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(54) Title: POLYPEPTIDES DERIVED FROM ENTEROCOCCUS AND THEIR USE FOR VACCINATION AND THE GENERATION OF THERAPEUTIC ANTIBODIES

(57) Abstract: Medicament for the treatment or the prevention of a bacterial infection, characterized in that it contains at least one polypeptide selected from the group of SEQ ID NO: 1 to SEQ ID NO: 9, and contiguous fragments thereof, wherein said at least one polypeptide or contiguous fragment thereof induces opsonic antibodies in a patient in need thereof. The polypeptides or the contiguous fragments thereof according to the present invention can be used for the preparation of a vaccine against an infection against *Enterococcus*.

Polypeptides derived from Enterococcus and their use for vaccination and the generation of therapeutic antibodies

The present invention relates to a medicament for the treatment or the prevention of a bacterial infection, characterized in that it contains at least one polypeptide selected from the group of SEQ ID NO: 1 to SEQ ID NO: 9, and contiguous fragments thereof, wherein said at least one polypeptide or contiguous fragment thereof induces opsonic antibodies in a patient in need thereof. The polypeptides or the contiguous fragments thereof according to the present invention can be used for the preparation of a vaccine against an infection against *Enterococcus*.

Background of the present invention

Enterococci are among the three most common nosocomial pathogens and due to their multiple antibiotic resistances cause substantial morbidity and mortality, especially among intensive care patients and the immunocompromised. While several new antibiotics have been introduced in the last decade, resistance against these new drugs is developing and spreading rapidly. Life-threatening systemic disease such as endocarditis caused by resistant strains may at times be untreatable. Therefore, alternative treatment and prevention strategies are desperately needed to counter the rise of multiply resistant clones in hospitals and nursing homes worldwide. A better understanding of the different enterococcal cell surface structures will help to target new therapeutic and prophylactic approaches.

It is known that all gram-positive bacteria (also those belonging to the genus of *Enterococcus)* contain in the cell wall several specific carbohydrates and proteins. In the course of the present invention several proteins have been identified that play a possible role in the dynamic equilibrium of the outer cell wall.

The humoral immune response is mediated by antibody molecules secreted by plasma cells. Antigen that binds to the B-cell antigen receptor signals B-cells and is at the same time internalized and processed into peptides that activate armed helper T-cells. Signals from the bound antigen and from the helper T-cell induce the B-cell to proliferate and differentiate into plasma cells secreting specific antibody. These antibodies protect the host from infection in three main ways. First, said antibodies can inhibit the toxic effects or infectivity of pathogens by binding to them. Such antibodies are termed neutralizing antibodies. Second, by coating the pathogens, said antibodies can enable accessory cells that recognize the Fc portions of arrays of antibodies to ingest and kill the pathogen. This process is called opsonisation. Third, antibodies can trigger the activation of the complement system. Complement proteins can strongly enhance opsonisation or can directly kill certain bacterial cells.

Genomics is a recently introduced new discipline that studies the functioning of an organism through its genome and has greatly accelerated the identification of new candidates for vaccine development. More than fifty genomic sequences of enterococci are currently available in different data bases and this number is constantly growing. The analysis of all these genes and genomes is an extremely fast and efficient approach to predict the cellular localization and/or function of proteins synthesized in an organism. This *in silico* approach combined with functional genomics data will in future years help to identify new targets for vaccination tests (Sette and Rappuoli, 2010).

EP2248533 discloses a medicament for the treatment or the prevention of a bacterial infection is disclosed which contains a polypeptide having a contiguous sequence of at least six amino acids of SEQ ID NO:1 as disclosed therein. Said polypeptide can be used for the preparation of a vaccine against an Enterococcus infection.

Similarly, EP2450053 discloses another polypeptide used for the preparation of a vaccine against an Enterococcus infection.

WO 2010/089340 relates to a protective peptide of Enterococcus faecalis (E. faecalis) or a functional active variant thereof, optionally further consisting of additional amino acid residue(s); a nucleic acid coding for the same; a pharmaceutical composition comprising said peptide or said nucleic acid; an antibody or functional active fragment thereof specifically binding to the antigen; a hybridoma cell line which produces said antibody; a method for producing said antibody; a pharmaceutical composition comprising said antibody; the use of said peptide or said nucleic acid for the manufacture of a medicament for the immunization or treatment of a subject; the use of said antibody or functional fragment thereof for the manufacture of a medicament for the treatment of an infection; a method of diagnosing an E. faecalis infection; and the use of said peptide for the isolation and/or purification and/or identification of an interaction partner of the peptide.

WO2007141278 relates to human binding molecules (e.g. antibodies) having killing activity against enterococci and uses thereof.

For the production of vaccines it is important that the antigen elicits antibodies which inhibit the pathogenic activity of the pathogenic microorganism. The opsonophagocytic assay has been used to simulate the immune response in vitro and to identify enterococcal virulence factors (see, for example, Romero-Steiner et al., Use of opsonophagocytosis for serological evaluation of pneumococcal vaccines. Clin Vaccine Immunol. 2006 Feb;13(2):165-9). Protective antibodies elicited by a vaccine have therefore the effect of neutralization, opsonisation and complement activation whereby antibodies induced by a specific antigen may also have two or even three of the protective activities. However, only few antigens have been identified so far that may offer the potential of inducing a protective immune response, and therefore would be promising vaccine targets.

It is therefore an object of the present invention to provide new polypeptides or parts thereof which can be used in order to produce protective antibodies, preferably IgG antibodies against said polypeptide or parts of said polypeptide. It is furthermore an object of the present invention, to provide vaccine compositions against gram-positive bacteria, and in particular Enterococci, based on said polypeptides or parts thereof.

Fang Teng et al. (in: Fang Teng et al. An Enterococcus faecium Secreted Antigen, SagA, Exhibits Broad-Spectrum Binding to Extracellular Matrix Proteins and Appears Essential for E. faecium Growth, Infection and Immunity, September 2003, p. 5033-5041, Vol. 71, No. 9) discloses the extracellular and secreted *E. faecium* SagA protein as apparently essential for growth, showing broad-spectrum binding to ECM proteins, forming oligomers, and antigenic during infection. Furthermore, antibodies produced against recombinant Sag A are described. Although the protein is described as secreted antigen, there is no disclosure of an actual antigenicity of Sag A in vivo, let alone the formation of opsonic and/or protective antibodies in a host. The publication merely describes a C-terminal domain of Sag A that is „similar to that found in various proteins", including P60 (52% similarity) of *L. monocytogenes*, which has cell wall hydrolase activity, and has also been shown to be involved in virulence. The publication is thus limited to the functional characterization of Sag A.

