

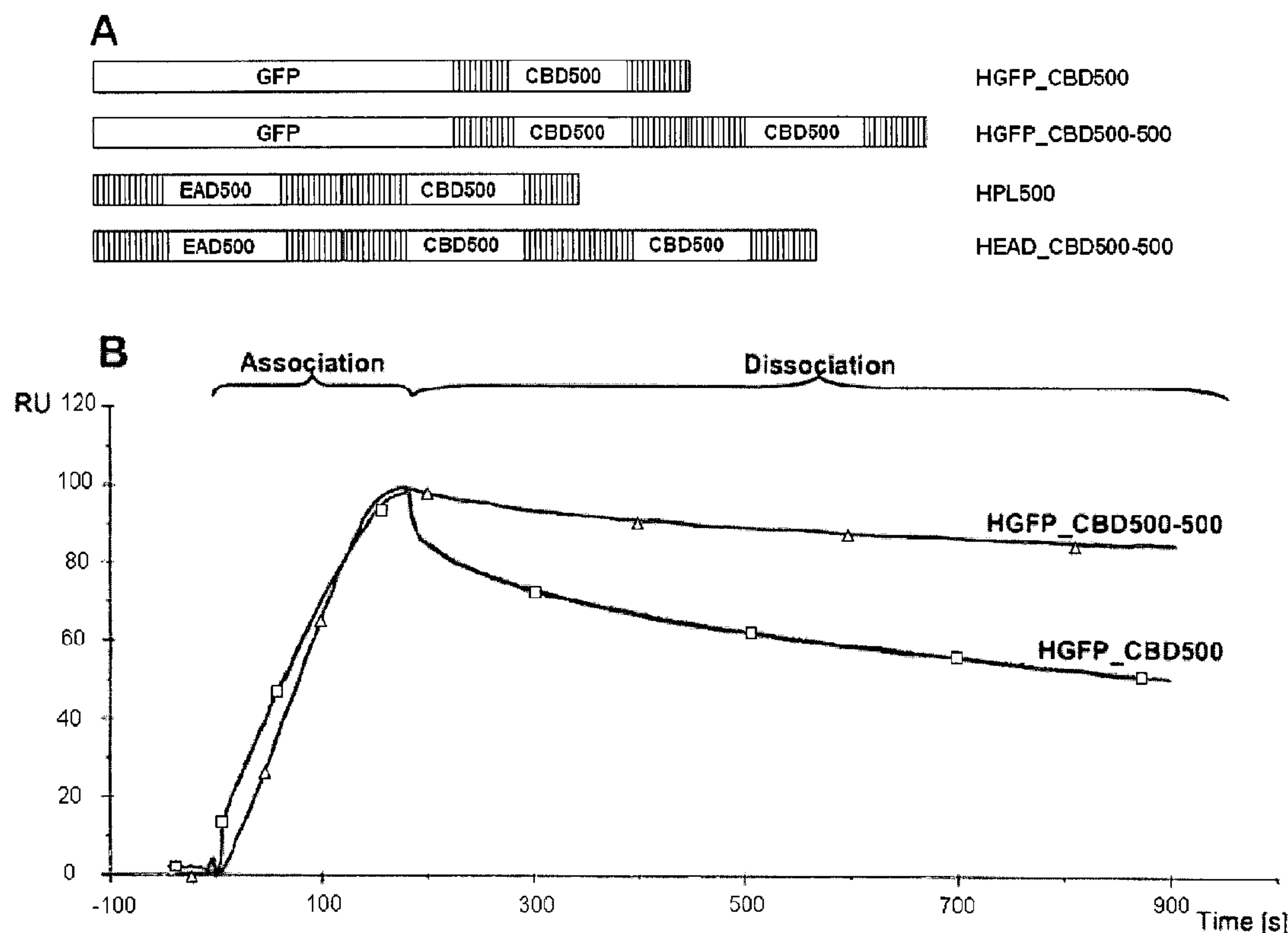


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(54) Titre : ENZYMES ARTIFICIELLES DE LYSE DE PEPTIDOGLYCANES ET PROTEINES DE LIAISON A DES PEPTIDOGLYCANES

(54) Title: ARTIFICIAL PEPTIDOGLYCAN LYSING ENZYMES AND PEPTIDOGLYCAN BINDING PROTEINS



(57) **Abrégé/Abstract:**

The present invention relates to recombinant polypeptides having the activity of binding and lysing of bacteria, comprising at least one enzymatically active domain and at least two bacterial cell binding domains. The present invention further relates to recombinant polypeptide having the activity of binding bacteria, comprising at least two bacterial cell binding domain. Further the present inventions relates to nucleic acid molecules comprising a nucleotide sequence encoding the recombinant polypeptides, vectors and host cells.

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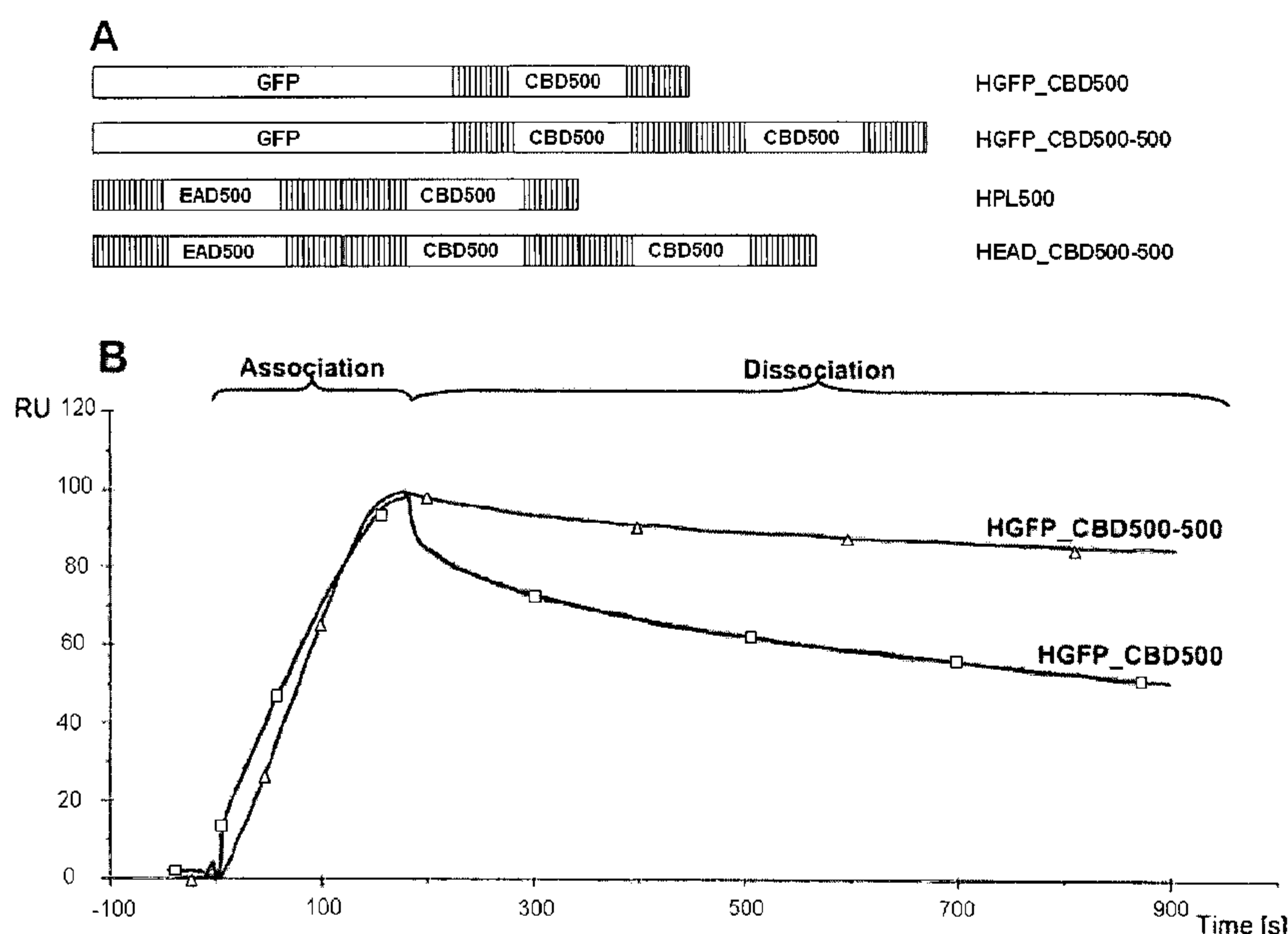
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(57) Abstract: The present invention relates to recombinant polypeptides having the activity of binding and lysing of bacteria, comprising at least one enzymatically active domain and at least two bacterial cell binding domains. The present invention further relates to recombinant polypeptide having the activity of binding bacteria, comprising at least two bacterial cell binding domain. Further the present inventions relates to nucleic acid molecules comprising a nucleotide sequence encoding the recombinant polypeptides, vectors and host cells.

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Artificial peptidoglycan lysing enzymes and peptidoglycan binding proteins

The present invention relates to recombinant polypeptides having the activity of binding and lysing of bacteria, comprising at least one enzymatically active domain and at least two bacterial cell binding domains. The present invention further relates to recombinant polypeptides having the activity of binding bacteria, comprising at least two bacterial cell binding domains. Further the present inventions relates to nucleic acid molecules comprising a nucleotide sequence encoding the recombinant polypeptides, vectors and host cells.

In recent years, peptidoglycan-degrading enzymes like bacteriophage endolysins have received increasing attention as antimicrobial agents. In view of emerging and spreading resistance of pathogenic bacteria against classical antibiotics, the demand for alternative ways of controlling these organisms is rising. Especially in case of Gram-positive bacteria, the application of phage endolysins as so called enzybiotics is a promising approach. Due to the absence of an outer membrane in Gram-positives, these enzymes also work as exolysins, i.e. they can cause lysis of susceptible cells from without. This property can be exploited e.g. in molecular biology for the efficient recovery of nucleic acids and proteins from bacterial cells, as it was demonstrated for endolysins from phages infecting *Listeria*. Aiming towards an application for control of the foodborne pathogen *Listeria monocytogenes*, genes coding for these lysins were introduced into a number of lactic acid bacteria including *Lactococcus lactis* and several lactobacilli, which are used as starter organisms in cheese production. Overexpressing and secreting the endolysins, these bacteria consequently showed lytic activity against *L. monocytogenes* cells. Medical applications of phage encoded peptidoglycan hydrolases reported so far include the detection and killing of *Bacillus anthracis*, and the control of *Streptococcus pneumoniae* in vitro and in mouse models.

Endolysins are cell wall lytic enzymes which are encoded in the late gene region of dsDNA phages and produced at the end of the lytic multiplication cycle. The same enzymes are also found within prophage genomes integrated into bacterial genomes. Their function is degradation of the bacterial peptidoglycan from within, resulting in lysis of the host cell and release of the phage progeny. According to their different target bonds within the

peptidoglycan, endolysins can be divided into 5 different classes: (i) N-acetyl- β -D-muramidases (also known as lysozymes) and (ii) N-acetyl- β -D-glucosaminidases, which are both glycosidases and cleave one of the two β -1,4 glycosidic bonds of the glycan strands each; (iii) lytic transglycosylases, which cleave the same bond as muramidases, but by a different mechanism; (iv) N-acetylmuramoyl-L-alanine amidases, which cut between the glycan and the peptide moieties; and (v) endopeptidases, which cleave within the peptide moiety. All endolysins except for lytic transglycosylases are hydrolases. The same enzymatic activities are also found in bacterial enzymes which lyse cell walls of its own or closely related bacteria, the so-called autolysins, and other bacterial cell wall lysing polypeptides like the bacteriocins. Bacterial autolysins are cell wall lytic enzymes, which play important roles in cell wall remodelling, cell division, transformation, or as virulence factors. Together they can be summarized as peptidoglycan lysing enzymes. The enzymatic degradation of the peptidoglycan due to the action of peptidoglycan lysing enzymes results in loss of integrity of the cell wall and finally in cell disruption caused by the high internal pressure.

The feature of killing bacterial cells makes the peptidoglycan lysing enzymes interesting candidates for a use as a prophylactic or therapeutic agent against bacterial infections in humans and animals, as an antimicrobial or enzybiotic for use as a disinfectant in medical, public or private environment, for use as a decontaminant of bacterial contamination in food industry, animal feed or cosmetic industry or as a general surfactant against bacterially contaminated surfaces. Peptidoglycan lysing enzymes are as well useful in bacterial diagnostics as they specifically bind and lyse bacterial cells from distinct bacterial groups, genera, species, strains or serovars. Specific cell lysis is often combined with additional detection methods relying on the cellular content of the bacterial cells to be detected like nucleic acid based methods or immunological methods.

Endolysins, autolysins and other peptidoglycan lysing enzymes from a Gram-positive background show a modular organization in which catalytic activity and substrate recognition are separated and localized in at least two distinct functional domains, the enzymatically active domain (EAD) and the cell binding domain (CBD). Most endolysins from phages infecting Gram-negative hosts are single-domain globular proteins. However, recently two lysins from a Gram-negative background that consist of two functional domains were reported: The endolysins of the *Pseudomonas aeruginosa* phages Φ KZ and EL consist of an N-terminal cell binding domain and a C-terminal catalytic domain (Briers et al. Molecular

Microbiology, Volume 65, Number 5, September 2007 , pp. 1334-1344(11)). In contrast, the majority of endolysins from phages infecting Gram-positive bacteria feature a reverse orientation of the domains, with an N-terminal EAD and a C-terminal CBD.

