyr Gly Phe Gln Cys Phe Asp Tyr Ala Asn Ala
 35 40 45
 Gly Trp Lys Val Leu Phe Gly His Thr Leu Lys Gly Leu Gly Ala Lys
 50 55 60
 Asp Ile Pro Phe Ala Asn Asn Phe Asp Gly Leu Ala Thr Val Tyr Gln
 65 70 75 80
 Asn Thr Pro Asp Phe Leu Ala Gln Pro Gly Asp Met Val Val Phe Gly
 85 90 95
 Ser Asn Tyr Gly Ala Gly Tyr Gly His Val Ala Trp Val Ile Glu Ala
 100 105 110
 Thr Leu Asp Tyr Ile Ile Val Tyr Glu Gln Asn Trp Leu Gly Gly Gly
 115 120 125
 Trp Thr Asp Arg Ile Glu Gln Pro Gly Trp Gly Trp Glu Lys Val Thr
 130 135 140
 Arg Arg Gln His Ala Tyr Asp Phe Pro Met Trp Phe Ile Arg Pro Asn
 145 150 155 160
 Phe Lys Ser Ala Thr Ala Pro Ala Ser Ile Gln Ser Pro Thr Gln Ala
 165 170 175
 Ser Lys Lys Glu Thr Ala Lys Pro Gln Pro Lys Ala Val Glu Leu Lys
 180 185 190
 Ile Ile Lys Asp Val Val Lys Gly His Asp Leu Pro Lys Arg Gly Gly
 195 200 205
 Asn Pro Lys Gly Ile Val Ile His Asn Asp Ala Gly Ser Lys Gly Ala
 210 215 220
 Thr Ala Glu Ala Tyr Arg Asn Gly Leu Val Asn Ala Pro Ser Ser Arg
 225 230 235 240
 Leu Glu Ala Gly Ile Ala His Ser Tyr Val Ser Gly Asn Thr Val Trp
 245 250 255
 Gln Ala Leu Asp Glu Ser Gln Val Gly Trp His Thr Ala Asn Gln Leu
 260 265 270
 Gly Asn Lys Tyr Tyr Tyr Gly Ile Glu Val Cys Gln Ser Met Gly Ala
 275 280 285
 Asp Asn Ala Thr Phe Leu Lys Asn Glu Gln Ala Thr Phe Gln Glu Cys
 290 295 300
 Ala Arg Leu Leu Lys Lys Trp Gly Leu Pro Ala Asn Arg Asn Thr Ile
 305 310 315 320
 Arg Leu His Asn Glu Phe Thr Ser Thr Ser Cys Pro His Arg Ser Ser
 325 330 335

EP 2 185 699 B1

Val Leu His Thr Gly Phe Asp Pro Val Thr Arg Gly Leu Leu Pro Glu
 340 345 350
 Asp Lys Arg Leu Gln Leu Lys Asp Tyr Phe Ile Lys Gln Ile Arg Ala
 355 360 365
 Tyr Met Asp Gly Lys Ile Pro Val Ala Thr Val Ser Asn Glu Ser Ser
 370 375 380
 Ala Ser Ser Asn Thr Val Lys Pro Val Ala Glu Leu Met Ser Asn Ser
 385 390 395 400
 Thr Ala Gln Asp Pro Met Pro Phe Leu Lys Ser Ala Gly Tyr Gly Lys
 405 410 415
 Ala Gly Gly Thr Val Thr Pro Thr Pro Asn Thr Gly Trp Lys Thr Asn
 420 425 430
 Lys Tyr Gly Thr Leu Tyr Lys Ser Glu Ser Ala Ser Phe Thr Pro Asn
 435 440 445
 Thr Asp Ile Ile Thr Arg Thr Thr Gly Pro Phe Arg Ser Met Pro Gln
 450 455 460
 Ser Gly Val Leu Lys Ala Gly Gln Thr Ile His Tyr Asp Glu Val Met
 465 470 475 480
 Lys Gln Asp Gly His Val Trp Val Gly Tyr Thr Gly Asn Ser Gly Gln
 485 490 495
 Arg Ile Tyr Leu Pro Val Arg Thr Trp Asn Lys Ser Thr Asn Thr Leu
 500 505 510
 Gly Val Leu Trp Gly Thr Ile Lys
 515 520

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 <213> artificial sequence

<220>
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<400> 21

EP 2 185 699 B1

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	ttaggtgcaa	aagatatacc	atgtgcaaac	aatttcgatg	gactagctac	tgtataccaa	240
5	aatacacogg	actttttggc	acaacccggc	gacatgggtg	tattcggtag	caattacggg	300
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	gagcagaatt	ggctaggcgg	tggtctggact	gacagaatcg	aacaacccgg	ctgggggttg	420
	gaaaaagtta	caagacgaca	acatgcttac	gatttcctta	tgtggtttat	cgttcctaac	480
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	agcaaagggg	caacagcaga	agcgtatcga	aacggattag	ttaacgcacc	ttcatcaaga	720
	ttagaagcgg	gtattgcgca	tagttatgta	tcaggtaaca	cagtgtggca	agcttttagat	780
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	agattacaca	acgaattcac	ttcaacatca	tgcacacaca	gaagctcagt	attgcacact	1020
	ggtttttgacc	cagtaactcg	cgggtctattg	ccagaagaca	agcggttgca	acttaaagac	1080
	tacttttatca	agcagattag	ggcgtacatg	gatggtaaaa	taccggttgc	cactgtctct	1140
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	gtgactccga	ccccgaacac	gggctggaaa	accaacaagt	acggtactct	ttacaaaagc	1320
	gagagcgcat	cttttacgcc	aaacacggac	atcatcacgc	gcaccaccgg	cccatttcgc	1380
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35 <220>
 <223> pitti26 ALE1

<400> 22

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EP 2 185 699 B1

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 Lys Lys Glu Phe Ile Glu Trp Leu Lys Thr Ser Glu Gly Lys Gln Phe
 20 25 30
 5 Asn Val Asp Leu Trp Tyr Gly Phe Gln Cys Phe Asp Tyr Ala Asn Ala
 35 40 45
 Gly Trp Lys Val Leu Phe Gly His Thr Leu Lys Gly Leu Gly Ala Lys
 50 55 60
 10 Asp Ile Pro Phe Ala Asn Asn Phe Asp Gly Leu Ala Thr Val Tyr Gln
 65 70 75 80
 Asn Thr Pro Asp Phe Leu Ala Gln Pro Gly Asp Met Val Val Phe Gly
 85 90 95
 Ser Asn Tyr Gly Ala Gly Tyr Gly His Val Ala Trp Val Ile Glu Ala
 100 105 110
 15 Thr Leu Asp Tyr Ile Ile Val Tyr Glu Gln Asn Trp Leu Gly Gly Gly
 115 120 125
 Trp Thr Asp Arg Ile Glu Gln Pro Gly Trp Gly Trp Glu Lys Val Thr
 130 135 140
 Arg Arg Gln His Ala Tyr Asp Phe Pro Met Trp Phe Ile Arg Pro Asn
 145 150 155 160
 20 Phe Lys Ser Ala Thr Ala Pro Ala Ser Ile Gln Ser Pro Thr Gln Ala
 165 170 175
 Ser Lys Lys Glu Thr Ala Lys Pro Gln Pro Lys Ala Val Glu Leu Lys
 180 185 190
 25 Ile Ile Lys Asp Val Val Lys Gly His Asp Leu Pro Lys Arg Gly Gly
 195 200 205
 Asn Pro Lys Gly Ile Val Ile His Asn Asp Ala Gly Ser Lys Gly Ala
 210 215 220
 Thr Ala Glu Ala Tyr Arg Asn Gly Leu Val Asn Ala Pro Ser Ser Arg
 225 230 235 240
 30 Leu Glu Ala Gly Ile Ala His Ser Tyr Val Ser Gly Asn Thr Val Trp
 245 250 255
 Gln Ala Leu Asp Glu Ser Gln Val Gly Trp His Thr Ala Asn Gln Leu
 260 265 270
 Gly Asn Lys Tyr Tyr Tyr Gly Ile Glu Val Cys Gln Ser Met Gly Ala
 275 280 285
 35 Asp Asn Ala Thr Phe Leu Lys Asn Glu Gln Ala Thr Phe Gln Glu Cys
 290 295 300
 Ala Arg Leu Leu Lys Lys Trp Gly Leu Pro Ala Asn Arg Asn Thr Ile
 305 310 315 320
 40 Arg Leu His Asn Glu Phe Thr Ser Thr Ser Cys Pro His Arg Ser Ser
 325 330 335
 Val Leu His Thr Gly Phe Asp Pro Val Thr Arg Gly Leu Leu Pro Glu
 340 345 350
 Asp Lys Arg Leu Gln Leu Lys Asp Tyr Phe Ile Lys Gln Ile Arg Ala
 355 360 365
 45 Tyr Met Asp Gly Lys Ile Pro Val Ala Thr Val Ser Asn Glu Ser Ser
 370 375 380
 Ala Ser Ser Asn Thr Val Lys Pro Val Ala Glu Leu Met Asn Ser Phe