Furthermore, the presence of a surface-exposed proteins does not automatically result in the formation of opsonic and/or protective antibodies in the host. In fact, to the knowledge of the inventors, only two protein antigens have been confirmed so far in *E. faecalis* as being the target of protective antibodies: an ABC transporter described by Burnie and colleagues (Burnie et al. Identification of ABC transporters in vancomycin-resistant Enterococcus faecium as potential targets for antibody therapy. FEMS Immunol Med Microbiol (2002) vol. 33 (3) pp. 179-89) and, only recently, the collagen adhesin ACE (Singh et al. Importance of the collagen adhesin ace in pathogenesis and protection against Enterococcus faecalis experimental endocarditis. PLoS Pathog (2010) vol. 6(1) pp. e1000716). Two other surface proteins have been shown to be *not* protective, namely the enterococcal aggregation substance (McCormick et al. Antibodies to a surface-exposed, N-terminal domain of aggregation substance are not protective in the rabbit model of Enterococcus faecalis infective endocarditis. Infect Immun (2001) vol. 69 (5) pp. 3305-14) and the enterococcal surface protein Esp (Sava et al., Sava IG Enterococcal surface protein contributes to persistence in the host but is not a target of opsonic and protective antibodies in Enterococcus faecium infection. J Med Microbiol. 2010 Sep;59(Pt 9):1001-4). Studies on

several other protein antigens have been reported in the literature as being involved in virulence, while no protective effect of either passive or active immunization has been documented. Therefore, to date, only very few vaccine targets, either proteinaceous or carbohydrates, have been identified in *E. faecium*.

In one aspect thereof, the object of the present invention is solved by providing a medicament for the treatment or the prevention of a bacterial infection, characterized in that said medicament contains at least one polypeptide selected from the group of SEQ ID NO: 1 to SEQ ID NO: 9, and contiguous fragments (active variants) thereof, wherein said at least one polypeptide or contiguous fragment thereof induces antibodies in a patient in need thereof. The present invention is based on the surprising finding that the polypeptides of SEQ ID NO: 1 to 9 or active fragments thereof can be used to provide such a vaccine target, and/or can form the basis to provide effective and preferably therapeutically effective (e.g. opsonic) antibodies.

In the context of the present invention, the inventors identified nine proteins (polypeptides) of *E. faecium* E155 and their homologues in *E. faecalis*, respectively, at least parts of which function as effective antigens (epitopes), and thus can be used to provide an active polypeptide and/or peptide-based vaccine or a passive antibody-based medicament for a prevention and/or treatment of a bacterial infection, such as, for example, infection caused by gram-positive bacteria, and in particular by Enterococci.

The main protective defense mechanism of the human immune system against enterococci is phagocytosis, which occurs through direct recognition of certain enterococcal surface structures or through opsonisation by antibody and complement. Therefore, in another aspect of the invention, the active vaccine according to the present invention can be administered to patients (preferably before the infection), in order to stimulate their immune response and to avoid an infection in a hospital or a nursing home.

The amino acid sequences of the polypeptides SEQ ID NOs: 1 to 9 are both disclosed in the attached Sequence Listing and Table 1 (see below). The polypeptides of SEQ ID NOs: 1 to

9 have been shown to be effective in opsonophagocytotic assays (see examples), a clear indication for their protective effect also *in vivo*.

Table 1: Polypeptides as identified according to the present invention.

Protein name and abbreviation	**SEQ ID No.**	**Locus Tag/Acc No.**	**Method for identification**
low affinity penicillin-binding protein 5 (PBP5)	1	EFAU004_00870; AAD26697	Biotin, High pH, Trypsin
Basic membrane lipoprotein (BML)	2	EFAU004_00080; YP_005353284	Biotin, High pH, Trypsin
peptidoglycan-binding protein LysM (LysM)	3	EFAU004_01059; WP_002337891	Biotin, High pH, Trypsin
D-alanyl-D-alanine carboxypeptidase (Dala)	4	EFAU004_01127; WP_016922432	Biotin, High pH, Trypsin
PpiC-type peptidyl-prolylcis-trans isomerase (PpiC)	5	EFAU004_02526; WP_002291335	Biotin, High pH, Trypsin
Enolase (Enol)	6	EFAU004_02073; YP_005355275	Biotin, High pH, Trypsin
SCP-like extracellular protein (serine protease) (SCP)	7	WP_002353118.1	Biotin, High pH, Trypsin
Adhesion lipoprotien (Adlip)	8	EFUG_02345; EEV57494	Biotin, High pH, Transcriptomic data
Periplasmic solute binding family (PSB)	9	EFAU004_00598	Biotin, High pH, Transcriptomic data

The nine polypeptides according to the invention were overexpressed and purified in order to inject said proteins into a rabbit. The animal then produced antibodies, based on the combination of proteins. The different sera as obtained showed a significant opsonic killing activity, confirmed by the stimulation of an *in vitro* immune response by opsonophagocytic assay. Moreover, the protein Sag A is used as a positive control in all the experiments, a promising vaccine target in *E. faecium* (EP 2 248 533 B1).

The person skilled in the art is aware that not necessarily the whole polypeptide has to be used for the production of a vaccine. Even shorter fragments based on contiguous amino acids of a polypeptide can be used. Such fragments are designated herein as “active variants” and comprise an (at least one) “epitope” that usually consist of at least six contiguous amino acids out of the SEQ ID NO: 1 to 9. Preferably, however, said polypeptides have at least 10,

more preferably at least 15 and more preferred at least 20 contiguous amino acids of the SEQ ID NO: 1 to 9. In a particularly preferred embodiment, the polypeptide has at least 30, more preferred at least 50 and especially preferred at least 100 contiguous amino acids of SEQ ID NO: 1 to 9. Preferred fragments are disclosed in table 2 and SEQ ID Nos. 10 to 167.

Active variants may also be obtained by changing the sequence of the polypeptide as defined herein and are characterized by having a biological activity similar to that displayed by the protective peptide of the sequence of SEQ ID NOs: 1 to 9 from which the variant is derived, including the ability to induce protective immune responses and/or to show protection against *E. faecium* or *E. faecalis* e.g. in an animal model as disclosed herein, wherein any variant may be tested as described in the Examples. In another preferred embodiment of the invention the polypeptide of the invention or an active variant, can consist of 1 to 400 additional amino acid residue(s), preferably 1 to 350, 1 to 300, 1 to 250, 1 to 200, 1 to 150, more preferably 1 to 100, even more preferably at most 1 to 50, most preferably 1, 2, 3, 4, 5, 10, 20, 30 or 40 additional amino acids residue(s).

In one preferred embodiment of the invention, the peptide and/or the antigens of the invention comprising additional amino acid residue(s) as defined herein is characterized in that it comprises at least 2, preferably at least 3, more preferably at least 4 epitopes as defined above. The antigenic peptide and/or the epitope may be flanked by the amino acid residue(s) C-terminally, N- terminally or C- and N-terminally.

The active variant of the polypeptide may have added at least one additional amino acid residue heterologous or homologous to the peptide of any of the SEQ ID NOs: 1 to 9. Homologous refers to any amino acid residue which is identical to the amino acid residue of the protein from *E. faecium* or *E. faecalis* from which the peptide is derived, wherein the peptide of any of the SEQ ID NO: 1 to 9 is derived from the polypeptide as listed in Table 1. Alternatively or additionally, the polypeptide may consist of the antigen, optionally the additional sequence as defined above and at least one amino acid residue heterologous to the antigen, preferably a marker protein.