For only very few endolysins, the ligands in the bacterial cell wall recognized by the binding domain are known. The pneumococcal phage Cpl-1 lysozyme specifically recognizes choline containing teichoic acids in the cell wall of *Streptococcus pneumoniae*, which places it in the family of Choline-Binding Proteins (CBP). The cholin binding modules (CBM) of these proteins are formed by a repeat of about 20 amino acid residues found in multiple tandem copies, ranging from 4 to 18. Cpl-1 exemplifies the modular design of these enzymes with two separate domains – an EAD and a CBD- usually connected via a short linker region. Although the majority of modular phage endolysins consist of one catalytic and one substrate binding domain, there are a number of proteins that harbor two different enzymatic activities, as e.g. the endolysins of *Streptococcus agalactiae* phage B30 (muramidase and endopeptidase), *Staphylococcus aureus* phage Φ 11 (endopeptidase and amidase), *Streptococcus agalactiae* phage NCTC 11261 (endopeptidase and muramidase), and *Staphylococcus warneri* M phage Φ WMY (endopeptidase and amidase).

The fact that peptidoglycan lysing enzymes with phage and bacterial origin, endolysins as well as autolysins or bacteriocins show similar modular architectures, and that high homologies between distinct domains of bacterial and phage derived lytic proteins can be found, suggests a common ancestry and co-evolution of these proteins by interchange of functional domains (Garcia et al., 1990, Gene 86, 81-88). Diaz et al. (1990, Proc. Natl. Acad. Sci., 87, 8125-8129) created chimaeras of phage and bacterial pneumococcal enzymes which exhibited combined biochemical properties. Recombinant chimaeras from genes lacking nucleotide homology were constructed in Diaz et al. (1991, J. Biol. Chem., 266, 5464-6571), confirming also the function of the CBDs in substrate recognition. Croux et al. (1993, Mol. Microbiol., 9, 1019-1025) even created chimaeras based on pneumococcal and clostridial cell wall lytic enzymes which led to the switch in enzymatic activity of endolysins towards cells from other bacterial families. Sanz et al. (1996, Eur. J. Biochem., 235, 601-605) constructed multifunctional pneumococcal murein hydrolases by module assembly which comprised two EADs and one CBD. Recently, fusion proteins consisting of lysostaphin, a peptidoglycan hydrolase from *Staphylococcus simulans*, and the *Streptococcus agalactiae* phage B30 endolysin, as well as a C-terminally truncated version thereof, were reported (Donovan et al. 2006, Appl. Environ. Microbiol., 72, 2988-2996). Also in this case, the artificial constructs

combined properties of both enzymes, lysing both *Staphylococcus* and *Streptococcus* cells. Loessner et al. (2002, Mol. Microbiol., 44, 335-349) described the concept of CBDs determining the specific recognition and high-affinity binding to bacterial cell wall carbohydrates using *Listeria monocytogenes* as a role model. US 2004/0197833 teaches the use of immobilized isolated CBDs in a method for the enrichment of target cells.

The object of the present invention is to provide improved and advantageous proteins which allow the reliable detection and enrichment and/or lysis of bacterial cells.

The object is solved by the subject matter as defined in the claims.

The following figures illustrate the present invention.

Figure 1: Schematic representation of the GFP-double CBD fusion proteins against *Listeria* cells, as well as the GFP-single CBD constructs serving as references. GFP = Green fluorescent protein; CBD500, CBD118, CBDP35 = Cell wall binding domains of *Listeria* phage endolysins Ply500, Ply118, and PlyP35, respectively; L = linker region of the PlyPSA endolysin.

Figure 2: Peptidoglycan binding proteins with duplicated CBD resulting in higher affinity due to reduced dissociation from the cell wall. (A) Schematic representations of double CBD500 fusion proteins as well as the respective single CBD500 constructs serving as references. GFP = Green fluorescent protein; EAD500 = Enzymatically active domain of *Listeria* phage endolysin Ply500; CBD500 = Cell wall binding domain of Ply500. (B) Overlay of the SPR sensograms of HGFP_CBD500 (black) and HGFP_CBD500-500 (grey), measured at a concentration of 50 nM. Association and dissociation phases are indicated. RU = relative response units.

Figure 3: Relative lytic activities of wild-type ply500 with N-terminal His-tag (open circles) and H_EAD_CBD500-500 (solid squares) against cells of *Listeria monocytogenes* WSLC 1042 measured with the photometric lysis assay at different NaCl concentrations. The optimum activity of wild-type ply500 at 200 mM NaCl corresponds to 1.0. All assays were carried out in triplicate.

Figure 4: Determination of the minimal bactericidal concentration (MBC) of peptidoglycan lysing enzymes against enterococci. The bacterial concentration of surviving cells of *Enterococcus faecalis* strain 17 is shown in dependence of the protein concentration of Fab25 VL (squares) or EADFab25_CBD25_CBD20 (circles) present in the cell lysis assay.

The term “peptidoglycan lysing enzyme” as used herein refers to an enzyme which is suitable to lyse bacterial cell walls. The enzyme comprises at least one of the following activities of which the “enzymatically active domains” (EADs) of the peptidoglycan lysing enzymes are constituted: endopeptidase, 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, the so-called “endolysins” or it is derived from related cell wall lysing enzymes coded by bacteria, the so-called “autolysins” or other bacterial peptidoglycan lysing enzymes like bacteriocins, virulence factors or other antimicrobial polypeptides (e.g. lysostaphin, ALE-1 lysin, mutanolysin, enterolysin). In addition, the peptidoglycan lysing enzymes 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 “peptidoglycan binding protein” as used herein refers to an artificially constructed bacterial cell binding protein which has none of the enzymatic activities described for the peptidoglycan lysing enzyme. The peptidoglycan binding protein comprises more than one CBD derived from a CBD. The peptidoglycan binding protein is constructed by shuffling of naturally occurring CBDs and/or by multiplication of naturally occurring CBDs.

The term “domain” as used herein refers to a subunit of a peptidoglycan lysing enzyme which is ascribed a specific function, and can also coincide with a structural or evolutionary conserved domain. Specific functions associated with a domain are for example bacterial peptidoglycan lysis or bacterial cell binding. The functional domains are sometimes also called “modules”.

The term “CBD” as used herein refers to the cell wall binding domain of a peptidoglycan lysing enzyme, 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 mediate binding of the peptidoglycan lysing enzyme to the bacterial cell wall. The term CBD as used herein

describes a segment within a polypeptide chain which is derived from a naturally occurring peptidoglycan lysing enzyme.

The term “EAD” as used herein refers to the enzymatically active domain of a peptidoglycan lysing enzyme which is responsible for hydrolysis of the bacterial peptidoglycan. It contains at least one of the enzymatic activities described for a peptidoglycan lysing enzyme. The term EAD as used herein describes a segment within a polypeptide chain which is derived from a naturally occurring peptidoglycan lysing enzyme.

A “CHAP” domain (cysteine, histidine-dependent amidohydrolases/peptidases) is a region of about 110 to about 140 amino acid residues 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 residue. These residues form part of the putative active site of CHAP domain containing proteins.

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

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 acid residues which is characteristic for proteins which interact with other binding partners. It is identified via a proline-rich consensus motif. The SH3 domain is located within the CBD. SH3 domains found in peptidoglycan lysing enzymes are often of the SH3b or SH3_5 type.

The term “wild-type” refers to the naturally occurring form of a protein or a nucleic acid with respect to the sequence.

The term “shuffling” as used herein refers to the combination of different fragments of polypeptides from different wild-type enzymes into new chimaeric polypeptide constructs. In this context, the enzymes are preferentially peptidoglycan lysing enzymes, and the fragments are preferentially EADs and CBDs. Usually, the fragments are combined by molecular biological methods on nucleic acid level. Additional linker sequences may be introduced between the fragments for structural or cloning reasons.

One object of the present invention refers to peptidoglycan lysing enzymes that are composed of at least one EAD and at least two CBDs. Artificially created peptidoglycan lysing enzymes according to the invention exhibit new properties like an extended or altered binding range compared to naturally occurring proteins or an increased binding affinity to the bacterial cell wall or an increased or altered lytic activity or combinations thereof.

Another object of the present invention refers to peptidoglycan binding proteins that are composed of at least two CBDs. Artificially created peptidoglycan binding proteins according to the invention exhibit new properties like an extended or altered binding range compared to naturally occurring proteins or an increased binding affinity to the bacterial cell wall or both.

In the peptidoglycan lysing enzymes or peptidoglycan binding proteins according to the invention the at least two CBDs may be derived from two different peptidoglycan lysing enzymes (domain shuffling) or by multiplication of one CBD naturally occurring in an endolysin. If more than one EAD is present in the peptidoglycan lysing enzyme according to the invention the EADs may be derived from two different peptidoglycan lysing enzymes.

Meanwhile, a large number of peptidoglycan lysing proteins against different genera, species or strains of gram positive and gram negative bacteria is described in the art. The modular nature of the peptidoglycan lysing proteins, and the distinction between EAD and CBD is well known. Lots of conserved domains existing in peptidoglycan lysing proteins are characterized functionally, and their existence within a polypeptide or nucleotide sequence can be predicted by suitable computer programs which use respective protein or nucleic acid databases, e.g. CDD (Marchler-Bauer et al., 2005; Nucleic Acids Research, 33, D192-D196); Pfam (Finn et al., 2006, Nucleic Acids Research 34, D247-D251) or SMART (Schultz et al., 1998, Proc. Natl. Acad. Sci. USA 95, 5857-5864, Letunic et al., 2006, Nucleic Acids Res 34, D257-D260) or by binding assays with deletion mutants (Loessner et al., 2002, Mol.

Microbiol., 44, 335-349). The artificial peptidoglycan lysing enzymes according to the invention are constructed by combining the desired enzymatic activity derived from an EAD with at least two CBDs for the cell binding activity using standard techniques for cloning and production of recombinant proteins as described in Sambrook et al. (Molecular cloning. A laboratory manual; 2nd ed. Cold Spring Harbor Laboratory Press 1989). The artificial peptidoglycan binding proteins according to the invention are constructed by combining at least two CBDs for the cell binding activity using standard techniques for cloning and production of recombinant proteins as described in Sambrook et al. (Molecular cloning. A laboratory manual; 2nd ed. Cold Spring Harbor Laboratory Press 1989). The at least two CBDs can derive from different peptidoglycan lysing enzymes which leads to shuffled chimaeric enzymes, or they can derive from a multiplication of CBDs from one naturally occurring enzyme, or combinations of both. Principally, all naturally occurring peptidoglycan lysing enzymes are potential candidates for the supply of EAD and CBD domains.

The peptidoglycan lysing enzymes preferably comprise at least one EAD selected from the group composed of Amidase_5 (bacteriophage peptidoglycan hydrolase, pfam05382), Amidase_2 (N-acetylmuramoyl-L-alanine amidase, pfam01510), Amidase_3 (N-acetylmuramoyl-L-alanine amidase, pfam01520), Transgly (transglycosylase, pfam00912), Peptidase_M23 (peptidase family M23, pfam01551), endolysin_autolysin (CD00737), Hydrolase_2 (cell wall hydrolase, pfam07486), CHAP (amidase, pfam05257), Transglycosylase (transglycosylase like domain, pfam06737), MtlB (membrane-bound lytic murein transglycosylase B, COG2951), MtlA (membrane-bound lytic murein transglycosylase A, COG2821), MtlE (membrane-bound lytic murein transglycosylase E, COG0741), bacteriophage_lambda_lysozyme (lysis of the bond between N-acetylmuramic acid and N-acetylglucosamine, CD00736), Peptidase_M74 (penicillin-insensitive murein endopeptidase, pfam03411), SLT (transglycosylase SLT, pfam01464), Lys (C-type lysozyme/alpha-lactalbumin family, pfam00062), COG5632 (N-acetylmuramoyl-L-alanine amidase, COG5632), MepA (murein endopeptidase, COG3770), COG1215 (glycosyltransferase, COG1215), AmiC (N-acetylmuramoyl-L-alanine amidase, COG0860), Spr (cell wall-associated hydrolase, COG0791), bacteriophage_T4-like_lysozyme (lysis of the bond between N-acetylmuramic acid and N-acetylglucosamine, cd00735), LT_GEWL (lytic transglycosylase (LT) and goose egg white lysozyme (GEWL) domain, cd00254), peptidase_S66 (LD-carboxypeptidase, pfam02016), Glyco_hydro_70 (glycosyl hydrolase

family 70, pfam02324), Glyco_hydro_25 (glycosyl hydrolase family 25), VanY (D-alanyl-D-alanine carboxypeptidase, pfam02557), and LYZ2 (lysozyme subfamily 2, smart 00047).

The peptidoglycan lysing enzymes preferably comprise at least one CBD selected from the group composed of SH3_5 (bacterial SH3 domain, pfam08460), SH3_4 (bacterial SH3 domain, pfam06347), SH3_3 (bacterial SH3 domain, pfam08239), SH3b (bacterial SH3 domain homologue, smart00287), LysM (LysM domain found in a variety of enzymes involved in cell wall degradation, pfam01476 and cd00118), PG_binding_1 (putative peptidoglycan binding domain, pfam01471), PG_binding_2 (putative peptidoglycan binding domain, pfam08823), MtlA (peptidoglycan binding domain from murein degrading transglycosylase, pfam03462), Cpl-7 (C-terminal domain of Cpl-7 lysozyme, pfam08230), CW_binding_1 (putative cell wall binding repeat, pfam01473), LytB (putative cell wall-binding domain, COG2247), and LytE (LysM repeat, COG1388).