EP 2 185 699 B1

	385		390		395		400
	Ser Asn Asn Thr Ala Gln Asp Pro Met Pro Phe Leu Lys Ser Ala Gly						
		405		410		415	
5	Tyr Gly Ser Asn Ser Thr Ser Ser Ser Asn Asn Asn Gly Tyr Lys Thr						
		420		425		430	
	Asn Lys Tyr Gly Thr Leu Tyr Lys Ser Glu Ser Ala Ser Phe Thr Ala						
		435		440		445	
	Asn Thr Asp Ile Ile Thr Arg Leu Thr Gly Pro Phe Arg Ser Met Pro						
		450		455		460	
10	Gln Ser Gly Val Leu Arg Lys Gly Leu Thr Ile Lys Tyr Asp Glu Val						
		465		470		475	
	Met Lys Gln Asp Gly His Val Trp Val Gly Tyr Asn Thr Asn Ser Gly						
		485		490		495	
	Lys Arg Val Tyr Leu Pro Val Arg Thr Trp Asn Glu Ser Thr Gly Glu						
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	Leu Gly Pro Leu Trp Gly Thr Ile Lys						
		515		520			
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	caatgctttg attatgccaa tgctgggtgg aaagttttgt ttggacatac actgaaaggt	180					
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	ttcaaaagcg ctacagctcc agcttcaata caatctccta cgcaagcatc taaaaaggaa	540					
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	gaatcgcaag taggttggca tactgctaac caattaggca ataaatatta ttacgggtatt	840					
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EP 2 185 699 B1

<213> unknown

<220>

<223> Lysostaphin

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<400> 24

Ser Asn Ser Thr Ala Gln Asp Pro Met Pro Phe Leu Lys Ser Ala Gly
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 Tyr Gly Lys Ala Gly Gly Thr Val Thr Pro Thr Pro Asn Thr Gly Trp
 20 25 30
 Lys Thr Asn Lys Tyr Gly Thr Leu Tyr Lys Ser Glu Ser Ala Ser Phe
 35 40 45
 Thr Pro Asn Thr Asp Ile Ile Thr Arg Thr Thr Gly Pro Phe Arg Ser
 50 55 60
 Met Pro Gln Ser Gly Val Leu Lys Ala Gly Gln Thr Ile His Tyr Asp
 65 70 75 80
 Glu Val Met Lys Gln Asp Gly His Val Trp Val Gly Tyr Thr Gly Asn
 85 90 95
 Ser Gly Gln Arg Ile Tyr Leu Pro Val Arg Thr Trp Asn Lys Ser Thr
 100 105 110
 Asn Thr Leu Gly Val Leu Trp Gly Thr Ile Lys
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<211> 372

<212> DNA

<213> unknown

30 <220>

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 tacaaaagcg agagcgcac ttttacgcca aacacggaca tcatcacgcg caccacggc 180
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 gaagttatga aacaggacgg ccatgtgtgg gtgggctata ccggcaacag cggccagcgt 300
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<210> 26

<211> 124

45 <212> PRT

<213> unknown

<220>

<223> ALE1

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<400> 26

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EP 2 185 699 B1

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 20 25 30
 Tyr Lys Thr Asn Lys Tyr Gly Thr Leu Tyr Lys Ser Glu Ser Ala Ser
 35 40 45
 Phe Thr Ala Asn Thr Asp Ile Ile Thr Arg Leu Thr Gly Pro Phe Arg
 50 55 60
 Ser Met Pro Gln Ser Gly Val Leu Arg Lys Gly Leu Thr Ile Lys Tyr
 65 70 75 80
 Asp Glu Val Met Lys Gln Asp Gly His Val Trp Val Gly Tyr Asn Thr
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 Asn Ser Gly Lys Arg Val Tyr Leu Pro Val Arg Thr Trp Asn Glu Ser
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 Thr Gly Glu Leu Gly Pro Leu Trp Gly Thr Ile Lys
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 <212> DNA
 <213> unknown

<220>
 <223> ALE1

<400> 27

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 ctgtataaaa gcgaaagtgc gagctttacc gcgaataccg acatcattac gcgattaacg 180
 ggtccgttcc gcagcatgcc gcagagcggg gttttacgca aaggcttaac gattaaatat 240
 gatgaagtta tgaaacagga cggccatgtg tgggtgggtt ataacacgaa cagcggcaaa 300
 cgcgtttact taccggtgcg tacgtggaac gagagcaccg gtgaattagg cccgctgtgg 360
 ggcaccatca aataa 375

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 <211> 497
 <212> PRT
 <213> artificial

<220>
 <223> pitti26 phage USA

<400> 28

EP 2 185 699 B1

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 Lys Lys Glu Phe Ile Glu Trp Leu Lys Thr Ser Glu Gly Lys Gln Phe
 20 25 30
 Asn Val Asp Leu Trp Tyr Gly Phe Gln Cys Phe Asp Tyr Ala Asn Ala
 35 40 45
 Gly Trp Lys Val Leu Phe Gly Leu Leu Leu Lys Gly Leu Gly Ala Lys
 50 55 60
 Asp Ile Pro Phe Ala Asn Asn Phe Asp Gly Leu Ala Thr Val Tyr Gln
 65 70 75 80
 Asn Thr Pro Asp Phe Leu Ala Gln Pro Gly Asp Met Val Val Phe Gly
 85 90 95
 Ser Asn Tyr Gly Ala Gly Tyr Gly His Val Ala Trp Val Ile Glu Ala
 100 105 110
 Thr Leu Asp Tyr Ile Ile Val Tyr Glu Gln Asn Trp Leu Gly Gly Gly
 115 120 125
 Trp Thr Asp Arg Ile Glu Gln Pro Gly Trp Gly Trp Glu Lys Val Thr
 130 135 140
 Arg Arg Gln His Ala Tyr Asp Phe Pro Met Trp Phe Ile Arg Pro Asn
 145 150 155 160
 Phe Lys Ser Glu Thr Ala Pro Arg Ser Ile Gln Ser Pro Thr Gln Ala
 165 170 175
 Ser Lys Lys Glu Thr Ala Lys Pro Gln Pro Lys Ala Val Glu Leu Lys
 180 185 190
 Ile Ile Lys Asp Val Val Lys Gly Tyr Asp Leu Pro Lys Arg Gly Gly
 195 200 205
 Asn Pro Lys Gly Ile Val Ile His Asn Asp Ala Gly Ser Lys Gly Ala