The active variant of the polypeptide may be obtained by sequence alterations in the peptide, wherein the peptide with the sequence alterations retains a function of the unaltered peptide, e.g. having a biological activity similar to that displayed by the unaltered peptide. Such sequence alterations can include, but are not limited to, (conservative) amino acid substitutions, deletions, mutations and insertions. The additional sequence or amino acid residue(s) as defined above consists of (an) amino acid residue(s), which may be any amino acid, which may be either an L-and/or a D-amino acid, naturally occurring and otherwise. Preferably the amino acid is any naturally occurring amino acid such as alanine, cysteine, aspartic acid, glutamic acid, phenylalanine, glycine, histidine, isoleucine, lysine, leucine, methionine, asparagine, proline, glutamine, arginine, serine, threonine, valine, tryptophan or tyrosine. However, the amino acid may also be a modified or unusual amino acid. Examples of those are 2-aminoadipic acid, 3-aminoadipic acid, beta-alanine, 2-aminobutyric acid, 4-aminobutyric acid, 6-aminocaproic acid, 2-aminoheptanoic acid, 2-aminoisobutyric acid, 3-aminoisobutyric acid, 2-aminopimelic acid, 2, 4-di aminobutyric acid, desmosine, 2,2'-diaminopimelic acid, 2,3-diaminopropionic acid, N-ethylglycine, N-ethylasparagine, hydroxylysine, allo-hydroxylysine, 3-hydroxyproloine, 4-hydroxyproloine, isodesmosine, allo-isoleucine, N-methylglycine, N-methylisoleucine, 6-N-Methyllysine, N-methylvaline, norvaline, norleucine or ornithine. Additionally, the amino acid may be subject to modifications such as posttranslational modifications. Examples of modifications include acetylation, amidation, blocking, formylation, γ-carboxyglutamic acid hydroxylation, glycosylation, methylation, phosphorylation and sulfatation. If more than one additional or heterologous amino acid residue is present in the peptide, the amino acid residues may be the same or different from one another.

Alternatively, the polypeptide of any of the SEQ ID NOs: 1 to 9 may be essentially identical to or even consists of the antigen, i.e. the full-length protein is used as the epitope and/or antigen.

The person skilled in the art is aware that with suitable computer programs the hydrophobicity and hydrophilicity of the areas of the polypeptide can be determined.

Therefore, the preferred fragments are mainly hydrophilic since the parts of the polypeptide which are located on the outer areas of the folded polypeptide are preferred for the preparation of a vaccine. Moreover, when longer parts of the polypeptide are used, it is more likely that not only linear epitopes are within the fragment but also conformational epitopes are present which occur in the course of three-dimensional folding of the polyprotein.

Preferred active peptides (active variants) – and thus epitopes - according to the present invention are listed in the following table 2, and are disclosed in SEQ ID Nos. 10 to 167.

Table 2: Preferred peptides (epitopes) according to the present invention

Polypeptide/ SEQ ID No./ Reference Acc No./size of polypeptide	**Position/sequence of epitope (residues) in decreasing likelihood**
PBP5/1 AAD26697; 679 AA	GTLVYPKLIAD (SEQ ID NO. 41); AYIAGAVILIAA (SEQ ID NO. 42); LKQLGVVPSK (SEQ ID NO. 43); SYQVTRVSDVSQVDLKTALIYSDN (SEQ ID NO. 44); NQAISQSWVQPDYFVPLKII (SEQ ID NO. 45); EKKVLIEHE (SEQ ID NO. 46); GDLLALASSPS (SEQ ID NO. 47); IQEVDGRYYPLGEAAAQLIGYVGDI (SEQ ID NO. 48); GGYFYYQHYQETQAVEA (SEQ ID NO. 49); LQTIVPDLREVVQDV (SEQ ID NO. 50); VEQFVQAL (SEQ ID NO. 51); SELLQYLNQ (SEQ ID NO. 52); HSLSALGIPLAAK (SEQ ID NO. 53); GELLINPIQQAAMYSVF (SEQ ID NO. 54); PNLVFP (SEQ ID NO. 55); AADVKGLQISNLKVD (SEQ ID NO. 56); PNEVLTIN (SEQ ID NO. 57); ITAAIG (SEQ ID NO. 58); SDILLAD (SEQ ID NO. 59); IKAIASSF (SEQ ID NO. 60); YSFSYK (SEQ ID NO. 61); SFLFAF (SEQ ID NO. 62); KVSLTTQ (SEQ ID NO. 63); GELKDLSYKG (SEQ ID NO. 64); PELPAGA (SEQ ID NO. 65); QPFISR (SEQ ID NO. 66); GSTVATT (SEQ ID NO. 67); LDKYQNIY (SEQ ID NO. 68)
BML/2 YP_005353284; 360 AA	KVWVIGVD (SEQ ID NO. 69); NFVIIDDVIDGLDNVVSAT (SEQ ID NO. 70); TSTLKAVGTVVEDL (SEQ ID NO. 71); SYLAGVAAAY (SEQ ID NO. 72); EIKVLNQY (SEQ ID NO. 73); TNVVGFIGG (SEQ ID NO. 74); EHTVYGL (SEQ ID NO. 75); SGDVKVPE (SEQ ID NO. 76); GIGYKLKPAIQE (SEQ ID NO. 77); NADIIFHA (SEQ ID NO. 78); KAGVDAG (SEQ ID NO. 79); EDGVGLTEG (SEQ ID NO. 80); KKAVDE(SEQ ID NO. 81)
LysM/3 WP_002337891; 197 AA	EHTYVAPVETVEVAPAAPAAATAP (SEQ ID NO. 82); VAEQYVTSR (SEQ ID NO. 83); RIYVGEQLTIP (SEQ ID NO. 84); GRYQLDASYLNGD (SEQ ID NO. 85); LSKISQK (SEQ ID NO. 86)
Dala/4 WP_016922432; 435 AA	STVPVVLKSPVKVWVR (SEQ ID NO. 87); SITKIIGLYIVLDQV (SEQ ID NO. 88); AGACFVGT (SEQ ID NO. 89); IITVVLNA (SEQ ID NO. 90); AQDVAIVARHLILDFPEILDVSST (SEQ ID NO. 91); NLSVTPDLSNVPLH (SEQ ID NO. 92); ASMVALAEK (SEQ ID NO. 93); KVNAKAAFAVDAQ (SEQ ID NO. 94); KVSISD (SEQ ID NO. 95);