Preferably, the domains described above have amino acid residue lengths in the range of about 15 to about 250 amino acid residues, preferred are lengths in the range of about 20 to about 200 amino acid residues. As an example, about 15 to about 40 amino acid residue long domains are found in peptidoglycan binding domains like the LysM domain or the CW_binding_1 motif which is responsible for cholin binding. These small domains are often found as naturally repeated motifs also in wild-type cell wall lysing enzymes. These domains can be combined with additional CBDs from other cell wall lysing enzymes in order to create chimaeric shuffled artificial peptidoglycan lysing enzymes or peptidoglycan binding proteins.

Usually, complete EAD or CBD domains of peptidoglycan lysing enzymes are larger than the conserved domains described above. Preferentially, an EAD or CBD is in the range of about 50 residues to about 400 residues long. Each EAD and CBD contains at least one functional domain in order to exhibit their functions of peptidoglycan lysis or bacterial cell binding, but can also comprise more than one functional domain and additional sequence segments with unknown function. EAD and CBD domains of peptidoglycan binding enzymes are not always defined by the conserved domains described above. There are also peptidoglycan binding enzymes known (e.g. Ply118) which bind and lyse bacterial cells although none of the above described conserved domains is found. Whether potential domains function as an EAD or CBD can be tested with suitable functional assays (e.g. photometric lysis assay, plate lysis assay or determination of minimal bactericidal concentration (MBC) for peptidoglycan lysis

(EAD), and cell binding assay, fluorescence microscopy or determination of binding affinity for cell binding (CBD)). The domain borders of EADs and CBDs can be defined by local alignment search tools (e.g. BLAST at the NCBI, Altschul et al., 1997, Nucleic Acids Res. 17, 3389-3402) which find regions of local similarity between sequences. In addition, a multitude of peptidoglycan lysing enzymes are already described with respect to their EAD and CBD domains.

Preferably, the peptidoglycan lysing enzymes and peptidoglycan binding proteins of the present invention are composed of EADs and CBDs derived from wild-type peptidoglycan lysing enzymes selected from the group consisting of Ply500, Ply511, Ply118, Ply100, PlyP40, Ply3626, phiLM4 endolysin, PlyCD119, PlyPSAa, Ply21, PlyBA, Ply12, PlyP35, PlyPH, PlyL, PlyB, phi11 endolysin, phi MR11 endolysin, phi12 endolysin, *S. aureus* phage PVL amidase, plypitti26, ΦSA2usa endolysin, endolysin of *Staphylococcus warneri* M phage ΦWMY PlyGBS, B30 endolysin, Cpl-1, Cpl-7, Cpl-9, PlyG, PlyC, pal amidase, Fab25, Fab20, endolysins from the *Enterococcus faecalis* V583 prophage, lysostaphin, phage PL-1 amidase, *S. capitis* ALE-1 endopeptidase, mutanolysin (N-acetylmuramidase of *Streptomyces globisporus* ATCC 21553), enterolysin A (cell wall degrading bacteriocin from *Enterococcus faecalis* LMG 2333), LysK, LytM, Ami autolysin from *L. monocytogenes*, endolysins of the *Pseudomonas aeruginosa* phages ΦKZ and EL, T4 lysozyme, gp61 muramidase, and STM0016 muramidase.

The wild-type peptidoglycan lysing enzyme PlyP40 has a length of 344 amino acid residues in its wild type form. It possesses two functional domains that have only a minimal homology with other known endolysins. The N-terminal amino acid residues at the positions from 1 to 200 represent the enzymatically active domain (EAD) which is depicted in SEQ ID NO: 103. The cell binding domain (CBD) of PlyP40 comprises the C-terminal located amino acid residues from 227 to 344 which are depicted in SEQ ID NO: 104. Thus, the EAD deriving from the wild-type peptidoglycan lysing enzyme PlyP40 comprises preferably an amino acid sequence according to SEQ ID NO: 103, whereas the CBD deriving from the wild-type peptidoglycan lysing enzyme PlyP40 comprises preferably an amino acid sequence according to SEQ ID NO: 104.

The fragments derived from naturally occurring peptidoglycan lysing enzymes in order to construct the enzymes and proteins according to the invention may not combine the mere

sequence segments determined from the prediction of the conserved functional domains as described above, but preferably add suitable linker sequences which connect the different functional modules. The linker sequences can be derived from the wild-type sequences in neighbourhood to the defined functional domains or can be external suitable linker sequences known from the art. A suitable linker is for example the short domain linker with the sequence AAKNPN or TGKTVAANKPNRHS (SEQ IDs No: 61 and 11) from the *Listeria* endolysin PlyPSA (Korndörfer et al., 2006, J. Mol. Biol., 364, 678-689) defined from the x-ray structure. Polyglycine linkers are also known in the art to serve as flexible domain linkers. Preferred linkers are also glycine and alanine rich linkers. Specific sequences for glycine and alanine rich linkers are given as SEQ ID NO:63, 64 and 65. Preferred are also proline and threonine rich sequences which occur as natural linkers, e.g. in enterolysin A SEQ ID NO:66. Proline and threonine rich linker sequences can be described by the consensus motif (PT)_xP or (PT)_xT, where x stands for an integer in the range of 1 to 10. Another linker possibility are the so-called "junction zones" between EADs and CBDs described in Croux et al. (1993, Molec. Microbiol., 9, 1019-1025). A skilled person knows several methods how to predict a suitable boundary for a functional domain to be taken out of a wild-type enzyme, e. g. secondary structure prediction, prediction of domain linkers, inspection of 3D-models of proteins or inspection of domain linkers and boundaries in highly resolved X-ray and NMR structures of proteins. Suitable methods are for example described in Garnier et al., 1996, Methods in Enzymology 266, 540-553; Miyazaki et al., 2002, J. Struct. Funct. Genomics, 15, 37-51; George und Heringa, 2003, Protein Eng. 15, 871-879; Bae et al., 2005, Bioinformatics, 21, 2264-2270, Altschul et al., 1997, Nucleic Acids Res. 17, 3389-3402; Schwede et al., 2003, Nucleic Acids Research 31, 3381-3385. Lund et al, CPHmodels 2.0: X3M a Computer Program to Extract 3D Models. Abstract at the CASP5 conference A102, 2002. The length of polypeptide linker between EAD and CBD domains or between CBD and CBD domains are in the range of about 5 to about 150 amino acid residues, preferentially of about 6 to about 60 amino acid residues.

Preferably, the order for the combination of EAD and CBDs in the peptidoglycan lysing enzymes according to the invention is EAD-CBD1-CBD2(-CBDN, N = 3 or more) from the N-terminus to the C-terminus. Preferred are also variants where an at least second EAD is added next to the EAD at the N-terminus or at the C-terminus. Preferred are also variants where the at least two CBDs are positioned at the N-terminus or at the N- and C-terminus with the EADs positioned in the middle. In addition, marker sequences or tags can be

included, which can both be positioned N-terminal, C-terminal or in the middle, but especially preferred at the N-terminus.

Peptidoglycan lysing enzymes and peptidoglycan binding proteins according to the invention exhibit new properties compared to the wild-type enzymes from which they are derived.

The binding range of a peptidoglycan lysing enzyme or peptidoglycan binding protein determines the bacterial host range which is recognized. Most of the naturally occurring peptidoglycan lysing enzymes exhibit a relatively narrow host range. For technical application of the peptidoglycan lysing enzymes or peptidoglycan binding proteins it is often advantageous to extend the host range of the proteins so that an increased number of bacterial strains or species can be killed, captured or detected depending on the respective application. An extended host range comprised within one protein avoids the use of two or more proteins for the same application which has the advantages of reduced costs for protein production, reduced effort to optimize conditions for different proteins, simpler medical approval proceedings, and reduced immunogenicity. An extended host range which combines for example *Staphylococci* and *Enterococci* is useful in the therapy or prevention of nosocomial infections where multiresistant strains of both genera are an increasing problem. An extended host range is also useful in bacterial detection or a method to remove harmful bacteria from food. For example, pathogenic strains are found within all serovars of *Listeria*. None of the naturally occurring *Listeria* endolysins, however, is able to lyse cells from all serovars. A peptidoglycan lysing enzyme combining more than one CBD according to the invention is able to lyse all serovars. A host range which is not extended, but somehow altered compared to naturally occurring proteins, may be useful for applications which need tailored proteins for a given set of bacterial cells to be lysed, captured or detected. The binding range of peptidoglycan lysing enzymes or peptidoglycan binding proteins can be determined with assays known from the art or with the plate lysis assay, photometric lysis assay, binding assay or fluorescence microscopy described in the examples.

An increased binding affinity of peptidoglycan lysing enzymes or peptidoglycan binding proteins compared to wild-type proteins helps to reduce the amount of protein needed for any technical application which relies on the binding of the bacterial cells like cell lysis, cell capture, and detection. This reduces costs and minimizes immunological reactions and potential side effects in therapeutical applications. In applications relying on bacterial cell

capture, the assays are less sensitive for washing steps which decreases background signals, incubation times can be reduced, and detection assays are more sensitive. An increased binding affinity can be measured with assays known from the art or with the surface plasmon resonance analysis or the assay for determination of the minimal bactericidal concentration described in the examples.

An increased lytic activity of peptidoglycan lysing enzymes compared to wild-type enzymes is useful in all applications relying on the lysis of bacterial cells like protection and therapy of infections, sanitation, cell lysis as an initial step in bacterial detection, or removal of pathogenic bacteria from food, feed, cosmetics etc.. The amount of protein needed for the respective application is reduced compared to the wild-type protein which reduces costs and minimizes immunological reactions and potential side effects in therapeutical applications. An altered lytic activity compared to wild-type could be for example a different pH-optimum of the artificial enzyme or a higher lysis activity at other buffer compositions (e.g. high ionic strength, activity in the presence of organic solvents, activity in the presence of specific ions). This also includes a higher activity in specific samples like blood, human serum, or other medical samples. A pH-optimum of an artificial enzyme which is shifted to lower pH is for example interesting for an application of the artificial enzyme in food industry as food products or intermediate products in food processing often have a low pH-value, e.g. in dairy farming. Enzyme function under high salt concentration is also important in food industry, e.g. in cheese production. An increased or altered lytic activity can be determined with assays known from the art or with the plate lysis assay, photometric lysis assay, or the assay for determination of the minimal bactericidal concentration described in the examples.

In one aspect the present invention relates to artificial peptidoglycan lysing enzymes and peptidoglycan binding proteins which can be used to lyse, capture and/or detect *Listeria* bacteria. The inventors combined domains of the *Listeria* endolysins ply500 (SEQ ID NO:1), ply118 (SEQ ID NO:3) and plyP35 (SEQ ID NO:5) using the method described above in order to create artificial peptidoglycan lysing enzymes and peptidoglycan binding proteins which exhibit new properties compared to the wild-type enzymes. Ply500 comprises a conserved D-alanyl-D-alanine carboxypeptidase (VanY; pfam02557) domain as an EAD. The CBD of ply500 begins with an amino acid residue in the range of about H133 to Q150 and ends with K289. For Ply118 no conserved domains were found within the amino acid sequence. From sequence alignments with homologous peptidoglycan lysing enzymes,

however, it was derived that the CBD of ply118 begins with an amino acid residue in the range of about D90 to K180 and ends with amino acid residue K289. Preferred N-terminal starting amino acid residues for CBD118 are D90, K100, G127, S151, N161 or K180. PlyP35 also comprises a conserved D-alanyl-D-alanine carboxypeptidase (VanY; pfam02557) domain as an EAD. The CBD of plyP35 begins with an amino acid residue in the range of about P130 to N156 and ends with an amino acid residue in the range of Y281 to K291. Preferred N-terminal starting amino acid residues for CBDP35 are P130, A134, K143 and N156. Preferred C-terminal amino acid residues for CBDP35 are Y281, L286, and K291.

Preferred peptidoglycan binding proteins according to the invention are CBD500-118 (SEQ ID NO:7) which comprise the CBD of ply500 (amino acid residues H133 to K289) in the N-terminal position and the CBD of ply118 (amino acid residues D90 to I281) in the C-terminal position connected without an additional linker sequence, and CBD500L118 (SEQ ID NO:9) which comprises the CBD of ply500 (amino acid residues Q150 to K289) and the CBD of ply118 (amino acid residues K100 to I281) with a linker (L) connecting the two domains. The domain linker in this case is the plyPSA linker region with the amino acid sequence TGKTVAANKPNRHS (SEQ ID NO:11), which correspond to amino acid residues 173 to 186 from the *Listeria* endolysin PlyPSA (Korndörfer et al., 2006, J. Mol. Biol., 364, 678-689).

Further preferred embodiments according to the invention are the artificial peptidoglycan binding proteins CBD118-500 (SEQ ID NO:13) which comprise the CBD of ply500 (amino acid residues H133 to K289) in N-terminal position and the CBD of ply118 (amino acid residues D90 to I281) in C-terminal position connected without an additional linker sequence, and CBD118L500 (SEQ ID NO:15) which comprises the CBD of ply118 (amino acid residues K100 to I281) and the CBD of ply500 (amino acid residues Q150 to K289) with a linker (L) connecting the two domains. The domain linker in this case is the plyPSA linker region (SEQ ID NO:11).