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210 215 220
 Thr Ala Glu Ala Tyr Arg Asn Gly Leu Val Asn Ala Pro Ser Ser Arg
 225 230 235 240
 Leu Glu Ala Gly Ile Ala His Ser Tyr Val Ser Gly Asn Thr Val Trp
 245 250 255
 Gln Ala Leu Asp Glu Ser Gln Val Gly Trp His Thr Ala Asn Gln Leu
 260 265 270
 Gly Asn Lys Tyr Tyr Tyr Gly Ile Glu Val Cys Gln Ser Met Gly Ala
 275 280 285
 Asp Asn Ala Thr Phe Leu Lys Asn Glu Gln Ala Thr Phe Gln Glu Cys
 290 295 300
 Ala Arg Leu Leu Lys Lys Trp Gly Leu Pro Ala Asn Arg Asn Thr Ile
 305 310 315 320
 Arg Leu His Asn Glu Phe Thr Ser Thr Ser Cys Pro His Arg Ser Ser
 325 330 335
 Val Leu His Thr Gly Phe Asp Pro Val Thr Arg Gly Leu Leu Pro Glu
 340 345 350
 Asp Lys Arg Leu Gln Leu Lys Asp Tyr Phe Ile Lys Gln Ile Arg Ala
 355 360 365
 Tyr Met Asp Gly Lys Ile Pro Val Ala Thr Val Ser Asn Glu Ser Ser
 370 375 380
 Ala Ser Ser Asn Thr Val Lys Pro Val Ala Glu Leu Met Pro Pro Val
 385 390 395 400
 Pro Ala Gly Tyr Thr Leu Asp Lys Asn Asn Val Pro Tyr Lys Lys Glu
 405 410 415
 Gln Gly Asn Tyr Thr Val Ala Asn Val Lys Gly Asn Asn Val Arg Asp
 420 425 430
 Gly Tyr Ser Thr Asn Ser Arg Ile Thr Gly Val Leu Pro Asn Asn Thr
 435 440 445
 Thr Ile Thr Tyr Asp Gly Ala Tyr Cys Ile Asn Gly Tyr Arg Trp Ile
 450 455 460
 Thr Tyr Ile Ala Asn Ser Gly Gln Arg Arg Tyr Ile Ala Thr Gly Glu
 465 470 475 480
 Val Asp Ile Ala Gly Asn Arg Ile Ser Ser Phe Gly Lys Phe Ser Ala
 485 490 495
 Val

REFERENCES CITED IN THE DESCRIPTION

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Patentkrav

1. Polypeptidvariant, som omfatter sekvensen som vist i SEQ ID NO:1, hvor polypeptidvarianten, der omfatter sekvensen som vist i SEQ ID NO:1, omfatter et endolysincellebindende domæne (CBD) udvalgt blandt endolysinet CBD af ply_USA som vist i SEQ ID NO:3 eller af ply_pitti20 som vist i SEQ ID NO:5; eller hvor polypeptidvarianten omfatter sekvensen som vist i SEQ ID NO:20 eller 22; hvilken polypeptidvariant desuden eventuelt omfatter:

a) en polypeptidvariant, der omfatter sekvensen af SEQ ID NO:1 med en eller flere punktmutationer eller aminosyresubstitutioner i sekvensen,

(i) hvor de substituerede rester er udvalgt blandt de følgende aminosyrerester af SEQ ID NO:1: F19, W22, W36, F42, Y44, L55, L56, F67, L74, Y78, W107, Y115, I116, Y119, W123, W128, W137, W139, W154, E163, R167, E179, E189, Y200, Y275, Y276, C282, F300 og C303 eller

(ii) hvor den ene eller flere substitutioner i forhold til sekvensen af SEQ ID NO:1 er udvalgt fra gruppen bestående af: W22R, F42A, F44A F67T, Y115S, W123M, W137A, W139A, W154H, E163A, E179Q, E179A, E187Q, Y200A, Y200H, Y275A, Y275M, Y276A, C282A, F300A, C303S, W310A og W310M eller

(iii) hvor en af de følgende substitutioner i forhold til SEQ ID NO:1 er udvalgt fra gruppen bestående af: F67T + Y115S, F67T + W137A, F67T + W139A, F67T + W154H, Y115S + W137A, Y115S + W139A, E163Q + R169A, E163A + R169A, E163Q + R167A + E189Q, E163A + R167A + E189Q, E163Q + R167A + E179Q + E189Q, E163Q + R167A + E179A + E189Q, E163A + R167A + E179Q + E189Q, E163A + R167A + E179A + E189Q, Y200A + Y275A, Y200A + Y276A, Y200A + C282A, Y200A + F300A, Y275A + Y276A, L55H + L56T + E163A + R167A + Y200H, E163A + R167A + E179A + E189Q + Y200H, L55H + L56T + E163A + R167A + E179A + E189Q + Y200H, S237L + R354Q + A367V, og L55H + L56T + E163A + R167A + Y200H + S237L + R354Q + A367V eller

b) en polypeptidvariant, som ud over sekvensen af SEQ ID NO: 1 omfatter sekvenser, der repræsenterer markørenheder, tags eller andre funktionelle polypeptidsekvenser, eller

c) en polypeptidvariant, der omfatter en eller flere yderligere aminosyrerester indført i aminosyresekvensen i henhold til SEQ ID NO:1 udvalgt fra gruppen bestående af SEQ ID NO:15, 18 og 28 eller

d) en polypeptidvariant, der omfatter en polypeptidsekvens, som repræsenterer en kombination af varianter a), b) og c).

- 5 **2.** Polypeptid ifølge krav 1, hvor polypeptidet lyserer *S. aureus*, *S. aureus* (MRSA), *S. epidermidis*, *S. haemolyticus*, *S. simulans*, *S. saprophyticus*, *S. chromogenes*, *S. hyicus*, *S. warneri* og/eller *S. xylosus*.
- 10 **3.** Polypeptid ifølge et af de foregående krav, hvor varianten af polypeptidet, der omfatter sekvensen som vist i SEQ ID NO:1, mangler 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27 eller 28 N-terminal-aminosyrerester af SEQ ID NO:1.
- 15 **4.** Polypeptid ifølge et af de foregående krav, hvor udskiftninger af aminosyrerester F, W, Y, I, L udskiftes med aminosyrerester R, D, E, N, K, Q, H, S, T, M, G eller A.
- 20 **5.** Polypeptid ifølge et af de foregående krav, hvor polypeptidet omfatter biotin eller Streptavidin som en yderligere markørenhed, eller hvor polypeptidet omfatter et HA-tag, His-tag, Strep-tag, Myc-tag eller GST-tag.
- 25 **6.** Polypeptid, der omfatter sekvensen som vist i SEQ ID NO: 7 eller 11, eller et polypeptid ifølge krav 5, hvor polypeptidet omfatter sekvensen som vist i SEQ ID NO: 9 eller SEQ ID NO:13.
- 30 **7.** Nukleinsyremolekyle omfattende en sekvens, der koder for et polypeptid ifølge et af kravene 1 til 6.
- 8.** Nukleinsyremolekyle ifølge krav 7, hvor nukleinsyremolekylet omfatter en sekvens udvalgt blandt SEQ ID NO:8, 10, 12, 14, 16, 17, 19, 21 eller 23.
- 9.** Polypeptid ifølge et af kravene 1 til 6 til anvendelse som et medikament.
- 35 **10.** Polypeptid ifølge et af kravene 1 til 6 til anvendelse som et medikament til behandling eller forebyggelse af Staphylococcus-infektioner.