	PGFVNYK (SEQ ID NO. 96); VKELFDSAIIQSA (SEQ ID NO. 97); MDYCYD (SEQ ID NO. 98); KANIFVIGWR (SEQ ID NO. 99); QSPVEM (SEQ ID NO. 100); ATIVNAS (SEQ ID NO. 101); ASIPSLKTID (SEQ ID NO. 102); GKILYD (SEQ ID NO. 103); TITLAED (SEQ ID NO. 104)
PpiC/5 WP_002291335; 336 AA	YATEYYVVKMV (SEQ ID NO. 105); FEAGLKAHVDI (SEQ ID NO. 106); QSLVQRMIIYKVFNN (SEQ ID NO. 107); ENVLSAF (SEQ ID NO. 108); KSFHPEVEAQIIKLS (SEQ ID NO. 109); TITVSDF (SEQ ID NO. 110); TTKVIGE (SEQ ID NO. 111); DKQVDAE (SEQ ID NO. 112); PAEVKEAAFKL (SEQ ID NO. 113); ESQLEAA (SEQ ID NO. 114); KDQLKDI (SEQ ID NO. 115); SKLAKD (SEQ ID NO. 116);
Enol/6 YP_005355275; 432 AA	ANAILGVSIAVARAAADYLEVPLYHYLG (SEQ ID NO. 117); GVYVLAD (SEQ ID NO. 118); YTAVVSHR (SEQ ID NO. 119); EVFHALASILKAR (SEQ ID NO. 120); YEELVSKYPIISIE (SEQ ID NO. 121); KAGYVPGKDVVLAMD (SEQ ID NO. 122); ITDVYAREI (SEQ ID NO. 123); LTDVLGDKVQLVGDDLFVTNT (SEQ ID NO. 124); SDIAVATN (SEQ ID NO. 125); EVEVYTE (SEQ ID NO. 126); NSILIKVNQI (SEQ ID NO. 127); AEAIIGYDV (SEQ ID NO. 128); FEVIIEAI (SEQ ID NO. 129); LGEVAEYKGLKSFY (SEQ ID NO. 130); TKVLPT (SEQ ID NO. 131); YNQLLRIE (SEQ ID NO. 132); TKAVDNV (SEQ ID NO. 133); IMPVGAP (SEQ ID NO. 134); YEAVEL (SEQ ID NO. 135)
SCP/7 WP_002353118; 685 AA	GGHLVYRLYNK (SEQ ID NO. 136); YNQVHQINLLCNN (SEQ ID NO. 137); QDLLESLGCYGA (SEQ ID NO. 138); KELQVSFSHY (SEQ ID NO. 139); ILGLGHNFVVDSA (SEQ ID NO. 140); GDSVTLTAPSIQGYVLDDR (SEQ ID NO. 141); IYRLFLPGVKSGSHHYTA (SEQ ID NO. 142); TGLYIDDL (SEQ ID NO. 143); VNILKEQIVNVT (SEQ ID NO. 144); NVTVNHV (SEQ ID NO. 145); LEALYTSV (SEQ ID NO. 146); VDNVFISANP (SEQ ID NO. 147); KKPVPTV (SEQ ID NO. 148); LQALYNRV (SEQ ID NO. 149); DHLVKI (SEQ ID NO. 150); NAVLSS (SEQ ID NO. 151); NYFLCRN (SEQ ID NO. 152); NDIVQQAADI (SEQ ID NO. 153); SQAQVN (SEQ ID NO. 154); GQVIATDQAKVTSG (SEQ ID NO. 155); EYTVTIN (SEQ ID NO. 156); NANLLYNNQ (SEQ ID NO. 157); TALSNAKKVLDDS (SEQ ID NO. 158); TFKYKKI (SEQ ID NO. 159); KPKVNKS (SEQ ID NO. 160); DAAFKGLQHK (SEQ ID NO. 161); QNGVAPM (SEQ ID NO. 162); QKQVDS (SEQ ID NO. 163); AKKVLND (SEQ ID NO. 164); RDHHYTA (SEQ ID NO. 165); KAKAFK (SEQ ID NO. 166); DSAYKGL (SEQ ID NO. 167)
Adlip/8	KLKVVVTNSILAD (SEQ ID NO. 10); KIDLHSIVPIGK (SEQ ID NO. 11); TTKVPSLFVESS (SEQ ID NO. 12); GIDVIYLEG (SEQ ID NO. 13); IVTSEGCFKYFSKAYNVPSAYIW (SEQ ID NO. 14); QIKHLVEKL (SEQ ID NO. 15); ADLIFYNGV (SEQ ID NO. 16); IPIYSTI (SEQ ID NO. 17); TKLVKN (SEQ ID NO. 18); YEPLPEDV (SEQ ID NO. 19); GIIYAK (SEQ ID NO. 20); YIEKLDSL (SEQ ID NO. 21)
PSB/9	QSVYPLLKDG (SEQ ID NO. 22); DADVFVYH (SEQ ID NO. 23); IKDQLVKLYPKKAKVFE (SEQ ID NO. 24); HQYTYKYVGYKILN (SEQ ID NO. 25); KSFVTQHAAFGYLALDYGLKQVPIAGL (SEQ ID NO. 26); NVDLMVPAGS (SEQ ID NO. 27); KLEVLNPLESL (SEQ ID NO. 28); GEEVVPEK (SEQ ID NO. 29); LEIVTTFYPMY (SEQ ID NO. 30); FYPASLSKHE (SEQ ID NO. 31); IDFVVNGE (SEQ ID NO. 32); FKYIQFSDHGIAPSKAEHFHIFF (SEQ ID NO. 33); LAELKEY (SEQ ID NO. 34); GPNVVEG (SEQ ID NO. 35); HTWVSPK (SEQ ID NO. 36); LDEVFDYK (SEQ ID NO. 37); MILLPG (SEQ ID NO. 38); NYIYFE (SEQ ID NO. 39); YLTKLKRLD (SEQ ID NO. 40)

Another aspect of the invention thus relates to the polypeptides of the invention or active fragments thereof as described herein, especially for use in medicine, and in particular for use in the prevention and/or treatment of bacterial infection, such as infection by Enterococci.

In a preferred embodiment the polypeptide according to the present invention (according to any of SEQ ID NO: 1 to 9) or the contiguous fragment thereof is used as conjugate, whereby the antigen is covalently bound to an immunocarrier. Such immunocarrier may be a polypeptide or a protein or a carbohydrate-containing molecule (such as for example a capsular polysaccharide or glycoconjugate) which improves the interaction between T- and B-cells for the induction of an immune response against the antigen. This may be preferred for vaccines intended for use in patients with reduced activity of the immune system. Since infections of Enterococci are frequently a problem in hospitals and nursing homes such conjugates are particularly preferred for such patients. Suitable immunocarriers according to the present invention comprise tetanus toxoid, diphtheria toxoid, Pseudomonas aeruginosa toxin A or its derivatives thereof. Carbodydrate-containing molecules such as capsular polysaccharides or teichoic acids may also serve as conjugation partner for the above-mentioned polypeptide or fragments thereof. In an especially preferred embodiment such fragments of the immunocarrier are used which stimulate the immune response in the patient to be treated without having, however, the undesired side effect which such proteins may elicit when used in an unmodified form.

The covalent bond between the antigen and the immunocarrier can be provided by a direct chemical bond or by a spacer. Sometimes short molecules having two reactive groups on both ends are reacted with the antigen and the immunocarrier in order to produce a covalently linked molecule.

In an alternative the molecule(s), preferably used as vaccine (antigen and immunocarrier), can be produced recombinantly wherein suitable gene fragments are linked together and inserted into an appropriate vector. The vector is introduced in a suitable host cell and the host cell (e.g. *E.coli*, bacillus, yeast, or insect cells) produces the polypeptide or fragment thereof as defined above together with the immunocarrier as one molecule.