The peptidoglycan binding proteins CBD500-118, CBD500L118, CBD118-500, and CBD118L500 all exhibit altered cell binding activities with respect to host range and binding activity compared to the wild-type enzymes ply500 and ply118 from which the CBD domains were derived. The cell binding activity of the constructs CBD500L118 and CBD118L500

discloses that a linker between two domains helps to achieve an extended host range which combines the binding specificities of the wt-enzymes.

Further preferred peptidoglycan binding proteins according to the present invention are CBD500-P35 (SEQ ID NO:17) which comprises the CBD of ply500 (amino acid residues Q150 to K289) in N-terminal position and the CBD of plyP35 (amino acid residues P130 to K291) in C-terminal position, and the protein with the inverse orientation of CBDs CBDP35-500 (SEQ ID NO:19) which comprises the CBD of plyP35 (amino acid residues P130 to K291) at the N-terminus, and the CBD of ply500 (amino acid residues Q150 to K289) at the C-terminus. In this case, the CBDs are not connected by an external linker sequence, as the fragment for the CBD of plyP35 includes the internal domain linker of plyP35.

The artificial peptidoglycan binding proteins CBD500-P35 and CBDP35-500 both exhibit an extended host range compared to the wild-type enzymes ply500 and plyP35 from which the CBD domains were derived. Both chimaeric proteins combine the different binding specificities of the two wt-enzymes within one protein. The orientation of the CBDs makes no difference in this case. Both CBDs can be positioned N-terminally as well as C-terminally.

Further preferred peptidoglycan binding proteins according to the present invention are CBD500-500 (SEQ ID NO:21) which exhibits a duplication of the CBD of ply500 (amino acid residues Q150 to K289), and the artificial peptidoglycan lysing enzyme EAD-CBD500-500 (SEQ ID NO:23) which exhibits a duplication of the naturally occurring CBD in ply500.

Both proteins according to the invention exhibit a higher binding affinity to *Listeria* cells compared to the wild-type, and EAD-CBD500-500 in addition exhibits an increased lysis activity under high salt conditions compared to ply500.

In another aspect the present invention relates to artificial peptidoglycan lysing enzymes which can be used to lyse, capture or detect *Enterococcus* bacteria. The inventors combined domains of the *Enterococcus* endolysins Fab25VL (SEQ ID NO:25) and Fab20VL (SEQ ID NO:27) using the method described above in order to create artificial peptidoglycan lysing enzymes and peptidoglycan binding proteins which exhibit new properties compared to the wild-type enzymes.

Fab25VL is an endolysin of 317 amino acid residues length which preferentially binds and lysis bacteria from the species *E. faecium*, but also some strains from the species *E. faecalis*. The N-terminally positioned EAD of Fab25VL (amino acid residues 1 to 167) exhibits a conserved Amidase_2 domain which functions as an N-acetylmuramoyl-L-alanine-amidase. The CBD comprises the amino acid residues 200 to 317. Between the two domains, a linker region comprising amino acid residues 168 to 199 is observed. Fab20VL is an endolysin of 365 amino acid residues length which preferentially binds and lysis bacteria from the species *E. faecalis*. The N-terminally positioned EAD (amino acid residues 40 to 194) of Fab20VL also exhibits a conserved Amidase_2 domain which functions as an N-acetylmuramoyl-L-alanine-amidase. The CBD of Fab20VL (amino acid residues 215 to 365) comprises a bacterial SH3 domain in its C-terminal part which shows homologies to peptidoglycan lysing enzymes from *Staphylococcus* and *Streptococcus* phages. An N-terminally truncated variant of Fab20VL with a deletion of amino acid residues 1 to 19 - Fab20K (SEQ ID NO:29) was constructed which showed better expression in *E. coli* compared to Fab20VL.

A preferred peptidoglycan lysing enzyme according to the present invention is SEQ ID NO:31 which combines the EAD and CBD of Fab25 (amino acid residues 1 to 317) with the CBD of Fab20VL (amino acid residues 215 to 365) and a short linker segment derived from Fab20VL (amino acid residues 200 to 214). The construct is denoted EADFab25_CBD25_CBD20. EADFab25_CBD25_CBD20 exhibits an extended host range, an increased lysis activity with respect to living cells and an increased binding affinity as new features compared to the wt-enzymes.

Further preferred peptidoglycan binding enzymes according to the present invention are composed of at least two EADs and at least two CBDs capable of detecting and binding *Staphylococcus* bacteria.

Preferably the peptidoglycan lysing proteins according to the present invention comprise tags such as His-tag (Nieba et al., 1997, Anal. Biochem., **252**, 217-228), Strep-tag (Voss & Skerra, 1997, Protein Eng., **10**, 975-982), Avi-tag (US 5,723,584; US 5,874,239), 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), cystein-tag (EP1399551, SEQ IDs No:6 and 7), HA-tag (amino acid sequence EQKLISEEDL), FLAG-tag (Hopp et al., Bio/Technology. 1988;6:1204-1210) or other tags known in the art. Preferably the tag is coupled to the C-

terminus or the N-terminus of the peptidoglycan lysing protein according to the invention, most preferably to the N-terminus. Tags can be useful to facilitate expression and/or purification of the peptidoglycan lysing protein, to immobilize the peptidoglycan lysing protein according to the invention to a surface or to serve as a marker for detection of the peptidoglycan lysing protein, e.g. by antibody binding in different ELISA assay formats.

Preferably the peptidoglycan lysing proteins according to the invention comprise marker or label moieties such as biotin, streptavidin, GFP (green fluorescent protein), YFP (yellow fluorescent protein), cyan fluorescent protein, RedStar protein or other fluorescent markers, alkaline phosphatase, horse radish peroxidase, immuno-gold labels, spin labels or other markers and labels known in the art. The markers can be attached in a recombinant way if they are of polypeptide nature or post-translationally by chemical modification of the polypeptide residues. The markers or labels are especially useful to detect the peptidoglycan lysing proteins according to the invention when they are used in diagnostics.

Further preferred peptidoglycan lysing proteins are the constructs HGFP-CBD118-500 (SEQ ID NO:33), HGFP-CBD500-118 (SEQ ID NO:35), HGFP-CBD118L500 (SEQ ID NO:37), HGFP-CBD500L118 (SEQ ID NO:39), HGFP-CBD500-P35 (SEQ ID NO:41), HGFP-CBDP35-500 (SEQ ID NO:43), HCBD500-GFP-CBD118 (SEQ ID NO:45), HCBD118-GFP-CBD500 (SEQ ID NO:47), HGFP-CBD500-500 (SEQ ID NO:49), and HEAD-CBD500-500 (SEQ ID NO:51). H denotes a his-tag including six histidines (SEQ ID NO:53), and GFP denotes green fluorescent protein introduced as a fluorescent marker (SEQ ID NO:55).

All of the above mentioned fusion constructs including a his-tag and a GFP marker show *Listeria* cell binding activity and the peptidoglycan lysing enzymes additionally lysis activity. The constructs HCBD500-GFP-CBD118 and HCBD118-GFP-CBD500 however show that an N-terminal position of the GFP marker is preferred compared to a positioning between the two CBD domains, as the cell binding activity, especially the cell binding activity of CBD118, is reduced in these constructs.

In summary, the object of the present invention is to alter and improve the properties of wild-type peptidoglycan lysing enzymes by artificial combination of functional domains by shuffling or by multiplication of naturally occurring domains. Peptidoglycan lysing enzymes according to the invention are composed of at least one EAD and at least two CBDs in order to extend the binding range of naturally occurring proteins, and/or to increase the binding

affinity to the bacterial cell wall, and/or to increase or modify the lytic activity. Peptidoglycan binding proteins according to the invention are composed of at least two CBDs in order to extend the binding range of naturally occurring proteins, and/or to increase the binding affinity to the bacterial cell wall. In the polypeptides according to the invention the at least two CBDs are derived from two different peptidoglycan lysing enzymes (domain shuffling) or by multiplication of naturally occurring CBDs.

Preferred is a recombinant polypeptide as depicted in SEQ ID NO: 7, 9, 13, 15, 17, 19, 21, 23, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 71, 73, 75, 77, 79, 81, 83, 85, 87, 89, 91, 93, 95, 97, 99, 101.

In another preferred embodiment of the present invention the recombinant polypeptides according to the present invention as listed above comprise modifications and/or alterations of the amino acid sequences. Such alterations and/or modifications may comprise mutations such as deletions, insertions and additions, substitutions or combinations thereof and/or chemical changes of the amino acid residues, e.g. biotinylation, acetylation, pegylation, chemical changes of the amino-, SH- or carboxyl- groups. Said modified and/or altered recombinant polypeptides exhibit the activity of the single domains of the respective recombinant polypeptide as listed above. However, said activity of the single domains can each be higher or lower as the activity of the single domains of the respective recombinant polypeptide as listed above. In particular said activity of the single domains can be about 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200 % or more of the activity of the single domains of the respective recombinant polypeptide as listed above. The activity of the single domains can be measured by the assays as already described herein for measuring the activity of the CBDs and EADs.

In a further aspect the present invention relates to a nucleic acid molecule comprising a nucleotide sequence encoding the polypeptides according to the present invention.

Preferred is a nucleic acid molecule, wherein the nucleic acid molecule comprises a nucleotide sequence as depicted in SEQ ID NO: 8, 10, 14, 16, 18, 20, 22, 24, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 52, 72, 74, 76, 78, 80, 82, 84, 86, 88, 90, 92, 94, 96, 98, 100, 102.

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.

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 bacterial infections in a subject, in particular for the treatment or prophylaxis of infections caused by gram positive bacteria like staphylococci (e. g. *S. aureus*, *S. aureus* (MRSA), *S. epidermidis*, *S. haemolyticus*, *S. simulans*, *S. saprophyticus*, *S. chromogenes*, *S. hyicus*, *S. warneri* and/or *S. xylosus*), enterococci (e.g. *Enterococcus faecium*, *E. faecium* (VRE) *Enterococcus faecalis*), streptococci (*Streptococcus pyogenes*, *S. pneumoniae*, *S. mutans*, *S. uberis*, *S. agalactiae*, *S. dysgalactiae*, *Streptococci of the Lancefield groups A, B, C*), clostridia (e. g. *C. perfringens*, *C. difficile*, *C. tetani*, *C. botulinum*, *C. tyrobutyricum*), bacilli (e. g. *Bacillus anthracis*, *B. cereus*), *Listeria* (e.g. *L. monocytogenes*, *L. innocua*), *Haemophilus influenza*, *Corynebacterium diphtheriae*, *Propionibacterium acne*, mycobacteria (e.g. *Mycobacterium tuberculosis*, *M. bovis*). Alternatively, the polypeptides and/or cells according to the invention are employed in a method for the treatment or prophylaxis of bacterial infections in a subject, in particular for the treatment or prophylaxis of infections caused by gram negative bacteria of bacterial groups, families, genera or species comprising strains pathogenic for humans or animals like *Enterobacteriaceae* (*Escherichia*, especially *E. coli*, *Salmonella*, *Shigella*, *Citrobacter*, *Edwardsiella*, *Enterobacter*, *Hafnia*, *Klebsiella*, especially *K. pneumoniae*, *Morganella*, *Proteus*, *Providencia*, *Serratia*, *Yersinia*), *Pseudomonadaceae* (*Pseudomonas*, especially *P. aeruginosa*, *Burkholderia*, *Stenotrophomonas*, *Shewanella*, *Sphingomonas*, *Comamonas*), *Neisseria*, *Moraxella*, *Vibrio*, *Aeromonas*, *Brucella*, *Francisella*, *Bordetella*, *Legionella*, *Bartonella*, *Coxiella*, *Haemophilus*, *Pasteurella*, *Mannheimia*, *Actinobacillus*, *Gardnerella*, *Spirochaetaceae* (*Treponema* and *Borrelia*), *Leptospiraceae*, *Campylobacter*, *Helicobacter*, *Spirillum*, *Streptobacillus*, *Bacteroidaceae* (*Bacteroides*, *Fusobacterium*, *Prevotella*, *Porphyromonas*), *Acinetobacter*, especially *A. baumannii*.

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.

In particular said method of treatment may be for the treatment or prophylaxis of infections of the skin, of soft tissues, the respiratory system, the lung, the digestive tract, the eye, the ear, the teeth, the nasopharynx, the mouth, the bones, the vagina, of wounds of bacteremia and/or endocarditis.

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*.

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, especially by antibiotic resistant strains like *Staphylococcus aureus* (MRSA), *Enterococcus faecium* (VRE), *Pseudomonas aeruginosa* (FQRP) or antibiotic resistant *Clostridium difficile* 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.

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 bacterial strains, in particular by strains resistant against one or more of the following antibiotics: penicillin, streptomycin, tetracycline, methicillin, cephalothin, gentamicin, cefotaxime, cephalosporin, vancomycin, linezolid, ceftazidime, imipenem 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.

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.

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 peptidoglycan lysing enzyme is protected from environmental influences such as proteases, oxidation, immune response etc., until it reaches the site of infection.

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.

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

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.

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.

All medical applications rely on the effect of the polypeptides of the present invention to lyse specifically and immediately pathogenic 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.

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.