11. Desinfektionssammensætning omfattende et polypeptid ifølge et af kravene 1 til 6.

5

12. Kosmetisk sammensætning omfattende et polypeptid ifølge et af kravene 1 til 6.

13. Farmaceutisk sammensætning omfattende et polypeptid ifølge et af kravene 1 til 6.

10

DRAWINGS

Fig. 1

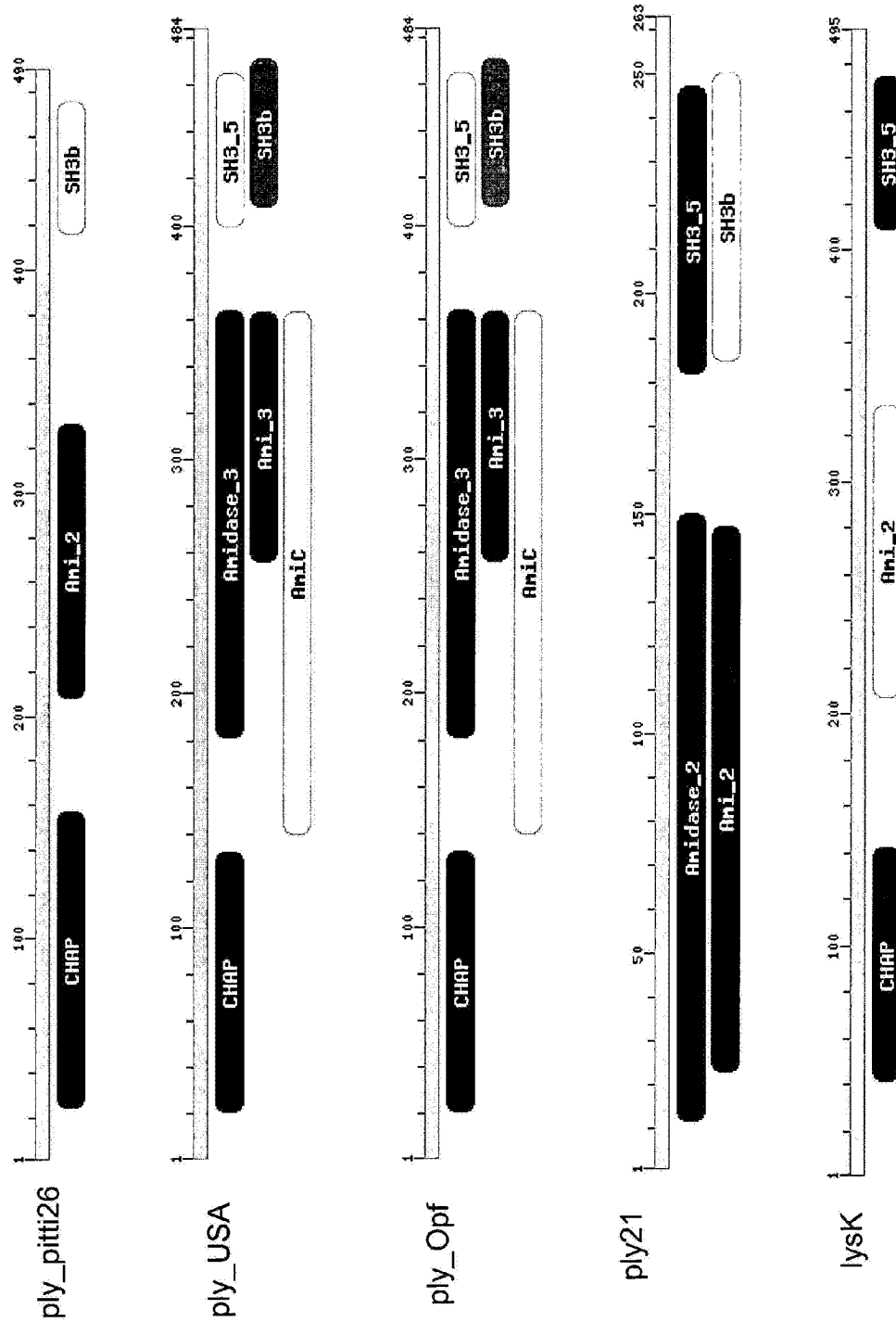


Fig. 2

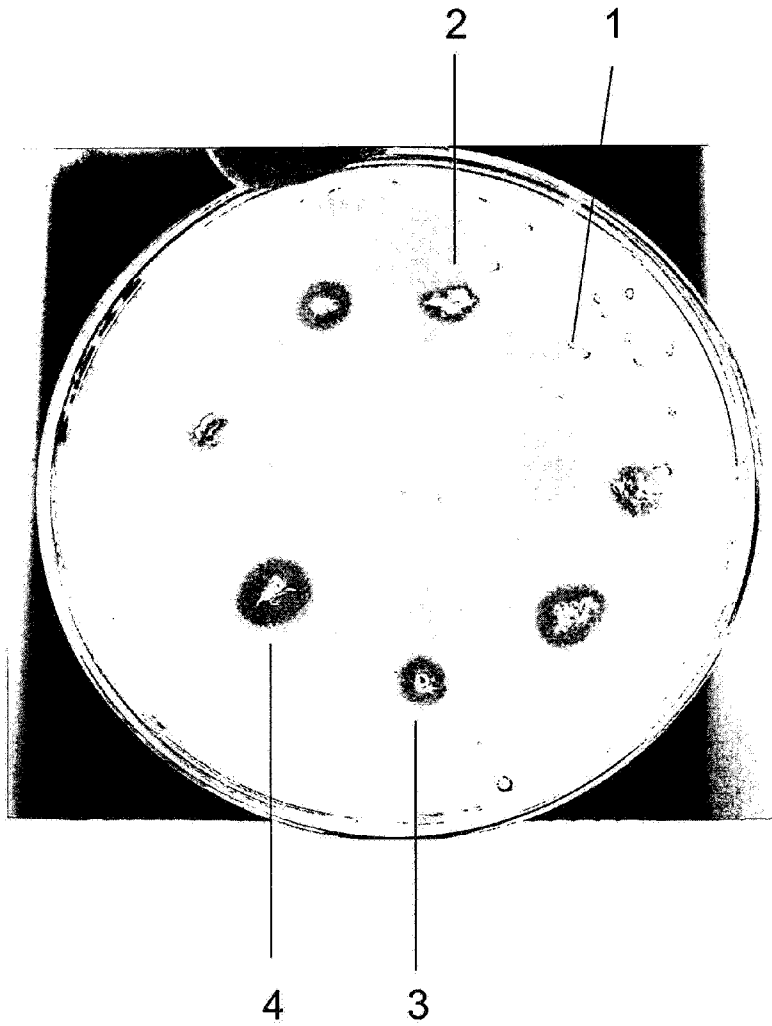


Fig. 3

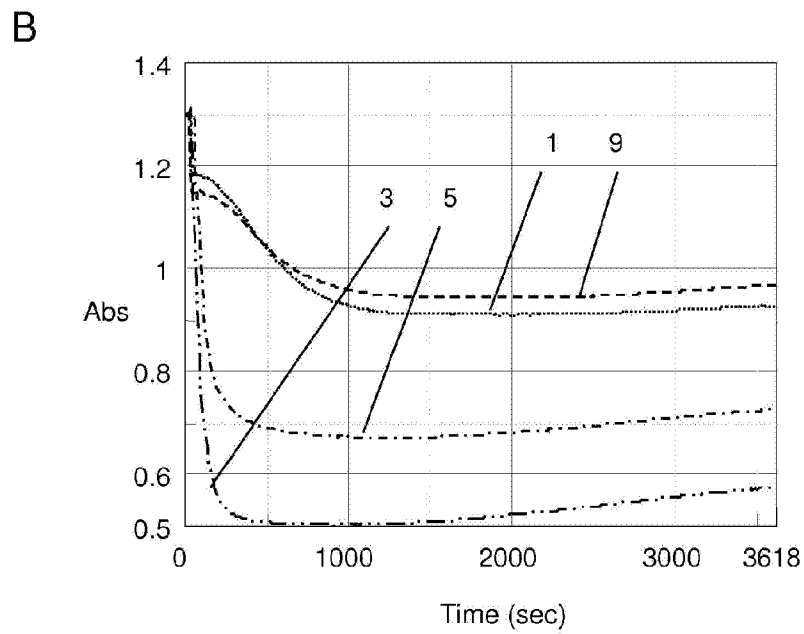
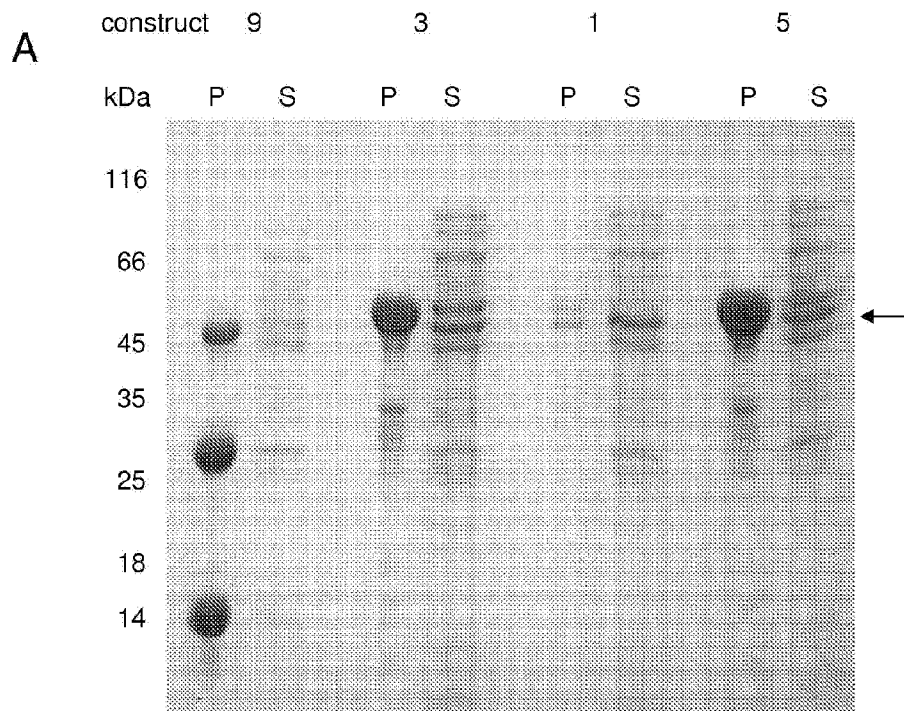