The polypeptides or fragments thereof either alone or coupled to an immunocarrier may be used for the treatment or the prevention of bacterial infections. Another aspect of the present invention thus is a method for the treatment or the prevention of bacterial infections, in particular of Enterococci, more preferably *Enterococcus faecium* based on the medicament as described herein. Said medicament preferably is a vaccine which comprises preferably also a pharmaceutically acceptable adjuvant. The adjuvant promotes the protective IgG subtype antibodies. Typical adjuvants include complete Freund's adjuvant (CFA), incomplete Freund's adjuvant (IFA), alum and other adjuvants suitable for human use (e.g. virus-like particles). Polymers like dextran sulfate have been shown to be also a potent stimulator of IgG antibodies against bacterial cell surface antigen. Other adjuvants include incomplete adjuvants; salt i.e. $AlK(SO_4)_2$, $AlNa(SO_4)_2$, $AlNH_4(SO_4)_2$, solica, kaolin, carbon polynucleotide, i.e. poly IC and poly AU. Preferred adjuvants include QuilA and Alhydrogel.

An active vaccine is administered to the patient preferably before an infection occurs. Such vaccination can therefore be applied regularly to patients at risk (e.g. elderly people, patients before solid organ or bone-marrow transplants) in order to stimulate their immune response and to avoid an infection in a hospital or a nursing home.

Medicaments and/or vaccines according to the present invention contain at least one polypeptide or active fragment thereof, but can contain 2 to up to 9 full length polypeptides according to the present invention. Preferred is a vaccine comprising at least one "set" of active fragments of at least one antigen according to the present invention, wherein said set is composed of 1, 2, 3 and up to 10 active fragments of said at least one antigen according to the present invention. The vaccine may also contain a mix of active fragments derived from antigens according to the present invention, i.e. SEQ ID Nos. 1 to 9. Preferred is a composition comprising at least one of the antigens (epitopes) as listed in table 2, above.

Under specific circumstances it may, however, also be possible to apply the vaccine at early stages of the infection in order to elicit protective antibodies which inactivate the bacteria belonging to the genus *Enterococcus*. In a preferred embodiment the vaccine of the present

invention provides protection against different *Enterococcus faecium* and possibly also against *Enterococcus faecalis* strains since there is extensive sequence homology between these species.

Antibodies induced by the protein of SEQ ID NO: 1 to 9 or suitable fragments thereof are protective and facilitate phagocytosis. Since such protective, and in particular opsonic antibodies, are preferred, it is desired to use those parts of the polypeptide of SEQ ID NO:1 to 9 which elicit antibodies having opsonic properties.

As mentioned above, still another subject of the invention is a pharmaceutical composition, especially a vaccine, comprising the protective peptide or active fragments thereof or the antibody or functional fragments thereof as defined by the invention, useful for the immunization of a subject against an infection or the treatment of a subject having an infection, wherein the infection is preferably caused by E. faecium and/or E. faecalis. This pharmaceutical formulation of a medicament to be used as a vaccine is known to the person skilled in the art, and described in the respective literature. Usually, a solution of the antigen possibly coupled to an immunocarrier is dissolved in a physiologically acceptable solution like a buffer. The solution must be stabilized in order to avoid an undesired precipitation of the immunologically active compounds. The vaccine is preferably produced in the form of a solution adapted to injection, preferably intramuscular injection. Other forms of pharmaceutical formulations like plasters or sprays are also acceptable provided the antigen comes in sufficient contact with the immune system and the formation of specific antibodies is elicited.

Alternatively, the peptide or active fragments thereof of the invention are used in a method of immunizing or treating a subject in need thereof, wherein an effective amount of the peptide or the nucleic acid of the invention is administered to the subject. The subject may be immunized in order to prevent an infection, particularly an E. faecium and/or E. faecalis infection, or may be treated to ameliorate or cure an infection, particularly an E. faecium and/or E. faecalis infection. The determination of the effective amount to be administered is within the knowledge of the skilled practitioner.

The polypeptides or fragment thereof either alone or coupled to an immunocarrier may be used for the treatment or the prevention of bacterial infections. The prevention of bacterial infection achieved by regularly application of the vaccine to patients of risk such as elderly people, infants and patients before organ or bone-marrow transplantation so antibodies had been generated though the stimulation of the immune response.

The vaccine is preferably produced in the form of a solution adapted to injection, preferably intramuscular injection. Other forms of pharmaceutical formulations like plasters or sprays are also acceptable provided the antigen comes in sufficient contact with the immune system and the formation of specific antibodies are elicited.

On the other hand, it is sometimes not possible to treat patients with an active vaccine since the immune system is severely impaired. In those circumstances the polypeptide of SEQ ID NO: 1 to 9 or fragments thereof (epitopes) as defined above can be used to produce either polyclonal antibodies or monoclonal antibodies that bind to or opsonize *Enterococcus*. The person skilled in the art is well aware how such antibodies can be prepared.

The inoculum for polyclonal antibody production is typically prepared by dispersing the antigen or the antigen-immunocarrier conjugate in a physiologically tolerable diluent such as saline, to form an aqueous composition. An immunostimulatory amount of the inoculum preferably with adjuvant is administered to a mammal and the inoculated mammal is then maintained for a time period sufficient for the antigen to induce protective anti-*Enterococcus* antibodies. After suitable periods of time, two weeks until four months, boosting doses of the antigen-immunocarrier may be applied and the antigen titer is monitored. At a suitable point, when the titer of the neutralizing or opsonic antibodies is at its peak, the antibodies are collected. Such antibodies can include antibody preparations from a variety of commonly used animals (such as mice, goats, primates, donkeys, rabbits or horses) and humans, whereby the antibodies are isolated from blood donations.

The antibodies induced in the mammal are harvested, isolated and purified to the extent desired by well-known techniques such as by alcohol fractionation and column chromatography or preferably by immunoaffinity chromatography whereby the antigen is bound to a chromatographic column. The antiserum passes the column whereby specific antibodies are retained and all other components of the serum are washed out. Then the purified antibodies are eluted with suitable gradients. A further purification may be required.

Alternatively, monoclonal antibodies can be prepared according to techniques well-known to the person skilled in the art. When a suitable monoclonal antibody is obtained, the binding regions can be identified and the whole antibody molecule as well as derivatives of the antibody like antibody fragments or subfragments can be provided. The general technique to produce monoclonal antibodies is amply described in textbooks. After having made the hybridomas or having selected the monoclonal antibody from libraries or genetically engineered animals it has to be determined to which part of the polypeptide of SEQ ID NO: 1 to 9 the mAb binds. Then, it has to be checked whether the antibody is opsonic and/or protective, preferably *in vivo*.

According to another preferred aspect of the present invention, it would be very beneficial to provide monoclonal or polyclonal antibody therapies which target antigenic polypeptides of E. faecium and/or E. faecalis as described herein and have the potential to support a therapy of an infection or eliminate the pathogen and the disease altogether. Therefore, another subject of the invention relates to an antibody or functional active fragment thereof which binds specifically to the antigens of the invention. In a preferred embodiment the antibody is a monoclonal, polyclonal, chimeric or humanized antibody or functional active variant thereof. In another preferred embodiment the functional active fragment comprises a Fab fragment. Antibodies generated against the antigens (polypeptides), fragments or variants thereof of the present invention can be obtained by direct injection of the antigens, fragments or variants thereof into an animal or by administering the antigens, fragments or variants thereof to an animal, preferably a non-human. The antibody so obtained will then bind the antigens, fragments or variants. Such antibodies can then be used to isolate reactive antigens, fragments or variants thereof from tissue expressing those.