In an even further aspect the polypeptides and/or cells are part of a cosmetics composition. As mentioned above, several bacterial species can cause irritations on environmentally exposed surfaces of the patient's body such as the skin. In order to prevent such irritations or in order to eliminate minor manifestations of said bacterial pathogens, special cosmetic preparations may be employed, which comprise sufficient amounts of polypeptides of the present invention in order to lyse already existing or freshly settling pathogenic bacteria.

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 pathogenic, facultative pathogenic or other undesirable bacteria can potentially infest food material.

A further aspect the present invention relates to the use of said polypeptides according to the present invention in diagnostics of bacterial infections. In this aspect the polypeptides according to the invention are used as a tool to specifically lyse pathogenic bacteria. The lysis of the bacterial cells by the polypeptides according to the present invention can be supported by the addition of detergents like Triton X-100 or other additives which weaken the bacterial cell envelope like polymyxin B. Specific cell lysis is needed as an initial step for subsequent specific detection of bacteria using nucleic acid based methods like PCR, nucleic acid hybridization or NASBA (Nucleic Acid Sequence Based Amplification), immunological methods like IMS, immunofluorescence or ELISA techniques, or other methods relying on the cellular content of the bacterial cells like enzymatic assays using proteins specific for distinct bacterial groups or species (e.g. β -galactosidase for enterobacteria, coagulase for coagulase positive strains).

Another aspect of the present invention is the use of a peptidoglycan binding protein according to the present invention for binding, enrichment, removing, capture and detection of pathogenic or otherwise undesirable bacteria from a sample. A sample with regard to the methods according to the present invention is any material supposed to or containing bacteria, whereas the bacteria are a target for detection, binding, enrichment, removing or capture. Samples can be e.g. food or feed materials, surface materials or human or veterinary diagnostic probes. Bacteria detection is performed via detection of markers attached to the peptidoglycan binding protein according to the present invention or by detection of said protein itself, e.g. by immunological methods like ELISA. For the methods according to the

present invention the peptidoglycan binding proteins according to the present invention may be immobilised on suitable supporting structures, e.g., microtiter plates, test stripes, slides, wafers, filter materials, reaction tubes, magnetic, glass or latex particles, pipette tips or flow-through cell chambers. The supporting structures may consist of, e.g., polystyrene, polypropylene, polycarbonate, PMMA, cellulose acetate, nitrocellulose, glass, silicon wafer, latex. The immobilisation may be accomplished by adsorption, by covalent binding or by further proteins, wherein the covalent binding is preferred. It is relevant that immobilisation is a functional one, that is, said peptidoglycan binding proteins exhibit structures accessible for bacteria although they are bound to the support material.

Examples

Example 1. DNA techniques and cloning procedures

DNA techniques and cloning procedures according to Sambrook *et al.* (Molecular cloning. A laboratory manual; 2nd ed. Cold Spring Harbor Laboratory Press 1989) were employed for construction of plasmids coding for endolysin based fusion proteins. The plasmid pQE-30 (QIAGEN) and its derivatives pHGFP, pHGFP_CBD118, pHGFP_CBD500 (Loessner et al. 2002), and pHEADPSA (Korndorfer et al. 2006), were used as vector backbones for the construction of plasmids coding for N-terminally 6xHis-tagged artificial fusion proteins (H stands for His-tag). Restriction sites needed for insertion of the fragments into the plasmids were introduced via the primers. Double CBD fusion constructs were created either by separate amplification of the two CBD fragments and subsequent ligation or alternatively by fusing the two fragments via the PCR based Gene Splicing by Overlap Extension (SOE PCR) method (Horton et al. 1990). CBD118 (SEQ ID NO:57) and CBD500 (SEQ ID NO:58) coding fragments were ligated via *EcoRI*/*MunI* sites in both orientations and then inserted into *SacI*/*SalI* sites of pHGFP, yielding pHGFP_CBD118-500 and pHGFP_CBD500-118. The plasmids pHGFP_CBDP35-500 and pHGFP_CBD500-P35 were created the same way. In case of pHGFP_CBD118L500 and pHGFP_CBD500L118, the fragment coding for the PlyPSA linker was introduced between the two CBDs by SOE PCR before insertion into pHGFP. As for pHCBD500_GFP_118 and pHCBD118_GFP_500, the 5' CBD and the GFP fragments were first fused via *KpnI* sites or by SOE PCR, and then ligated into *BamHI*/*SacI* sites of pHGFP_CBD118 and pHGFP_CBD500, respectively, replacing the mere GFP fragments of these plasmids. For construction of pHGFP_CBD500-500, the CBD500 fragment was cloned into the *SacI* site of pHGFP_CBD500, resulting in a duplication of

CBD500. pHEAD_CBD500-500 was created by inserting the complete *ply500* gene into *Bam*HI/*Sac*I sites of pHGFP_CBD500, replacing the GFP fragment. For all constructs, all stop codons except the ones at the 3' ends were omitted to allow genetic fusions. TAA was generally introduced as stop codon at the 3' ends. All constructs were verified by nucleotide sequencing.

Example 2. Overexpression and purification of His-tagged recombinant proteins

Overexpression of His-tagged (abbreviated in the respective constructs by "H") fusion proteins was performed in *E. coli* XL1-Blue MRF' (Stratagene). The respective strains were grown in modified LB medium (15 g/l tryptose, 8 g/l yeast extract, 5 g/l NaCl) containing 100 µg/ml ampicillin and 30 µg/ml tetracycline for plasmid selection at 30 °C, with 0.1 to 1 mM IPTG added as inducer once an OD₆₀₀ of 0.5 was reached. After further incubation at 30 °C for 4 h cultures producing proteins that contain a GFP domain were stored overnight at 4°C before harvesting and resuspension in 5 ml buffer A (500 mM NaCl, 50 mM Na₂HPO₄, 5 mM imidazole, 0.1 % Tween 20, pH 8.0) per 250 ml culture. If no GFP was present, cells were pelleted 4 h after induction. The cells were disrupted by two passages through a French Press 20K cell (SLM Aminco) at 100 MPa, and cell debris was removed by centrifugation and filtration (0.2 µm PES membrane, Millipore).

The 6xHis-tagged target proteins in the raw extracts were purified by Immobilized Metal Affinity Chromatography (IMAC) with Ni-NTA Superflow resin (QIAGEN) using Micro Biospin columns (BIORAD). Buffer B (500 mM NaCl, 50 mM Na₂HPO₄, 250 mM imidazole, 0.1 % Tween 20, pH 8.0) served as elution buffer. The purified proteins were dialyzed against two changes of dialysis Buffer (100 mM NaCl, 50 mM NaH₂PO₄, 0.005% to 0.1% Tween 20, pH 8.0), filtered (0.2 µm PES membrane, Millipore), and stored at -20 °C after addition of 50 % (v/v) of glycerol. For each protein, the course of overexpression and purification was analyzed by SDS-PAGE and the protein concentration was determined spectrophotometrically (NanoDrop ND-1000 Spectrophotometer).

Example 3. Binding assays and fluorescence microscopy

The binding properties of GFP-CBD fusion proteins were examined by binding using a representative set of *Listeria* strains (table 1) of all species and serovars. Late log phase cells of each strain in PBST buffer (50 mM NaH₂PO₄, 120 mM NaCl, pH 8.0, 0.01% Tween 20) were incubated with GFP-CBD protein in excess for 5 min at room temperature. After washing twice with buffer, the cells were prepared for fluorescence microscopy, using an Axioplan microscope and a filter set with excitation BP 450-490 nm, beamsplitter FT 510 nm,

and emission LP 520 nm (Carl Zeiss AG). Pictures of labelled cells were obtained by using a Leica DFC320 camera. For each assay, binding intensity was evaluated by visual inspection, using a four score system: ++, +, (+), and – indicates strong, weak, very weak and no binding, respectively.

The constructs HGFP_CBD500-118 and HGFP_CBD118-500, in which both cell wall binding domains were directly fused to each other in both orientations, and attached to an N-terminal GFP domain, both showed weak binding to all strains of serovars 4, 5, and 6 strains tested. As this corresponds to the binding pattern of the mere CBD500, these results suggested that CBD118 is not functional in these constructs. On the other hand it showed that CBD500 does not need to be located at the C-terminus of a protein to retain functionality. Assuming that enhanced flexibility of both binding domains in a fusion construct might render CBD118 functional, we created the proteins HGFP_CBD500L118 and HGFP_CBD118L500, which include the linker peptide of PlyPSA separating the CBDs. Again, both constructs labelled all strains belonging to serovar 4, 5, and 6 strains, but additionally also four out of seven serovar 1/2 strains were tested. From fluorescence microscopy it was observed that the latter were predominantly marked at the poles and septa as observed for HGFP_CBD118. In contrast, both proteins decorated strains of serovars 4, 5, and 6 in even distribution over the cell surfaces like HGFP_CBD500. Thus, these double CBD constructs combined properties of both CBDs, although their binding ranges within serovars 1/2, 3, and “7” were narrower than that of HGFP_CBD118. Introduction of a short linker not only enabled CBD118 to access its ligands, but it also enhanced binding of CBD500 in C-terminal position. The fusion protein HGFP_CBD118L500 displayed equally strong decoration of most of the serovar 4, 5, and 6 cells as HGFP_CBD500. In addition, two fusion constructs were generated in which the GFP was placed in central position, whereas CBD500 and CBD118 were either N- or C-terminally located. In HCBD500_GFP_CBD118, CBD118 was directly attached to the C-terminus of the GFP, placing it in the same environment as in HGFP_CBD118. This protein was able to mark all serovar 1/2 strains tested, although the decoration was very weak. The construct HCBD118_GFP_CBD500, in which the CBDs were inversely oriented, strongly bound to most of the serovar 4, 5, and 6 strains, but only weakly labelled one serovar 1/2 strain. Again, CBD500 showed stronger binding when located at the C-terminus. However, it was demonstrated to be functional also in N-terminal position (HCBD500_GFP_CBD118).

Table 1: Binding of GFP-tagged CBDs and double CBD fusion proteins from different *Listeria* endolysins to *Listeria* cells from different species and serovars. 500G118 and 118G500 stand for HCB500_GFP_118 and HCB118_GFP_500, respectively. “L” stands for a linker introduced between the shuffled CBDs. ++ strong, + weak, (+) very weak, - no binding; WLSC: Weihenstephan *Listeria* Strain Collection; SV: *Listeria* serovar

Species	WLSC code	Source	SV	Binding of HGFP_CBD										
				500	118	P35	500-118	118-500	500L118	118L500	500G118	118G500	500-P35	P35-500
<i>L. monocytogenes</i>	EGDe	J. Kreft	1/2a	-	++	++	-	-	+	(+)	(+)	-	++	++
<i>L. monocytogenes</i>	10403S	D. Portnoy	1/2a	-	++	++	-	-	-	-	(+)	-	++	+
<i>L. monocytogenes</i>	1442	Food	1/2a	-	++	-	-	-	+	+	(+)	+	-	-
<i>L. monocytogenes</i>	1066	SLCC 8800	1/2b	-	++	++	-	-	-	-	(+)	-	++	++
<i>L. monocytogenes</i>	1001	ATCC 19112	1/2c	-	++	++	-	-	-	-	(+)	-	++	++
<i>L. seeligeri</i>	4007	ATCC 35967	1/2b	-	++	++	-	-	+	+	(+)	-	++	++
<i>L. welshimeri</i>	50149	SLCC 5877	1/2b	-	++	+	-	-	+	+	(+)	-	(+)	(+)
<i>L. monocytogenes</i>	1485	soft cheese	3a	-	+	+	-	-	-	-	-	-	++	++
<i>L. monocytogenes</i>	1031	SLCC1694	3b	-	+	++	-	-	-	-	-	-	++	++
<i>L. monocytogenes</i>	1032	SLCC 2479	3c	-	+	++	-	-	-	-	-	-	++	++
<i>L. seeligeri</i>	40127	SLCC 8604	3b	-	+	++	-	-	-	-	-	-	++	++
<i>L. monocytogenes</i>	1034	SLCC 2482	“7”	-	+	-	-	-	-	-	-	-	-	-
<i>L. monocytogenes</i>	1020	ATCC 19114	4a	++	-	++	+	+	+	++	+	++	++	++
<i>L. monocytogenes</i>	1042	ATCC 23074	4b	++	-	-	+	+	+	++	+	++	++	++
<i>L. monocytogenes</i>	ScottA	J. Jay	4b	++	-	-	+	+	+	++	+	++	++	++
<i>L. monocytogenes</i>	1019	ATCC 19116	4c	++	-	++	+	+	+	++	+	++	++	++
<i>L. monocytogenes</i>	1033	ATCC 19117	4d	++	-	+	+	+	+	++	+	++	++	++
<i>L. monocytogenes</i>	1018	ATCC 19118	4e	++	-	(+)	+	+	+	++	+	++	++	++
<i>L. ivanovii</i>	3009	SLCC 4769	5	++	-	++	+	+	+	++	+	++	++	++
<i>L. ivanovii</i> (ssp. <i>Ivanovii</i>)	3010	ATCC 19119	5	++	-	++	+	+	+	++	+	++	++	++
<i>L. ivanovii</i> (ssp. <i>londoniensis</i>)	3060	SLCC 3765	5	++	-	-	+	+	+	+	(+)	+	+	+
<i>L. innocua</i>	2011	ATCC 33090	6a	++	-	-	+	+	+	+	(+)	+	++	++
<i>L. innocua</i>	2012	ATCC 33091	6b	++	-	++	+	+	+	++	+	++	++	++
<i>L. welshimeri</i>	50146	SLCC 7622	6a	++	-	+	+	+	+	++	+	++	++	++
<i>L. grayi</i> (ssp. <i>Grayi</i>)	6036	ATCC 19120	-	(+)	(+)	++	-	-	-	-	-	-	+	++
<i>L. grayi</i> (ssp. <i>Murrayi</i>)	6037	ATCC 25401	-	(+)	(+)	++	-	-	-	-	-	-	++	++

In a further approach, CBD118 in double CBD fusion constructs was replaced by CBDP35 (SEQ ID NO:59). The CBD of the endolysin of phage P35 strongly labelled most strains of serovars 1/2 and 3 as well as some strains of serovars 4, 5, and 6, binding in even distribution over the complete cell surface. The binding patterns of the newly constructed proteins HGFP_CBD500-P35 and HGFP_CBDP35-500 represented almost exact combinations of those of the single CBDs of Ply500 and PlyP35: They displayed strong binding to all strains which were either bound by CBD500 or by CBDP35 or by both, regardless of the location of the single CBDs within the fusions. These results proved that a combination of two cell wall binding domains from different peptidoglycan lysing enzymes can be fully functional in artificial fusion proteins, even when they are not in C-terminal position.