Fig. 4

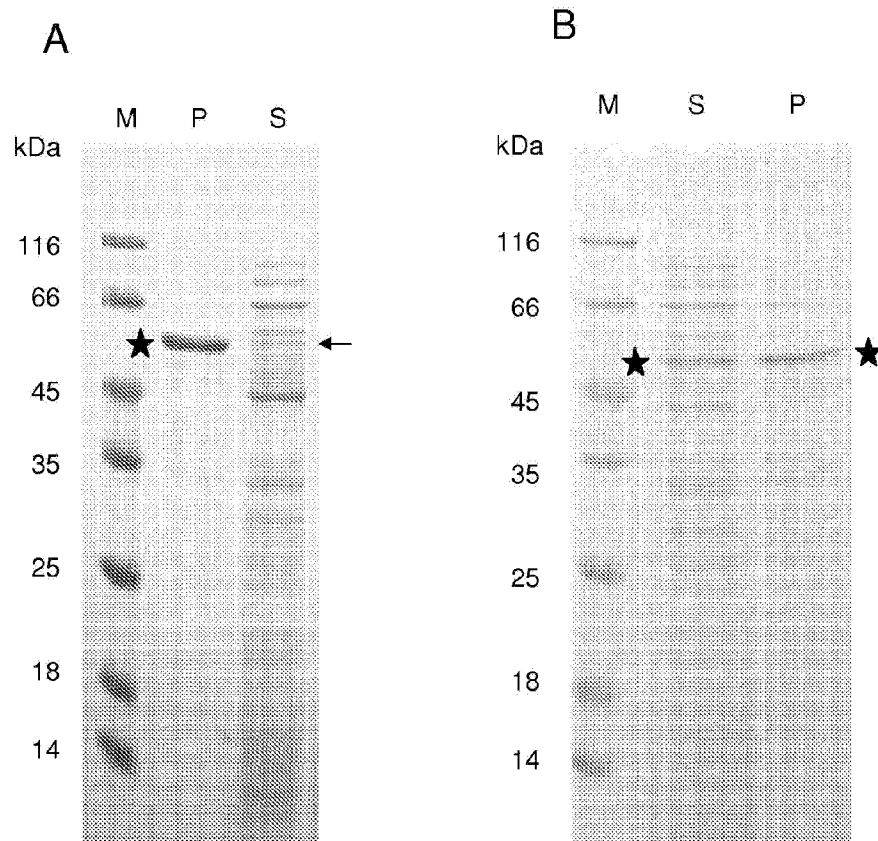


Fig. 5

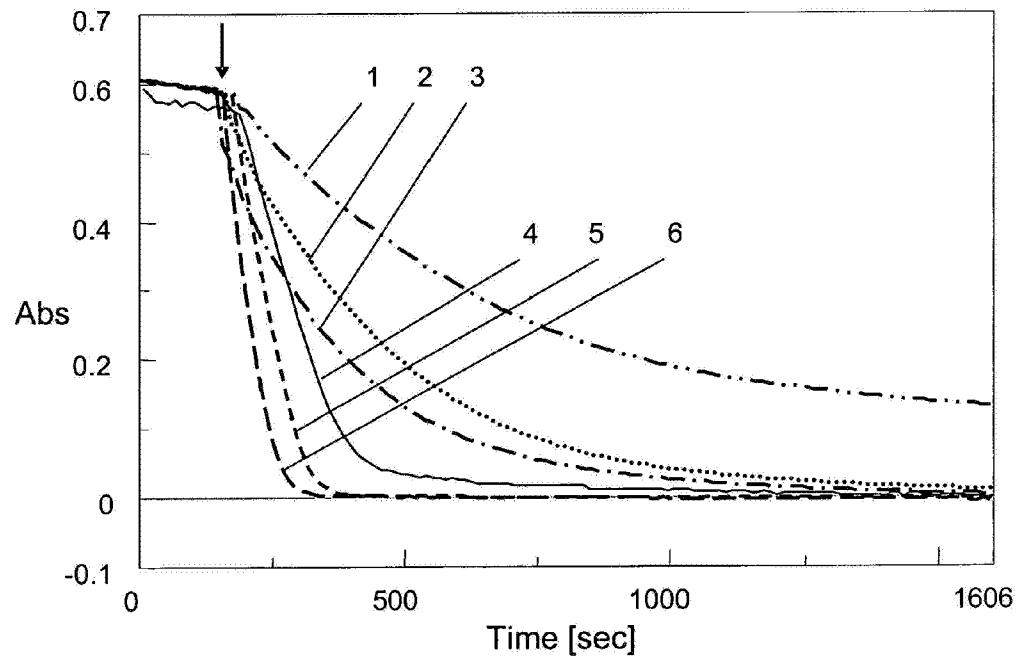


Fig. 6

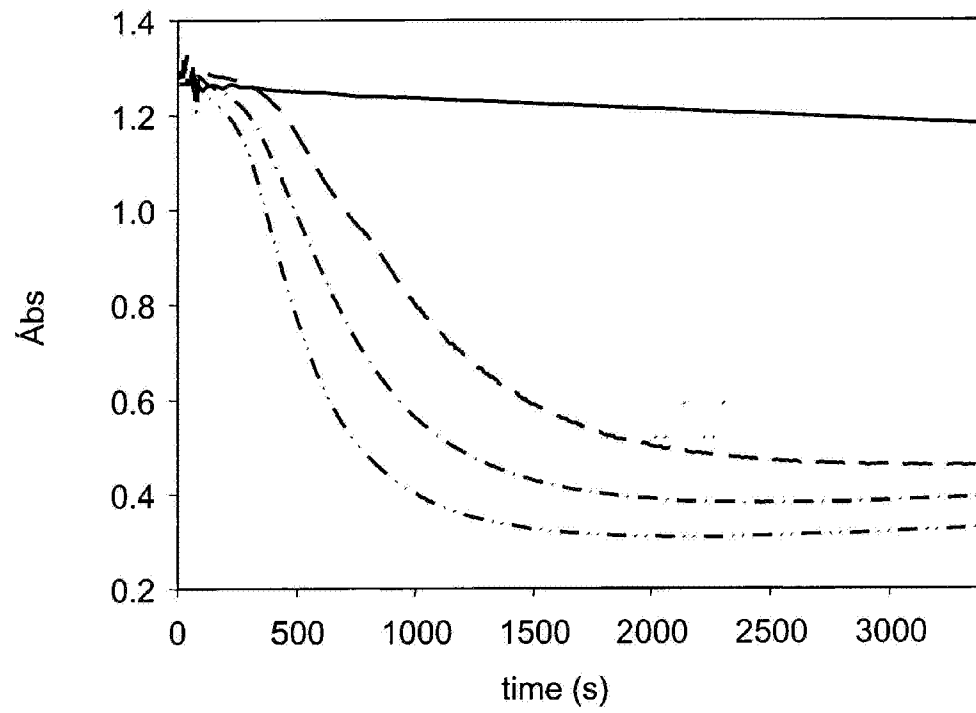


Fig. 7

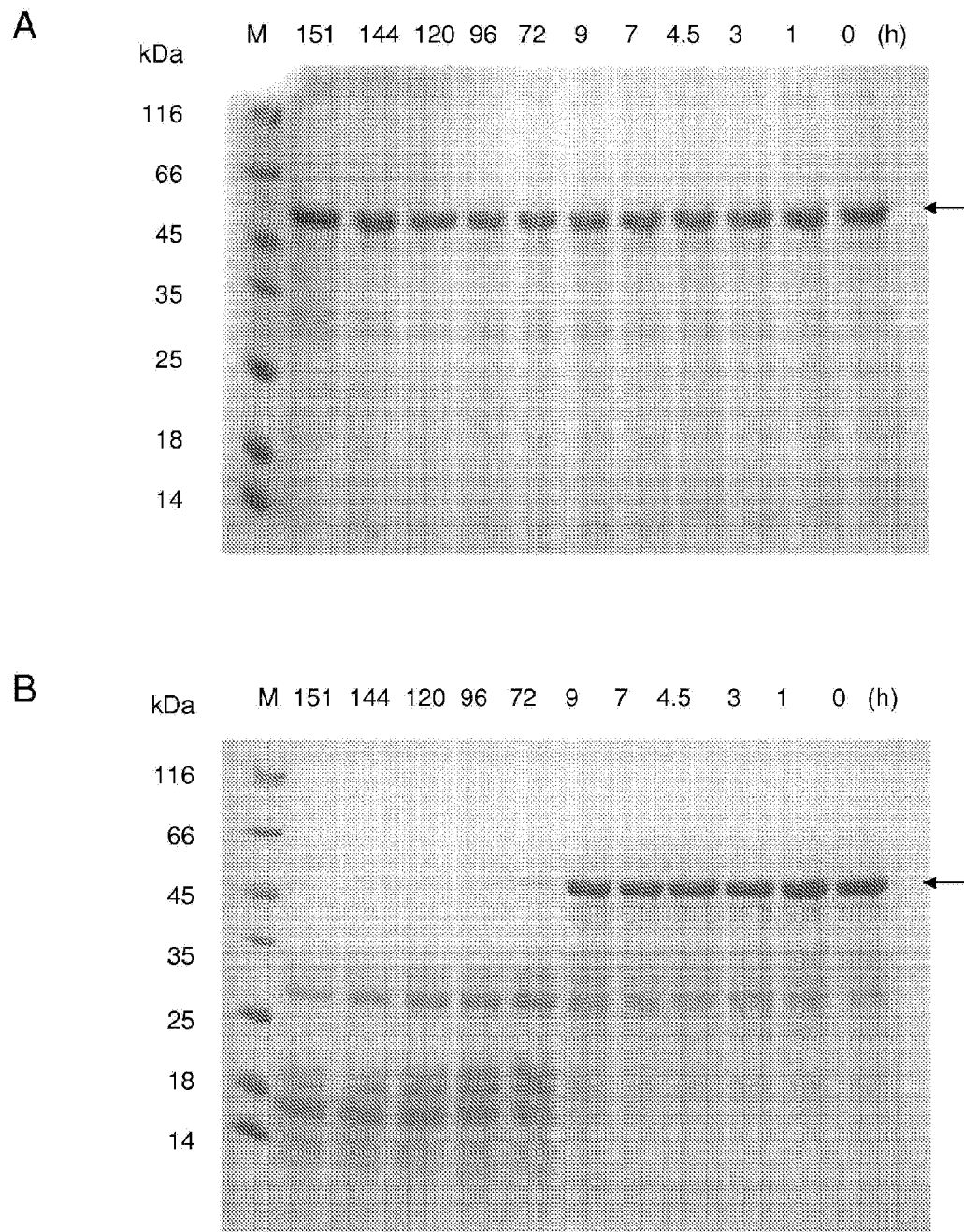


Fig. 8

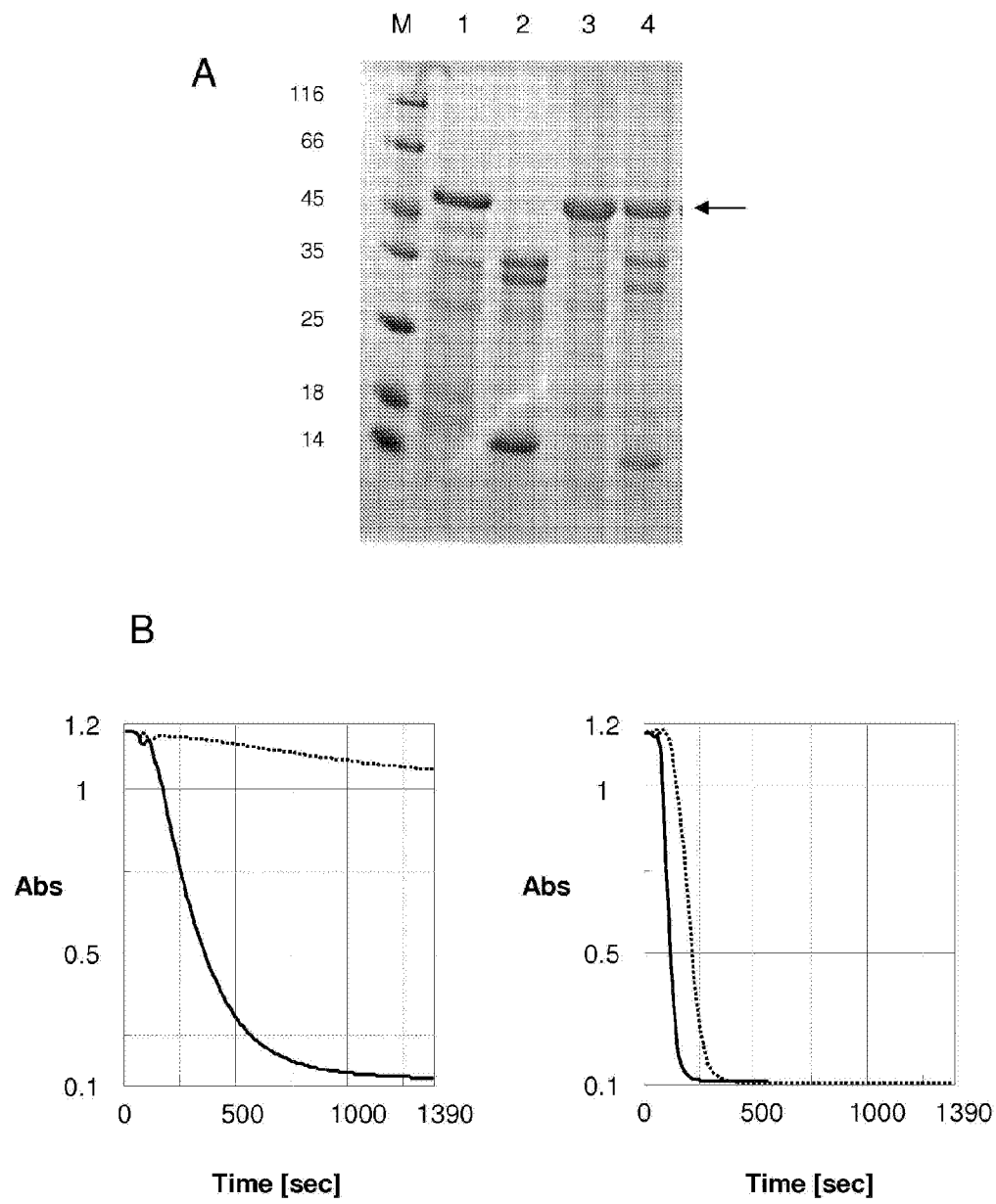


Fig. 9

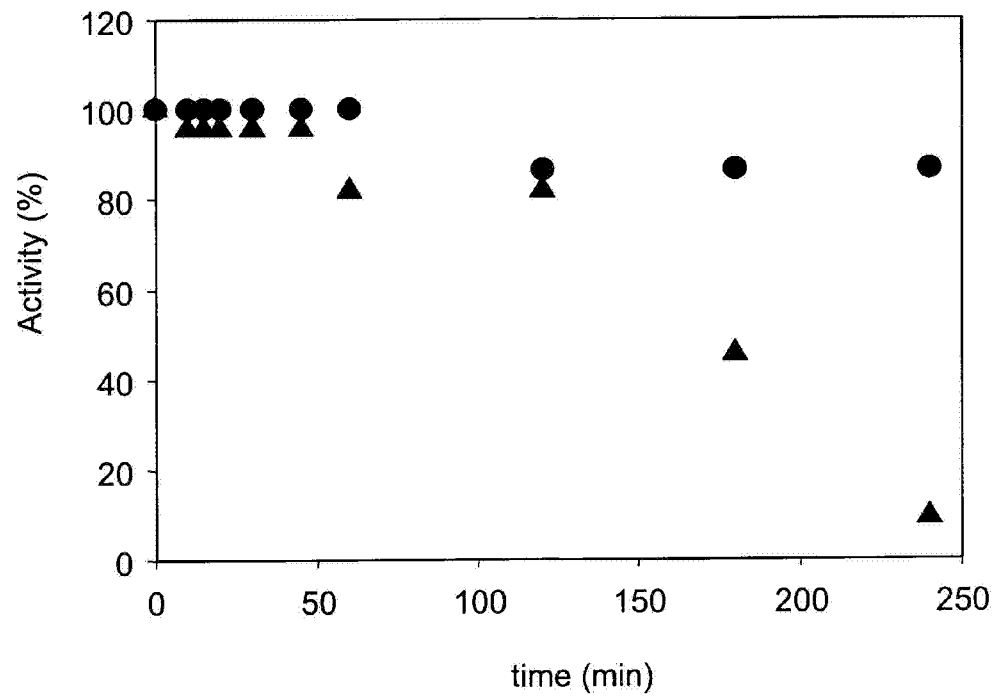


Fig. 10 A

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 TAKPQPKAVELKIIKDVVKGHDLPKRGGNPKGIVIHNDAGSKGATAEAYRNLVNPSSR
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 FQBCARLLKKWGLPANRNTIRLHNEFTSTSCPHRSSVLHTGFDVPVTRGLLPEDKRLQLKD
 YFIKQIRAYMDGKIPVATVSNESASSNTVKPVAELMPPVPAGYTLDKNNVPYKKEQGNV
 TVANVKGNNVRDGYSTNSRITGVLPNNNTITYDGAYCINGYRWITYIANSQRRYIATGE
 VDIAGNRISSEFGKFSV