For the preparation of monoclonal antibodies, any technique known in the art, which provides antibodies produced by continuous cell line cultures, e.g. a hybridoma cell line, can be used. Techniques described for the production of single chain antibodies (U. S. Patent No. 4,946,778) can be adapted to produce single chain antibodies to the antigens, fragments or variants thereof according to this invention. Also, transgenic mice or other organisms such as other mammals may be used to express humanized antibodies to antigens, fragments or variants thereof according to this invention. Still another subject of the invention relates to a hybridoma cell line which produces the antibody of the invention. Hybridoma cell lines expressing desirable monoclonal antibodies are generated by well-known conventional techniques. Similarly, desirable high titer antibodies are generated by applying known recombinant techniques to the monoclonal or polyclonal antibodies developed to these antigens (see, e.g., PCT Patent Application No. PCT/GB85/00392; British Patent Application Publication No. GB2188638A; Amit et al., Science, 233:747-753 (1986); Queen et al., Proc. Natl. Acad. Sci. USA, 86: 10029-10033 (1989); WO 90/07861 ; Riechmann et al., Nature, 332:323-327 (1988); Huse et al., Science, 246:1275-1281 (1988)).

The present invention also provides a method for producing an antibody according to the invention, characterized by the following steps:
(a) administering an effective amount of the peptide according to the invention to an animal; and
(b) isolating the antibody produced by the animal in response to the administration of step (a) from the animal.

Another subject of the invention relates to a method for producing an antibody according to the invention, characterized by the following steps:
(a) contacting a B cell with an effective amount of the peptide according to the invention;
(b) fusing the B cell of step (a) with a myeloma cell to obtain a hybridoma cell; and
(c) isolating the antibody produced by the cultivated hybridoma cell. More particularly, the antibody may be produced by initiating an immune response in a non- human animal by administrating a peptide of the invention to an animal, removing an antibody containing body

fluid from said animal, and producing the antibody by subjecting said antibody containing body fluid to further purification steps. Alternatively, the antibody may be produced by initiating an immune response in a non-human animal by administrating an antigen, fragment or variant thereof, as defined in the present invention, to said animal, removing the spleen or spleen cells from said animal and/or producing hybridoma cells of said spleen or spleen cells, selecting and cloning hybridoma cells specific for said antigen, fragment or variant thereof and producing the antibody by cultivation of said cloned hybridoma cells.

The antibody may be used in methods for treating an infection. Accordingly, still another subject of the invention relates to a pharmaceutical composition comprising the antibody of the invention. The pharmaceutical composition may encompass further components as detailed above for the vaccine. The composition may further encompass substances increasing their capacity to stimulate T cells. These include T helper cell epitopes, lipids or liposomes or preferred modifications as described in WO 01/78767. Another way to increase the T cell stimulating capacity of epitopes is their formulation with immune stimulating substances for instance cytokines or chemokines like interleukin-2, -7, -12, -18, class I and II interferons (IFN), especially IFN-gamma, GM-CSF, TNF-alpha, flt3-ligand and others.

Medicaments according to the present invention contain at least one antibody or active fragment thereof, but can contain 2 to up to 9 antibodies according to the present invention. Preferred is a medicament comprising at least one "set" of antibodies or active fragments thereof specifically directed against at least one antigen according to the present invention, wherein said set is composed of 1, 2, 3 and up to 10 antibodies or active fragments thereof specifically directed against said at least one antigen according to the present invention. Of course, also mixtures of antibodies or active fragments thereof specifically directed against 2, 3, and up to 9 of the antigens according to the present invention can be formulated into a medicament according to the present invention.

The polypeptides according to the present invention do not show significant differences in the percentage of killing, but preferred are the polypeptides according to the present invention showing a higher or equal activity at a higher dilution, namely Adlip (SEQ ID No.

8), PBP5 (SEQ ID No. 1), and PpiC (SEQ ID No. 5) (which are thus preferred). It is expected that a lower concentration of the antigen decreases and/or reduces the risk of prospective side effects.

The present invention will now be described further in the following examples with reference to the accompanying Figures and the Sequence Listing, nevertheless, without being limited thereto. For the purposes of the present invention, all references as cited herein are incorporated by reference in their entireties.

Description of the Figures

Figure 1 shows an SDS-PAGE gel stained with Coomassie Brilliant Blue of proteins purified by Protino® Ni-NTA Agarose. Lanes: 1 and 9: MW Standard; 2: Sag A; 3: Enol; 4: PpiC; 5: Dala; 6: LysM; 7: BML; 8: PBP5; 10: Adlip; 11: PSB and 12: SCP.

Figure 2 shows opsonic killing of a polyclonal rabbit antisera produced in rabbits against the whole bacterial cell of *Enterococcus faecium* E155. Different dilutions (1:50 and 1:100) of the sera were tested. The substantial opsonic activity of the antibodies as raised is represented by the bars. Bars represent data means for the observations, and the error bars indicates the SEM for each protein. SagA was used as positive control known as promising vaccine target in *E. faecium*.

Figure 3 shows an opsonophagocytic inhibition assay with serum raised against recombinant proteins using 100µg of purified protein for absorptions. Bars represent data means for the observations, and the error bars indicates the SEM for each protein. SagA was used as a positive control. However, the opsonic killing activity of the polypeptides of the present invention is notably stronger.

SEQ ID NOs: 1 to 9 show the amino acid sequence of the polypeptides according to the invention derived from strain *Enterococcus faecium* E155, and *Enterococcus faecalis*.

SEQ ID NOs: 10 to 167 show the amino acid sequence of preferred peptide fragments (epitopes) as active fragments derived from the polypeptides according to the invention.

Examples

The bacterial strain used for all experiments was the Vancomycin-resistant *E. faecium* E155 strain, a clinical isolate that belongs to a genetic subpopulation of hospital-associated *E. faecium* responsible for worldwide emergence due to its multidrug-resistance and especially high level resistance to quinolone and ampicillin.

Example 1:

Identification and extraction of the polypeptides according to the present invention

1. Surface protein extraction using trypsin shaving method

Extractions were performed as described by Tjalsma et al. (2008). Briefly, two aliquots of 50 ml of bacterial cultures of the E155 strain were harvested at OD_{600} = 0.4 by centrifugation (10,000 rpm., 2 min) and washed twice with 4 ml Bicam (triethylammonium bicarbonate buffer 100 mM pH 8). The first aliquot was then mixed with a solution of trypsin in Bicam at final concentration of 10µg/mL. The other aliquot was resuspended in Bicam without any trypsin. All the samples were incubated for 1 h at 37 °C under gentle shaking. After centrifugation (7,500 rpm., 5 min), the cell pellets were removed, and the supernatants were treated with 1 mM DTT for 30 min, followed by 1 mM iodoacetamide (IAA), also for 30 min at room temperature. Finally, fresh trypsin (0.5 mg) was added to all samples and tryptic cleavage was continued for 18 h at 37 °C. In this way two samples were obtained for each experiment. These "shaved" proteins were identified by LC-MS/MS. For this protocol, the inventors analyzed 25 different conditions using different amounts of trypsin, different times of incubation with the enzyme and additional combined treatments with lysozyme and/or mutanolysin.