Example 4. Determination of binding affinity by surface plasmon resonance analysis (SPR) Affinities of HGFP_CBD500 (SEQ ID NO:60) and HGFP_CBD500-500 to the cell wall of *L. monocytogenes* WSLC 1042 were determined by surface plasmon resonance analysis, using a BIAcore X instrument and C1 sensor chips (BIAcore, Uppsala, Sweden). The chip surface was activated with the amine coupling method and coated with HGFP-CBD500 molecules in both flow cells (70 µl of 0.5 mg/ml protein in 10 mM sodium acetate buffer, pH 5, at a flow rate of 5 µl/min). Heat inactivated WSLC 1042 cells in HBS buffer (10 mM HEPES, 150 mM NaCl, 3.4 mM EDTA, 0.005% Tween 20, pH 7.8) were then bound to the immobilized CBDs in flow cell Fc2 (3.0×10^{10} cells per ml; 15 µl at a flow rate of 3 µl/min). Finally, interactions between the immobilized cells and 3 different concentrations of both HGFP_CBD500 (50 nM, 100 nM, 200 nM) and HGFP_CBD500-500 (12.5 nM, 25 nM, 50 nM) in HBS buffer were measured (30 µl at 10 µl/min), Fc1 serving as reference cell. The association phase was measured for 3 min, the dissociation phase for 12 min. All steps were carried out at 25 °C. Evaluation of kinetic data was performed with the BIAevaluation software, version 4.1 (BIAcore), employing a “1:1 binding with mass transfer” model. The equilibrium association constants obtained for three concentrations measured for each protein are given in table 2.

Table 2: Equilibrium affinity constants (K_A) of HGFP_CBD500 and HGFP_CBD500-500 binding to the cell wall of *Listeria monocytogenes* WSLC 1042.

HGFP_CBD500		HGFP_CBD500-500	
Concentration (nM)	K_A (M^{-1})	Concentration (nM)	K_A (M^{-1})
200	5.61×10^8	50	1.00×10^{10}
100	6.50×10^8	25	5.61×10^{10}
50	5.96×10^8	12.5	2.19×10^{10}
mean	6.02×10^8	mean	2.93×10^{10}

The construct HGFP_CBD500-500 comprising the artificial double CBD was shown to bind to the immobilized *Listeria* cells with an approximately 50 fold higher affinity compared to HGFP_CBD500 comprising the natural CBD of the endolysin of phage A500 - ply500. Comparing sensograms of both the single and double CBD protein constructs, it was obvious that both constructs mainly differed in the dissociation phase. Once bound to the cell surface, HGFP_CBD500 detached much more rapidly than HGFP_CBD500-500, resulting in the higher overall affinity of the double CBD construct.

Example 5. Photometric lysis assays

The lytic activity of wild-type and chimaeric peptidoglycan lysing enzymes was determined by a photometric lysis assay. Substrate cells of *Listeria monocytogenes* strains WSLC 1001 (serovar 1/2 c) and WSLC 1042 (serovar 4 b) were prepared by growing the bacteria in TB medium until late log phase and freezing them in 50-fold concentration in PBS buffer (50 mM NaH₂PO₄, 120 mM NaCl, pH 8.0). The assay was carried out in a total volume of 1 ml, with cells diluted to an initial OD₆₀₀ of approximately 1.0 in PBS. All purified native endolysins and chimeric proteins to be compared were added to the cells in equimolar amounts in a volume of 20 µl, and the OD at 600 nm was measured at intervals of 15 s for maximum 10 minutes. Enzymatic concentrations used ranged from 30 to 152 pmol/ml. For negative control, 20 µl buffer were added to the cells. All assays were carried out in triplicate. Loessner et al. (2002, Mol. Microbiol. 44, 335-349) suggested ionic interaction as the molecular basis for binding of CBDs to their ligands in the cell wall. The CBD of endolysin ply500 showed optimum binding at a NaCl concentration of approximately 100 mM and decreasing binding capacity with increasing salt concentration. Based on that, the lytic activity of his-tagged wild-type ply500 (HPL500) and a construct according to the invention using a duplication of the naturally occurring CBD500 of ply500 H_EAD_CBD500-500 was compared under high salt conditions. The assays were carried out as described above, but using NaCl concentrations (between 1M and 2 M). Photometric curves were normalized and corrected by the data of the control assays (corrected value = value + (1 – control value)). The resulting curves were fitted with the following sigmoid function, using the software SigmaPlot 9.0 (Systat Software, Inc.): $f = y_0 + a / (1 + \exp(-(x - x_0) / b))^c$. The steepest slope of the function was determined, which corresponds to the relative enzymatic activity.

Surprisingly, at salt concentrations of 1 M NaCl or higher, the peptidoglycan lysing enzyme according to the invention H_EAD_CBD500-500 showed higher lytic activity than the naturally occurring enzyme ply500.

Example 6. Protein expression and purification of *Enterococcus* endolysins

The *Enterococcus* endolysins Fab25VL, Fab20VL, Fab20K, and the peptidoglycan lysing enzyme according to the invention EADFab25_CBD25_CBD20 were expressed in and isolated from *E. coli* HMS174 DE3. Protein expression was performed for 3 h at 37 °C after induction with 1 mM IPTG. The bacterial cell pellet was harvested by centrifugation (5000 rpm, 15 min, 4 °C), resuspended in 25 ml buffer A (25mM Tris, pH 8.0, 500 mM NaCl, 20 mM imidazol, 0.1 % Tween 20, 10 % glycerol), and the cells disrupted in a microfluidizer. Bacterial cell debris was removed by centrifugation (12000 rpm, 5 min, 4 °C). The supernatant was submitted to an ammonium sulphate precipitation to 30 % saturation. The precipitate was collected by centrifugation (12000 rpm, 5 min, 4 °C). The supernatant including the endolysins was applied to hydrophobic chromatography using a 5 ml phenylsepharose column (High Sub FF, Amersham). The column was washed with 10 volumes of buffer B (25mM Tris, pH 7.0, 500 mM NaCl, 30 % ammonium sulphate, 10 % glycerol). The endolysins were eluted with 10 column volumes of buffer C (25mM Tris, pH 7.0, 500 mM NaCl, 10 % glycerol). Protein containing fractions were analyzed for endolysin on Coomassie stained SDS-gels. Endolysin containing fractions were pooled and analyzed for lysis activity in plate lysis assays according to example 7.

Example 7. Plate lysis assay to test the lysis activity and host range of peptidoglycan lysing proteins against *Enterococcus* bacteria.

A variety of *Enterococcus* bacteria from the medically relevant species *Enterococcus faecium* and *Enterococcus faecalis* were grown over night at 37 °C in precultures of 3 ml BHI medium. For each strain, 2 ml of the preculture was inoculated into 25 ml fresh medium and incubated up to an OD_{600 nm} of around 1. Bacterial cells were harvested by centrifugation at 4500 rpm for 15 min at 4 °C. The cell pellet was resuspended in 500 µl BHI medium. For the test of lysis activity against heat inactivated cells (table 3), the cells were incubated at 85 °C for 45 min and collected by centrifugation at 1400 rpm. The cell pellet was resuspended in 10 ml LB top agar, and the top agar poured onto LB plates. For the test of lysis activity against living cells (table 4), *Enterococcus* precultures were grown in 1 ml BHI medium over night, the preculture mixed with 10 ml BHI top agar, poured onto LB plates, and incubated for 2 h at 30 °C. The *Enterococcus* endolysins Fab25VL, Fab20K, an equimolar combination of the endolysins Fab25VL and Fab20K, and the artificial enzyme EADFab25_CBD25_CBD20 according to the invention were used. 5 µl peptidoglycan lysing protein solution each were

pipetted in spots onto the bacterial lawn immersed in the top agar. The appearance of lysis zones around the spotted protein solutions was analysed after 18 h of incubation of the plates at 30 °C using a four score system: +++, ++, +, and – indicates strong, medium, weak, weak and no lysis, respectively.

Table 3. Plate lysis assay using peptidoglycan lysing enzymes against heat inactivated *Enterococcus* cells

Species	Strain (ProCC)	Source	Fab25VL	Fab20K	1:1 25VL:20K	EADFab25_CBD25_CBD20
E. faecium	880	Profos	+++	-	+++	+++
E. faecium	1177	University hospital	+++	-	+++	+++
E. faecium	S1506	ATCC 20477	+++	-	+++	+++
E. faecium	S1553	DSMZ 2146	+++	-	+++	+++
E. faecium	S1563	University hospital	+++	-	+++	+++
E. faecium	S1564	University hospital	+++	-	+++	+++
E. faecium	S1565	University hospital	+++	-	+++	+++
E. faecium	S1568	University hospital	+++	-	+++	+++
E. faecium	S1570	University hospital	+++	-	+++	+++
E. faecium	S1634	Robert Koch Institute	+++	-	+++	+++
E. faecalis	17	Prof. Stetter	++	+++	+++	+++
E. faecalis	1176	ATCC 19433	-	+++	+++	+++
E. faecalis	S1505	University hospital	++	+++	+++	+++
E. faecalis	S1507	University hospital	++	+++	+++	+++
E. faecalis	S1552	DSMZ 2570	-	+++	+++	+++
E. faecalis	S1566	University hospital	+++	+++	+++	+++
E. faecalis	S1567	University hospital	++	+++	+++	+++
E. faecalis	S1569	University hospital	+++	+++	+++	+++
E. faecalis	S1571	University hospital	++	+++	+++	+++
E. faecalis	S1578	University hospital	-	+++	+++	+++
E. faecalis	S2465	University hospital	+++	+++	+++	+++

Table 4. Plate lysis assay using peptidoglycan lysing enzymes against living *Enterococcus* cells

Species	Strain (ProCC)	Source	Fab25VL	Fab20K	1:1 25VL:20K	EADFab25_CBD25_CBD20
<i>E. faecium</i>	880	Profos	+++	-	++	++
<i>E. faecium</i>	1177	University hospital	++	-	++	+++
<i>E. faecium</i>	S1506	ATCC 20477	+	-	+	++
<i>E. faecium</i>	S1553	DSMZ 2146	++	-	+	+++
<i>E. faecium</i>	S1563	University hospital	-	-	-	+++
<i>E. faecium</i>	S1564	University hospital	++	-	++	+++
<i>E. faecium</i>	S1565	University hospital	++	-	++	++
<i>E. faecium</i>	S1568	University hospital	++	-	++	++
<i>E. faecium</i>	S1570	University hospital	+	-	-	++
<i>E. faecium</i>	S1634	Robert Koch Institute	++	-	++	+++
<i>E. faecalis</i>	17	Prof. Stetter	+	-	-	+++
<i>E. faecalis</i>	1176	ATCC 19433	-	-	-	+
<i>E. faecalis</i>	S1505	University hospital	+	-	+	+++
<i>E. faecalis</i>	S1507	University hospital	-	-	-	+++
<i>E. faecalis</i>	S1552	DSMZ 2570	-	-	-	+++
<i>E. faecalis</i>	S1566	University hospital	+	-	+	+
<i>E. faecalis</i>	S1567	University hospital	+	-	+	+++
<i>E. faecalis</i>	S1569	University hospital	+	-	-	+++
<i>E. faecalis</i>	S1572	University hospital	+	-	-	+++
<i>E. faecalis</i>	S1578	University hospital	-	-	-	+++

It turned out that using heat inactivated *Enterococcus* cells (table 3), the endolysin Fab20K lysed all *E. faecalis* strains with high efficiency, but no strains of *E. faecium*. Fab20 Endolysin seemed to be specific for *E. faecalis* strains. Fab25VL lysed all strains of *E. faecium* with high efficiency, but only two strains of *E. faecalis*. The other *E. faecalis* strains were lysed with medium efficiency or were not lysed at all. The naturally occurring endolysin Fab25VL therefore was not strictly specific to *E. faecium*, but lysed strains from this species more reliably than strains from the species *E. faecalis*. A 1:1 mixture of the two

endolysins Fab25VL and Fab20K lysed all strains tested with high efficiency, but the same was achieved using only one enzyme according to the invention, namely EADFab25_CBD25_CBD20 which combines the CBDs of Fab25VL and Fab20K, but has only the EAD of Fab25VL. Using only one enzyme instead of two facilitates enzyme production, reduces costs, and minimizes immunological reactions in therapeutic applications. Performing the assay using living cells (table 4), the advantages using the enzyme according to the invention EADFab25_CBD25_CBD20 were more pronounced. Whereas Fab25VL lysed mainly *E. faecium* cells under these assay conditions, and only some *E. faecalis* cells with low efficiency, the endolysin Fab20K did not work at all. None of the living cells were lysed at all, suggesting that maybe the cell surface receptors for endolysin binding or the substrate molecules for peptidoglycan lysis were not accessible in living cells. A combination of the two enzymes did not improve the situation, but cell lysis using EADFab25_CBD25_CBD20 gave much better results. All strains tested were lysed at least with low efficiency, but most of the strains were lysed with high efficiency. This unexpected result shows that the artificial peptidoglycan lysing enzymes according to the invention can have favourable effects in specific uses even if a first characterization suggests a pure additive effect of the more than one CBD used in terms of the bacterial host range.