Fig. 10 B

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Fig. 10 C

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Fig. 11

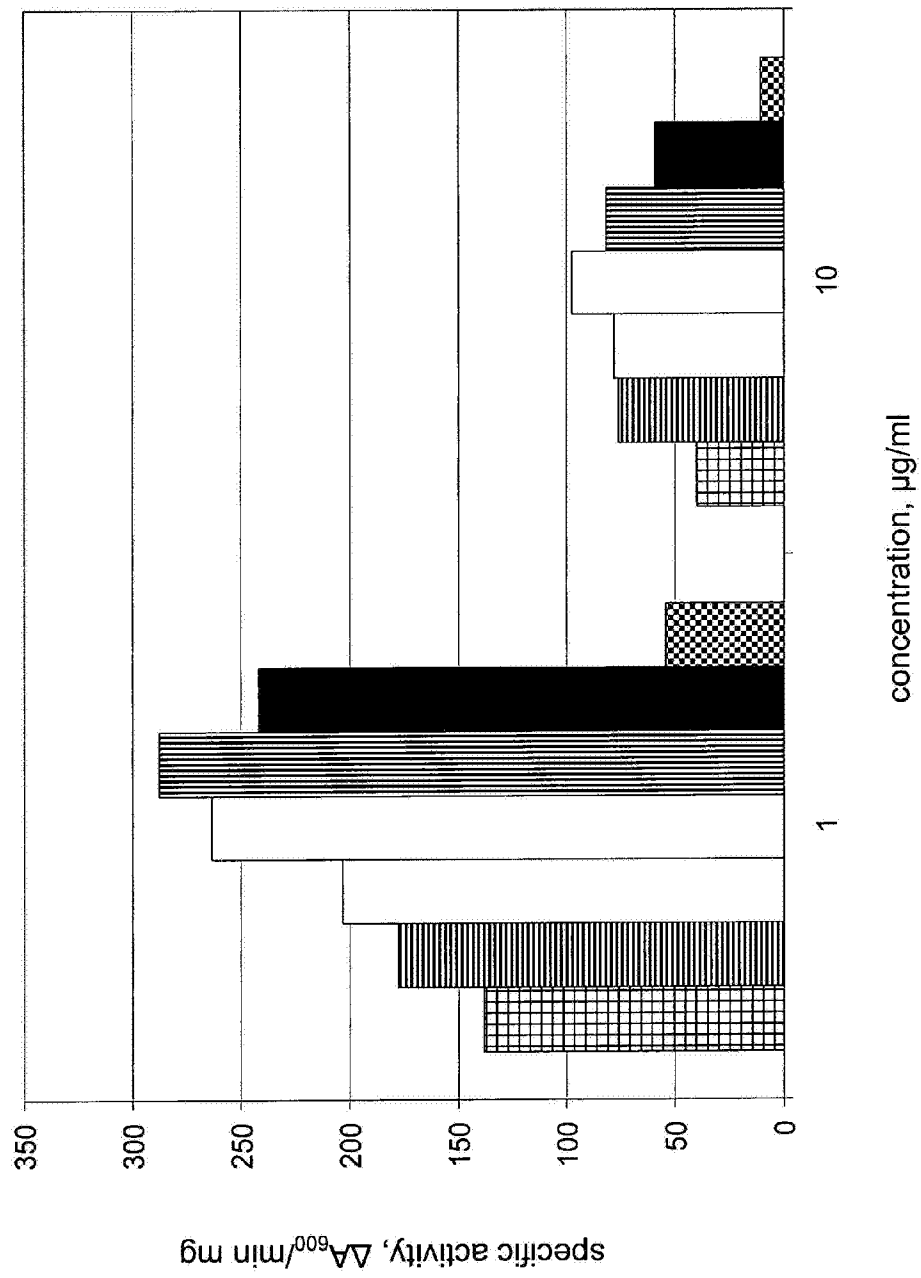