After analysis of the 25 samples as obtained using the different conditions by nanoLC-MS/MS, a total of 401 proteins was identified using the Mascot software databases. Overall,

34 proteins were identified as surface proteins, 29 as present in both intra- and extracellular location, 315 as cytoplasmic proteins and 23 of unknown location. The results of this method demonstrate that this procedure is not an appropriate strategy to obtain mostly surface proteins, since only around 16% of the identified proteins belong to this category. Furthermore, this procedure is not an appropriate strategy as the sole strategy to obtain target polypeptides.

2. Extraction of surface proteins under strong alkaline conditions

Surface-exposed proteins were extracted by exposure of cells to high pH using a protocol described by Hempel et al. (2011). Briefly, a cell pellet from a 50 ml culture (OD_{600} = 0.5) was washed with a PBS sucrose solution [NaCl 100 mM, sucrose 60 mM, sodium phosphate 55 mM (pH 7.2)] and then shaken gently for 1 h at room temperature in 2 ml NaOH glycine sucrose [glycine 50 mM, sucrose 60 mM (pH 12.4)]. After centrifugation (30 min, 10,000 g), 108 ml 1 M HCl and 100 ml 1 M Tris/HCl (pH 7.0) were added to 1 ml supernatant. Proteins were precipitated at 4 °C by addition of 8 ml cold acetone. The protein pellet obtained after centrifugation (10 min, 10,000 g) was resuspended in 20 ml Tris/HCl (pH 7.5).

This protocol was used to extract peripheral proteins that are loosely attached to the membrane or cell wall. These proteins can be detached using treatment with a polar reagent like an alkaline pH solution since they are non-covalently attached to either the lipid layer or to integral membrane proteins by hydrophobic, electrostatic, or other interactions. Two different media where tested for this procedure, TSB and GM17, which are both rich laboratory mediums. The samples obtained under the different conditions were analyzed by nanoLC-MS/MS. A total of 329 proteins were identified using the mascot software databases. Overall, 47 proteins were identified as surface proteins, 16 as present in both intra- and extracellular location, 246 as cytoplasmic proteins and 20 of unknown location. As this method let to the isolation of intact proteins, the inventors were able to test their immunogenicity by immunodotblot and western blot. However, the results demonstrate that this method is also not the best strategy to obtain samples enriched in surface proteins because just around 19% of the predicted proteins belong to this category. Thus, this

procedure is also not an appropriate strategy as the sole strategy to obtain target polypeptides.

3. Surface proteins extraction using biotinylation

Surface-exposed proteins were labeled and extracted by exposure of cells to Sulfo-NHS-SS-Biotin using a protocol described by Hempel et al. (2011). Briefly, 100 mL of bacterial cultures at OD_{600} = 0.5 were harvested at 8,000 x g for 5 min at 4°C. 0.2 g of cells (wet cell weight) were resuspended in 5 mL ice-cold PBS (pH 8.0) with 1 mM PMSF on ice. The biotinylation reaction was performed by adding 100 µL fresh Sulfo-NHS-SS-Biotin solution to 1 mL of intact cells, to give a final concentration of 1.5 mM Sulfo-NHS-SS-Biotin. A 1% (w/v) solution of Sulfo-NHS-SS-Biotin was prepared by adding 5 mg to 500 µL PBS (pH 8.0) immediately before use. Cells were incubated by gentle shaking for 1 h on ice. To stop the reaction and to remove nonreacted biotinylation reagent, cells were centrifuged at 8500× g for 1 min at 4 °C and washed three times with ice-cold PBS (pH 8.0)/ 500 mM glycine. A pellet of 1 mL reaction volume was resuspended in 500 µL PBS (pH 8.0) with 1 mM PMSF on ice and transferred to a 1.5 mL tube containing glass beads. The disruption of cells was performed mechanically in a FastPrep cell disruptor at 6 m/s^2 twice for 30 s. The cell debris was recovered from the glass beads with a total of 3 mL of PBS (pH 8.0). The lysate was centrifuged (100,000× g for 1h at 4 °C). The supernatant was then discarded. The cell debris were resuspended in a total of 400µL of [PBS (pH 8.0), IAA (5%)] and homogenized in a FastPrep cell disrupter at 6 m/s^2 twice for 30 s with 0.25 ml of glass beads. The proteins were the solubilized by addition of 100 µL of PBS (pH 8.0) with 1 mM PMSF, 4% CHAPS and 2% ASB-14. A second homogenization step was performed after detergent addition under the same conditions as mention above. Cell debris was removed by centrifugation (14,000 rpm, 15 min) after 1h of incubation with the detergent.

The biotinylated proteins were isolated and purified by NeutrAvidin agarose affinity-purification. For a reaction volume of 500 µL protein mixture 150 µL of NeutrAvidin agarose resin were washed twice with PBS (pH 8.0)/ 1% NP-40 and centrifuged at 1,000 rpm for 1 min at 4°C. The resin was mixed with the cell lysate for 1h by gently shaking on ice. The supernatant was removed and the resin-bound complex washed 6 times with PBS

(pH 8.0)/ 1% NP-40. Biotinylated proteins were eluted twice by incubation with 1mL of elution buffer (5% mercaptoethanol in H_2O) for 1h with gentle shaking; supernatant was recovered after centrifugation at 1,000 rpm for 1 min and poured to 8mL of cold acetone (-20°C, overnight). The precipitated proteins were harvested by centrifugation (8,500 rpm, 30 min, 4°C) and washed twice with 1 mL of cold 98% ethanol (4°C). The pellets were dried in a SpeedVac for 2 min and dissolved in 15µl 6M urea / 2M thiourea for 2 min at 80°C. The samples were loaded on a SDS-PAGE gel and the corresponding bands were excised from the gel and dehydrated with acetonitrile. Afterwards, samples were reduced and alkylated in two successive steps of 20 min with 0,5% Dithiothreitol and 5% Iodoacetamide. Samples were washed twice with 30% acetonitrile, 200mM ammonium bicarbonate and subsequently digested overnight with 0,2 µg of trypsin (Promega). Peptides were obtained by covering the gel bands with water and incubating them in an ultrasonic bath for 15min. Finally peptides samples were analyzed by nanoLC-MS/MS, sequence data were compared to the NCBI and MASCOT databases.

Three different media where tested for this approach, the rich laboratory medium TSB, ccM17 MOPS, a carbon depleted laboratory medium and BHI (brain hearth infusion) medium supplemented with 30 % of horse serum to mimic *in vivo* conditions. The samples obtained under the different conditions were analyzed by nanoLC-MS/MS. A total of 45 proteins were identified using the Mascot software databases. Overall, 27 proteins were identified as surface proteins, 6 as present in both intra and extra cellular location, and 12 as cytoplasmic proteins. The combined results demonstrated that this procedure is the best strategy to target preferentially surface proteins since 73% of the predicted proteins belonged to this category. Nevertheless, for the purposes of the present invention, all three approaches were combined.