Example 8. Determination of the minimal bactericidal concentration (MBC) of peptidoglycan lysing enzymes against enterococci.

Enterococcus faecalis strain 17 was grown over night at 37 °C in BHI medium. The preculture was diluted 1:10 into 25 ml fresh medium and incubated at 37 °C up to an OD_{600 nm} of around 1. Bacterial cells were harvested by centrifugation at 4500 rpm for 5 min at 4 °C, and the cell pellet was resuspended in lysis buffer (PBS (2.25 mM NaH₂PO₃, 7.75 mM Na₂HPO₃, 150 mM NaCl) including, 2 mM CaCl₂, 10 mM BSA) to a concentration of 10⁵ cfu/ml. Protein solutions of Fab25VL and EADFab25_CBD25_CBD20 (1 mg/ml) were serially diluted to 50 µg/ml, 5 µg/ml, 0.5 µg/ml, 0.05 µg/ml, 0.005 µg/ml, 0.0005 µg/ml, and 0.00005 µg/ml in lysis buffer. 450 µl cell suspension and 50 µl protein solution of each concentration were mixed and incubated for 1 h at 37 °C. As a control, lysis buffer without protein added was incubated. 100 µl of 1:10 and 1:100 dilutions of the lysis samples were plated to LB agar plates, incubated for 1 day at 37 °C and counted for cells surviving the lysis by the peptidoglycan lysing enzymes. The MBC99.9 %, for example, is defined as the lowest

enzyme concentration at which the initial bacterial cell concentration is reduced by a factor of 1000. In this case, the cfu/ml of surviving cells had to be lower than 10^2 .

It was observed that the MBC against *Enterococcus faecalis* strain 17 was lower using the peptidoglycan lysing enzyme according to the invention EADFab25_CBD25_CBD20 than the naturally occurring enzyme Fab25 VL. The MBC99.9 % was 0.05 µg/ml with EADFab25_CBD25_CBD20 whereas it was 5 µg/ml using Fab25VL. This result suggests a higher binding affinity of EADFab25_CBD25_CBD20 to the bacterial cells and/or an increased lysis activity. Using the polypeptide according to the invention a factor 100 less protein has to be used in order to kill the pathogenic bacteria.

Claims

1. Recombinant polypeptide having the activity of binding and lysing of bacteria, comprising at least one enzymatically active domain and at least two bacterial cell binding domain.
2. Recombinant polypeptide having the activity of binding bacteria, comprising at least two bacterial cell binding domain.
3. Recombinant polypeptide according to claim 1, comprising two enzymatically active domains.
4. Recombinant polypeptide according to anyone of claims 1, 2 or 3, wherein the enzymatically active domain(s) and/or bacterial cell bindings domain are derived from two different peptidoglycan lysing enzymes.
5. Recombinant polypeptide according to anyone of claims 1 to 4, wherein the enzymatically active domain(s) is/are selected from the group consisting of Amidase_5 (bacteriophage peptidoglycan hydrolase, pfam05382), Amidase_2 (N-acetylmuramoyl-L-alanine amidase, pfam01510), Amidase_3 (N-acetylmuramoyl-L-alanine amidase, pfam01520), Transgly (transglycosylase, pfam00912), Peptidase_M23 (peptidase family M23, pfam01551), endolysin_autolysin (CD00737), Hydrolase_2 (cell wall hydrolase, pfam07486), CHAP (amidase, pfam05257), Transglycosylase (transglycosylase like domain, pfam06737), MtlB (membrane-bound lytic murein transglycosylase B, COG2951), MtlA (membrane-bound lytic murein transglycosylase A, COG2821), MtlE (membrane-bound lytic murein transglycosylase E, COG0741), bacteriophage_lambda_lysozyme (lysis of the bond between N-acetylmuramic acid and N-acetylglucosamine, CD00736), Peptidase_M74 (penicillin-insensitive murein endopeptidase, pfam03411), SLT (transglycosylase SLT, pfam01464), Lys (C-type lysozyme/alpha-lactalbumin family, pfam00062), COG5632 (N-acetylmuramoyl-L-alanine amidase, COG5632), MepA (murein endopeptidase, COG3770), COG1215 (glycosyltransferase, COG1215), AmiC (N-acetylmuramoyl-L-alanine amidase, COG0860), Spr (cell wall-associated hydrolase, COG0791), bacteriophage_T4-like_lysozyme (lysis of the bond between N-acetylmuramic acid and N-acetylglucosamine, cd00735), LT_GEWL (lytic transglycosylase (LT) and goose egg

white lysozyme (GEWL) domain, cd00254), peptidase_S66 (LD-carboxypeptidase, pfam02016), Glyco_hydro_70 (glycosyl hydrolase family 70, pfam02324), Glyco_hydro_25 (glycosyl hydrolase family 25), VanY (D-alanyl-D-alanine carboxypeptidase, pfam02557), and LYZ2 (lysozyme subfamily 2, smart 00047).

6. Recombinant polypeptide according to anyone of claims 1 to 4, wherein the bacterial cell bindings domains are selected from the group consisting of SH3_5 (bacterial SH3 domain, pfam08460), SH3_4 (bacterial SH3 domain, pfam06347), SH3_3 (bacterial SH3 domain, pfam08239), SH3b (bacterial SH3 domain homologue, smart00287), LysM (LysM domain found in a variety of enzymes involved in cell wall degradation, pfam01476 and cd00118), PG_binding_1 (putative peptidoglycan binding domain, pfam01471), PG_binding_2 (putative peptidoglycan binding domain, pfam08823), MtlA (peptidoglycan binding domain from murein degrading transglycosylase, pfam03462), Cpl-7 (C-terminal domain of Cpl-7 lysozyme, pfam08230), CW_binding_1 (putative cell wall binding repeat, pfam01473), LytB (putative cell wall-binding domain, COG2247), and LytE (LysM repeat, COG1388).
7. Recombinant polypeptide according to anyone of claims 1 to 6, wherein said domains are in the range of about 15 to about 250 amino acid residues long, particular in the range of about 20 to about 200 amino acid residues long and more particular about 15 to about 40 amino acid residues long.
8. Recombinant polypeptide according to anyone of claims 1 to 7, wherein the enzymatically active domain(s) and/or the bacterial cell binding domains are derived from wild-type peptidoglycan lysing enzymes selected from the group consisting of Ply500, Ply511, Ply118, Ply100, PlyP40, Ply3626, phiLM4 endolysin, PlyCD119, PlyPSAa, Ply21, PlyBA, Ply12, PlyP35, PlyPH, PlyL, PlyB, phi11 endolysin, phi MR11 endolysin, phi12 endolysin, *S. aureus* phage PVL amidase, pypitti26, ΦSA2usa endolysin, endolysin of *Staphylococcus warneri* M phage ΦWMY PlyGBS, B30 endolysin, Cpl-1, Cpl-7, Cpl-9, PlyG, PlyC, pal amidase, Fab25, Fab20, endolysins from the *Enterococcus faecalis* V583 prophage, lysostaphin, phage PL-1 amidase, *S. capitis* ALE-1 endopeptidase, mutanolysin (N-acetylmuramidase of *Streptomyces globisporus* ATCC 21553), enterolysin A (cell wall degrading bacteriocin from *Enterococcus faecalis* LMG 2333), LysK, LytM, Ami

autolysin from *L. monocytogenes*, endolysins of the *Pseudomonas aeruginosa* phages Φ KZ and EL, T4 lysozyme, gp61 muramidase, and STM0016 muramidase.

9. Recombinant polypeptide according to claim 8, wherein the enzymatically active domain derived from PlyP40 is encoded by an amino acid sequence according to SEQ ID NO: 103 and/or wherein the bacterial cell binding domain derived from PlyP40 is encoded by an amino acid sequence according to SEQ ID NO: 104.
10. Recombinant polypeptide as depicted in SEQ ID NO: 7, 9, 13, 15, 17, 19, 21, 23, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 71, 73, 75, 77, 79, 81, 83, 85, 87, 89, 91, 93, 95, 97, 99, 101.
11. Nucleic acid molecule comprising a nucleotide sequence encoding for a recombinant polypeptide according to anyone of claims 1 to 10.
12. Nucleic acid molecule according to claim 11, wherein the nucleic acid molecule comprises a nucleotide sequence as depicted in SEQ ID NO: 8, 10, 14, 16, 18, 20, 22, 24, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 52, 72, 74, 76, 78, 80, 82, 84, 86, 88, 90, 92, 94, 96, 98, 100, 102.
13. Vector comprising a nucleic acid molecule according to claims 11 or 12.
14. Recombinant polypeptide according to anyone of claims 1 to 9 for use as a medicament.
15. Use of the recombinant polypeptide according to anyone of claims 1 to 9 as a disinfectant in medical, public or private environment, as a decontaminant of bacterial contamination in food industry, animal feed or cosmetic industry or as a surfactant against bacterially contaminated surfaces.
16. Use of the recombinant polypeptide according to anyone of claims 1 to 9 as a diagnostic means in the medicine, food or feed diagnostic or environmental diagnostic.