Fig. 12

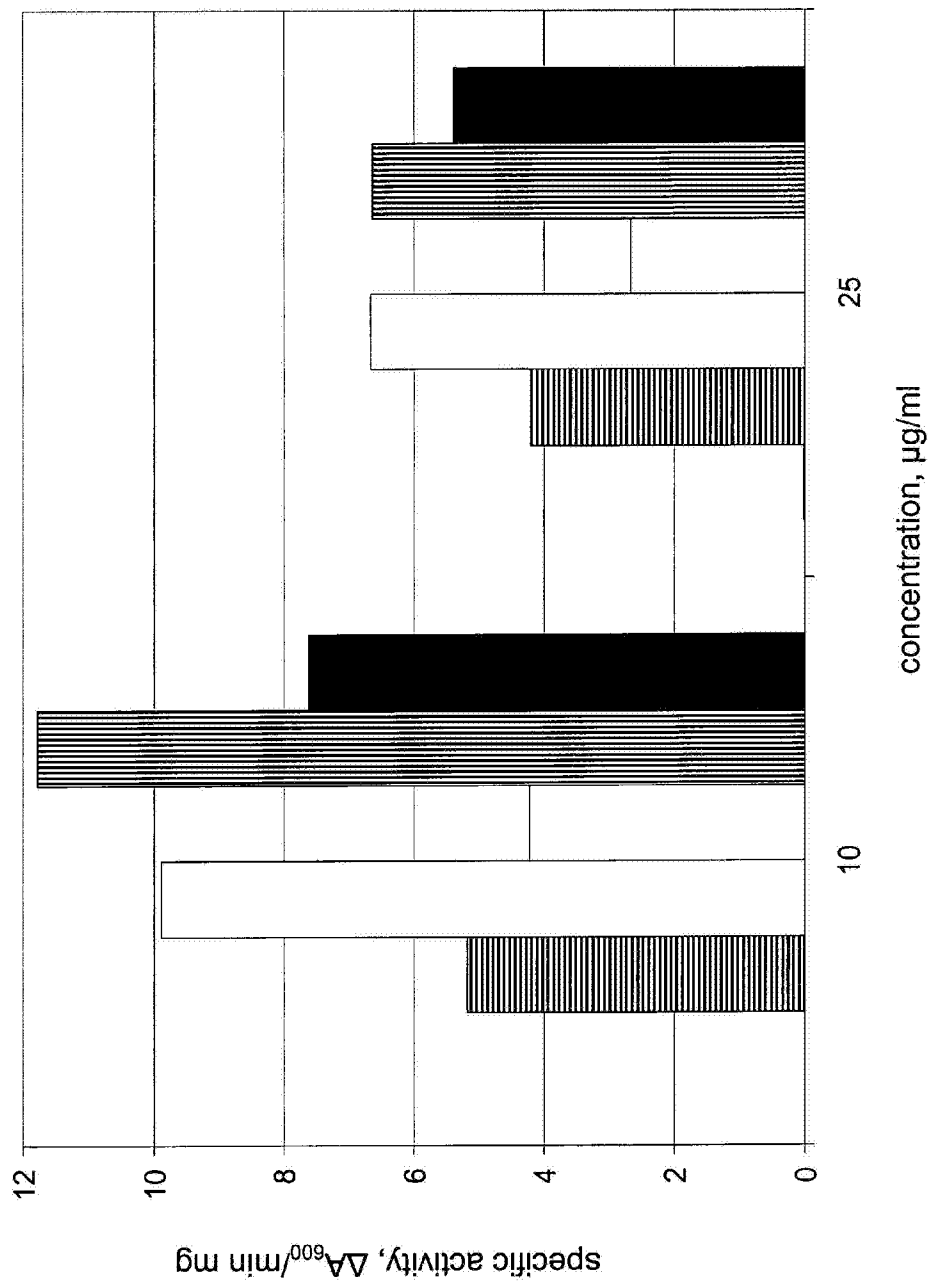


Fig. 13

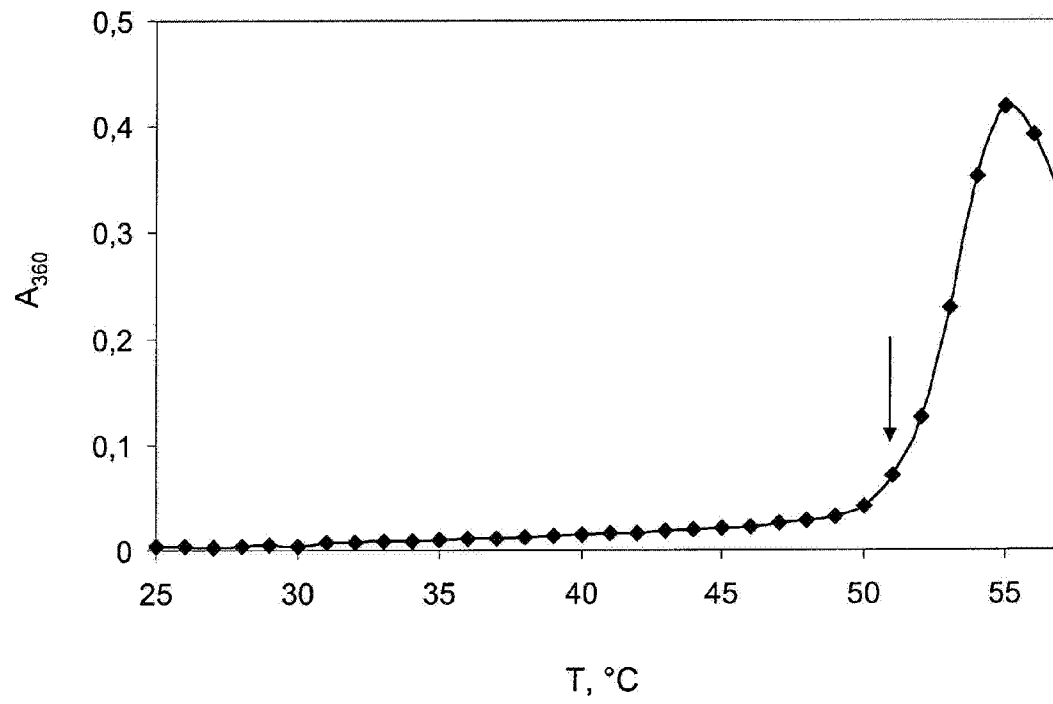
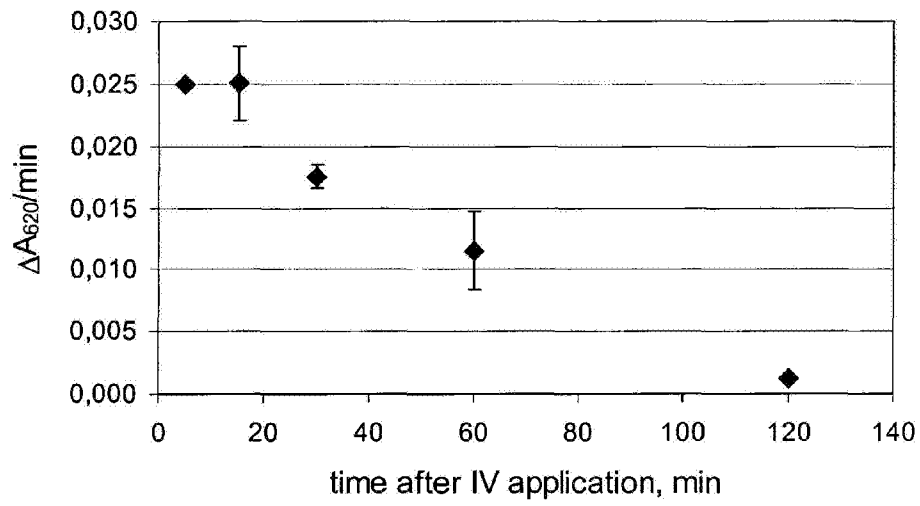


Fig. 14

A



B