Fig. 1

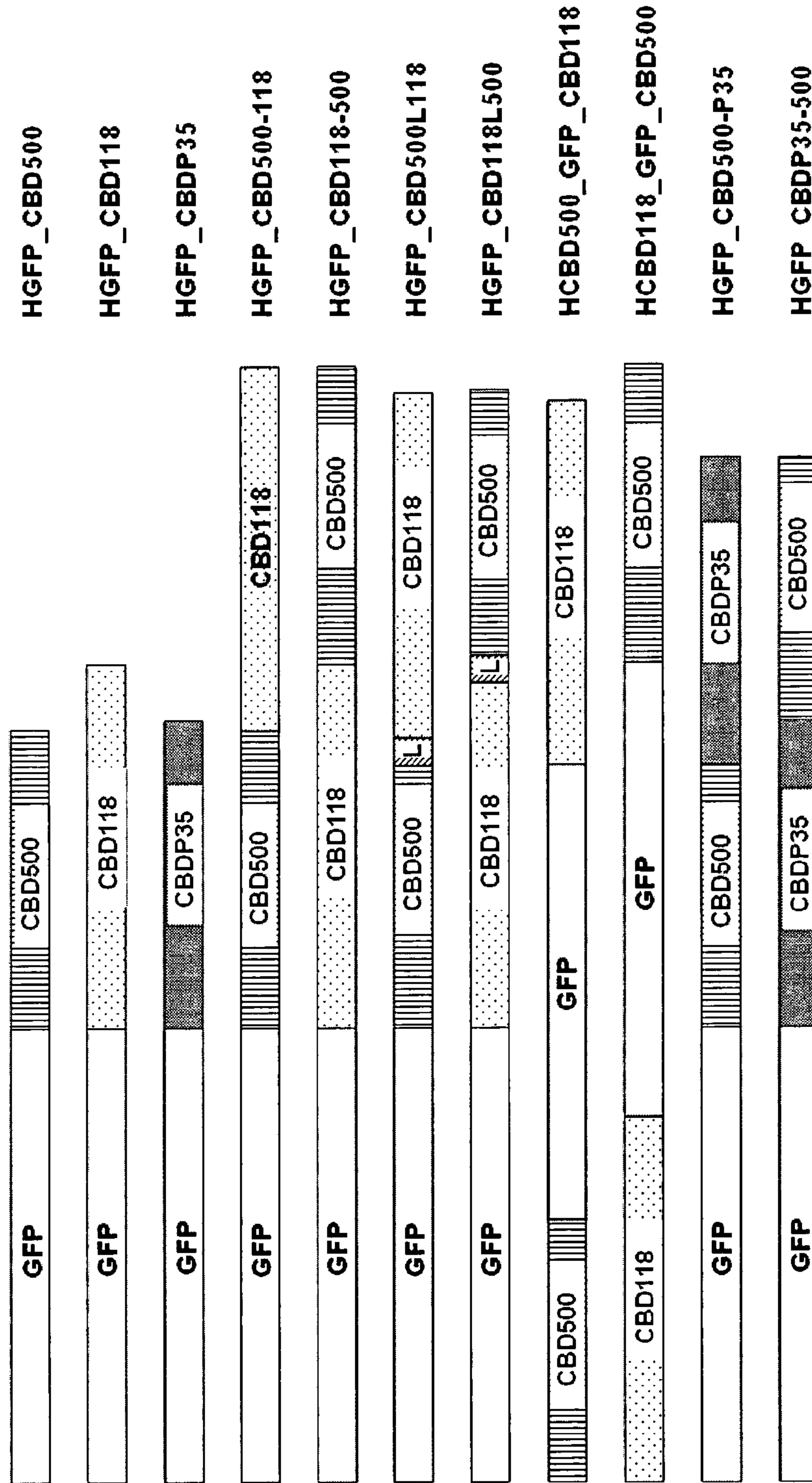


Fig. 2

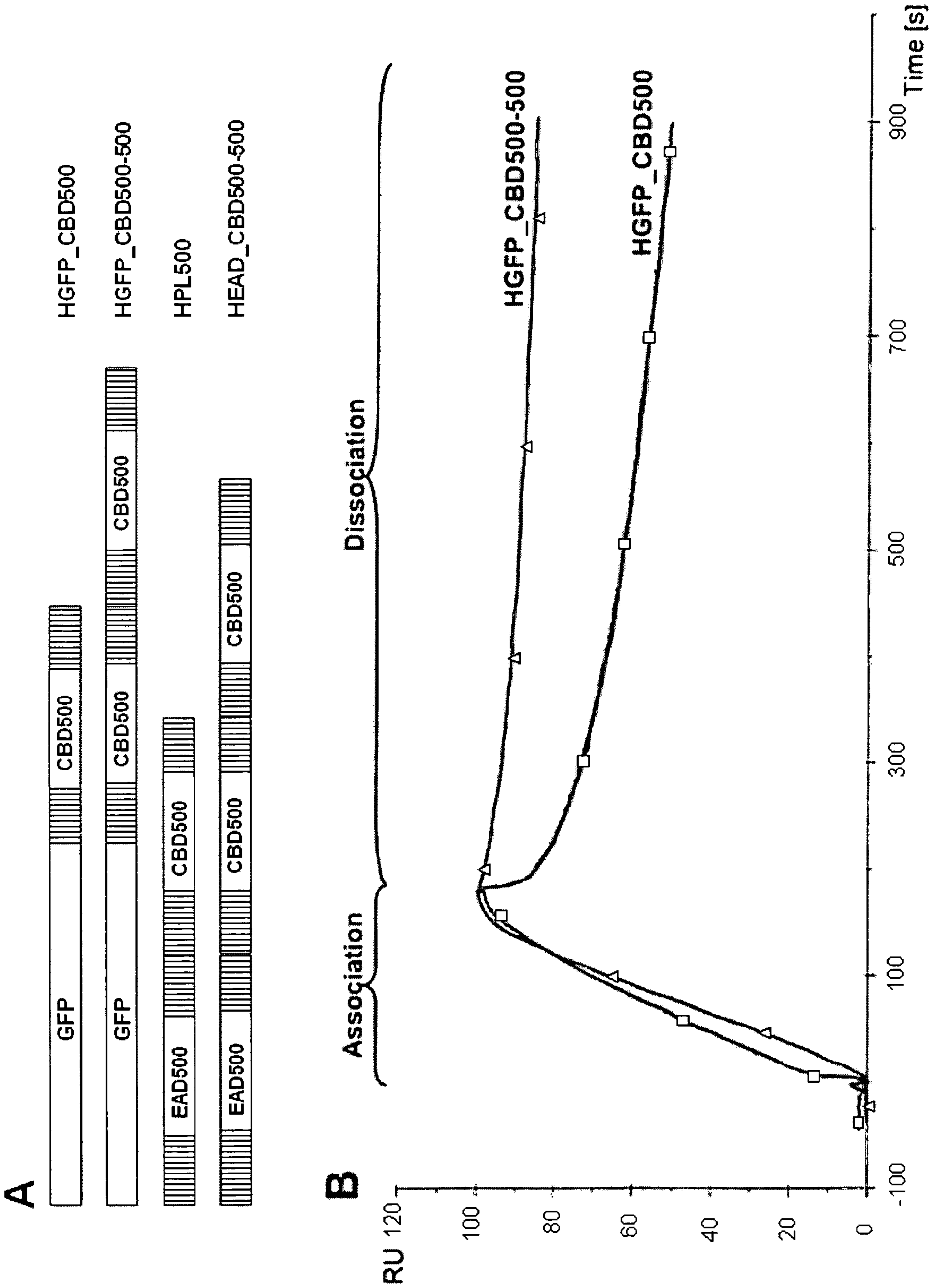


Fig. 3

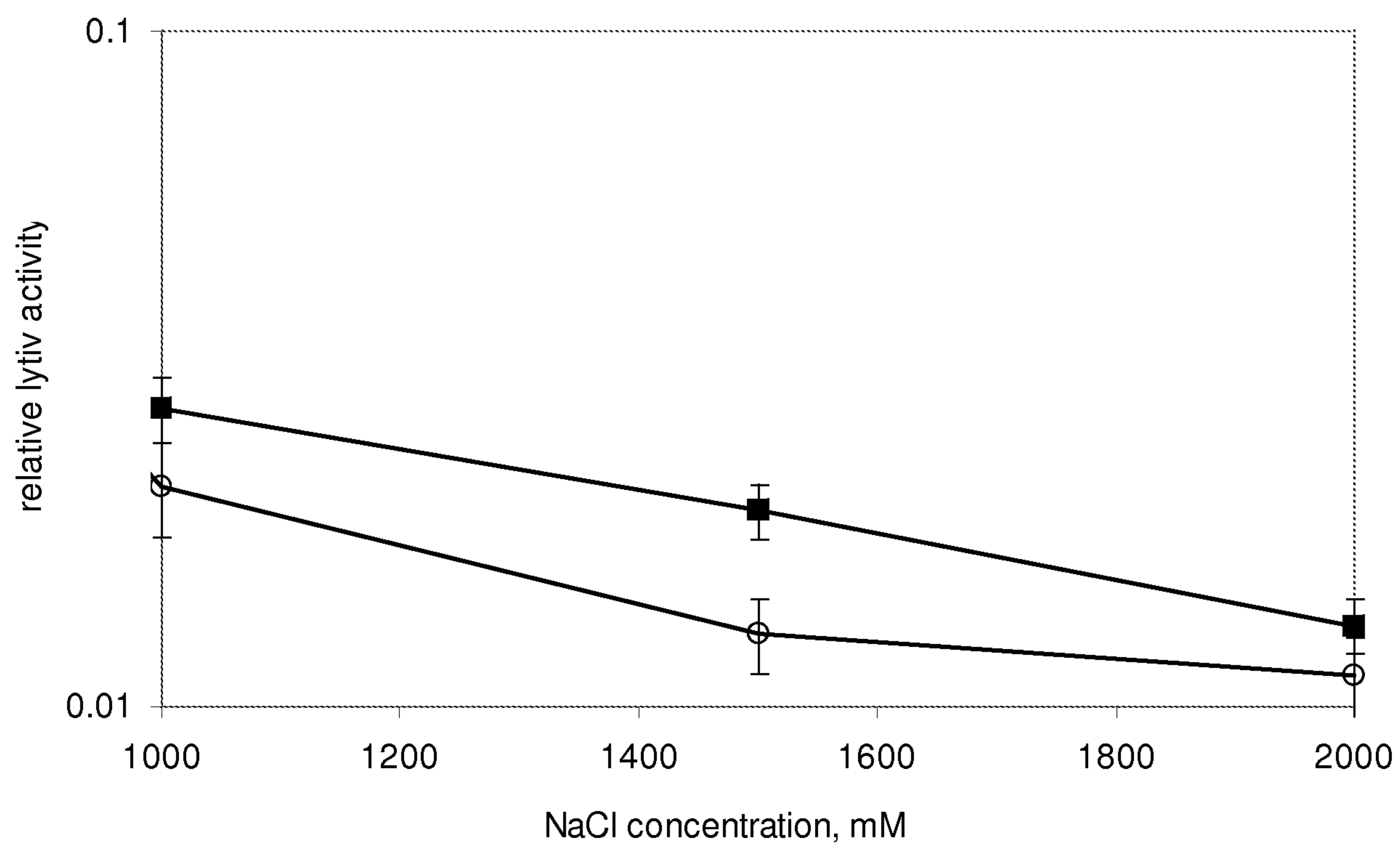
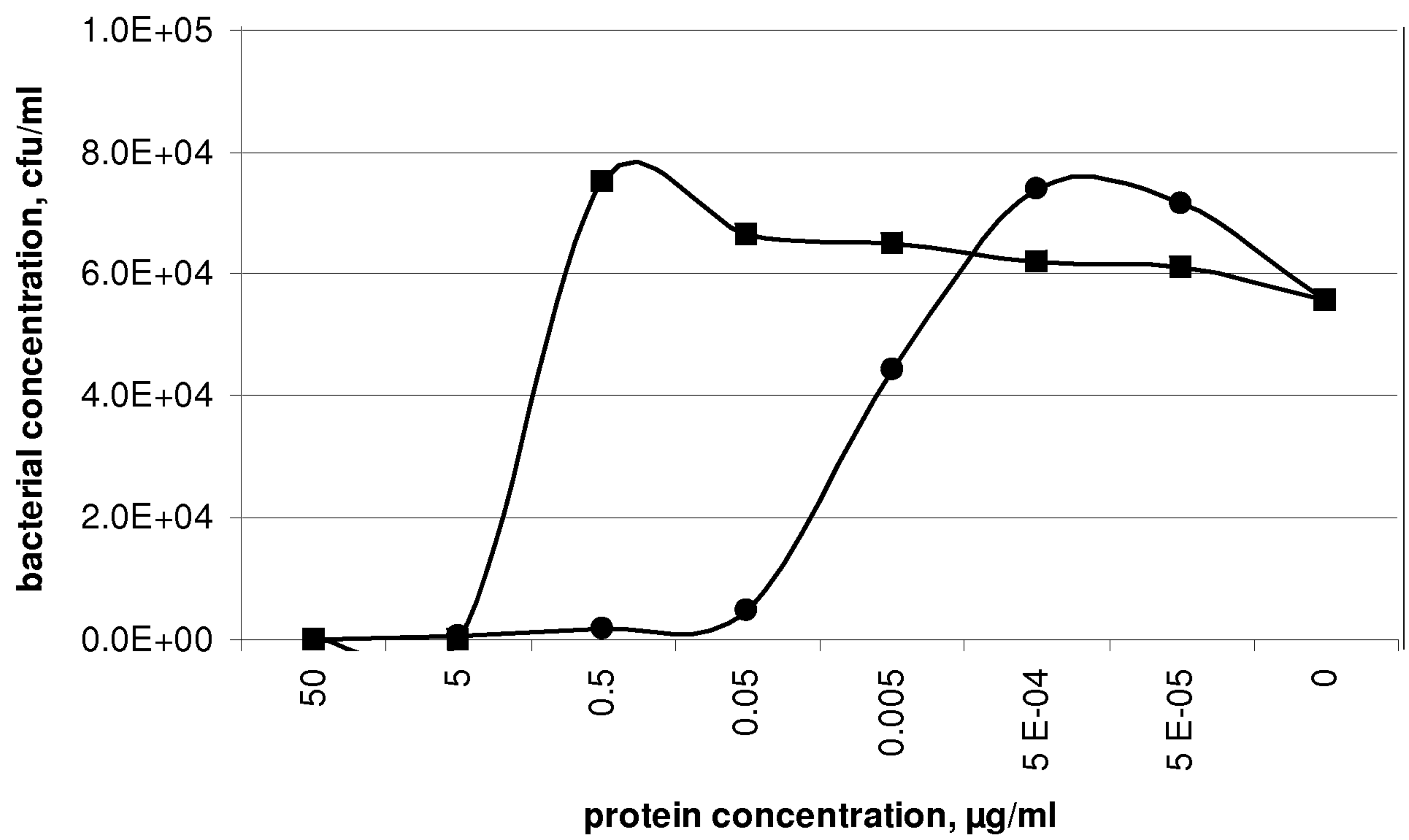
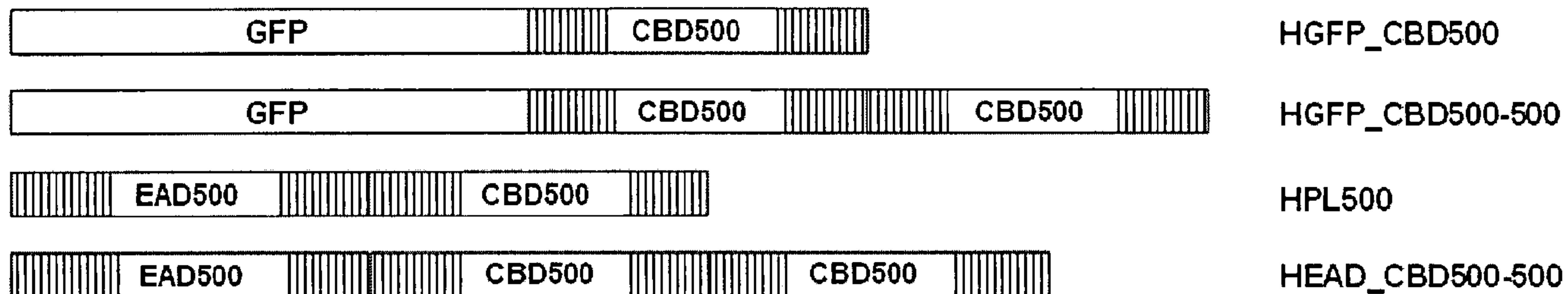


Fig. 4



A**B**