4. Additional surface protein identification from transcriptional analysis

Real-time PCR experiments performed with cDNA synthesized from RNA extracted from an *in vivo* endocarditis model in *E. faecalis* closely related species revealed that over 300 genes were up-regulated under these conditions. Among these, 19 genes were identified to encode surface related proteins. These proteins were analyzed by online BLAST in the J. Craig

Venter Institute database comparing the sequences in *E. faecium* completely sequenced strains. The adhesion lipoprotein (Adlip) and a protein showing homology to a periplasmic solute binding protein (PSB) were identified as the closest surface proteins related between the two strains and selected as targets for the overexpression experiments (see below).

6. MS analyses

MS analyses were performed after the overnight tryptic cleavage of protein samples obtained by shaving extraction. Trypsin-cleaved samples were desalted and concentrated on a tipmicroC18 Omix (Varian) before nano-liquid chromatography (LC)-MS-MS analysis. The chromatography step was performed using a Prominence nano-LC system (Shimadzu).

7. Summary of all the extraction methods

A comparison between the proteins identified with the three methods allowed the inventors to establish that 22 extracellular proteins were detected by two of the three methods as used, and seven by all of them. Sag A was used as a control. Finally, nine proteins were selected for overexpression (see table 2). Seven of them were identified by the three extraction methods and the remaining two identified to be induced *in vivo* in the closely related species *E. faecalis.*

Table 2. Comparison of the proteins identified by the different extraction methods

Protein	Abbreviation	Locus Tag	Method
low affinity penicillin-binding protein 5 (PBP5)	PBP5	EFAU004_00870	Biotin, High pH, Trypsin
Basic membrane lipoprotein	BML	EFAU004_00080	Biotin, High pH, Trypsin
peptidoglycan-binding protein LysM	LysM	EFAU004_01059	Biotin, High pH, Trypsin
D-alanyl-D-alanine carboxypeptidase	Dala	EFAU004_01127	Biotin, High pH, Trypsin
PpiC-type peptidyl-prolyl cis-trans isomerase	PpiC	EFAU004_02526	Biotin, High pH, Trypsin
Enolase	Enol	EFAU004_02073	Biotin, High pH, Trypsin
SCP-like extracellular	SCP	WP_002353118.1	Biotin, High pH,

protein (serine protease)			Trypsin
Adhesion lipoprotein	Adlip	EFUG_02345	Biotin, High pH, Transcriptomic data
Periplasmic solute binding family	PSB	EFAU004_00598	Biotin, High pH, Transcriptomic data

Example 2:

Overexpression of the polypeptides and production of polyclonal antibodies

1. Cloning of the genes encoding the protein candidates

The genes encoding the selected proteins were identified by *in silico* analysis using the *E. faecium* E155 genome sequence. Overexpression of the proteins was performed by cloning the corresponding genes into the expression vectors pQE30 or pET28a+. For the PBP5, BML, LysM, Dala, PpiC, Enol, PSB and Adlip the vector pQE30 was used, and the pET28a+ for the SCP. In addition, one more protein was overexpressed and purified, the Sag A protein supposed to be a promising vaccine target in *E. faecium* (Kropec *et. al*, 2011) was included as a positive control.

Overexpression and purification of the H6-proteins. A QIAexpress system was used for the expression of a six-His-tagged recombinant proteins as follows. First, the genes were amplified by PCR using primers designed at the beginning of each gene (excluding the signal peptide base pairs) and one at the end of it. The PCR product was digested using the endonucleases BamHI and PstI for the genes encoding SagA, LysM, Dala, PpiC, Enol, Adlip and PSB; and BamHI and SacI for SCP, PBP5, BML and PpiC; and cloned into the corresponding restriction sites of the respective plasmid: pQE30 or pET28a+. The resulting plasmid, were then introduced in *E. coli* M15(pREP4) cells for the pQE30 and in *E. coli* BL21 for the pET28a+. Colonies were screened by PCR and the integrity of the constructions was controlled by sequencing.

2. Overexpression and purification of the protein

The overexpression of all the proteins was carried out by inoculating an overnight culture in fresh LB media supplemented with the corresponding antibiotic. Bacteria were grown during 2 hours at 37°C and shaking at 160 rpm before induction of protein expression by 0,5 mM

IPTG, then, the culture was incubated for two additional hours under the same conditions. Cells were harvested by centrifugation and later disrupted using lysozyme and the FastPrep cell disrupter. Proteins were purified under denaturing conditions using Protino® Ni-NTA Agarose following the instructions of the manufacturer (Macherey-Nagel).

Purified proteins were subject of SDS-PAGE with 10% acrylamide/bisacrylamide resolving gels (NuPAGE, Invitrogen) and stained with Coomassie brilliant blue (SimplyBlue SafeStain, Invitrogen) for protein detection and molecular size confirmation (see Figure 1). Coomassie blue-stained bands were excised from the gel and treated in the same way as described before for nano LC-MS/MS analysis (see biotinylation procedure).

Example 3:

1. Production of polyclonal antibodies

In order to produce anti-protein hyperimmune serum, eight New Zealand White rabbits (2.5 to 3.5 kg; Charles River Laboratories) were vaccinated with each protein (Enol, PpiC, Dala, LysM, BML, PBP5, Adlip and PSB) according to the immunization schedule (see Table 3). Preimmune serum was collected from the rabbits on days 0 and 7 prior to the first vaccination to be used as a negative control.

Table 3. Immunization schedule for purified polypeptides

Schedule for the immunization with the polypeptide	
Day No.	**Procedure**
0	Pre Bleed 10 -15 mL
7	Pre Bleed 10 -15 mL
14	Immunization 1 s.c. (1FIA) 10 ug
28	Immunization 2 s.c. (1FIA) 10 ug
35	Boost 1 i.v. 5 ug (without FIA)
37	Boost 2 i.v. 5 ug (without FIA)
39	Boost 3 i.v. 5 ug (without FIA)
53	Test Bleed 2-10 mL
60	Boost 4 i.v. 5 ug (without FIA)
67	Boost 5 i.v. 5 ug (without FIA)
74	Terminal Bleed

The sera were heat inactivated at 56°C for 30min and were then absorbed 1h with heat killed

cells of *E. faecium* E1162 treated with proteinase K.

2. Opsonophagocytic Assays

An in vitro opsonophagocytic assay (OPA) was performed as described elsewhere (Huebner J, 1999) using baby rabbit serum as complement source and rabbit serum raised against purified proteins. Polymorphonuclear neutrophils (PMN's) were freshly prepared from human blood collected from healthy adult volunteers. Bacterial strains were grown to mid-log phase in TSB. For the assay, the following components were mixed: 100 µl of PMNs; 100 µl of 1:10 and 1:50 serum dilution, 100 µl of absorbed baby rabbit complement 1:30 dilution, and 100 µl of 1:150 dilution of bacteria *E. faecium* E155. The mixture was incubated on a rotor rack at 37°C for 90 min, and samples were plated in duplicate at time 0 and after 90 min. Percent killing was calculated by comparing the colony counts of a control without PMN's to the colony counts after a 90-minute incubation at 37°C (T90). For inhibition studies, rabbit serum was diluted 1:50 or 1:100 and incubated for 60 min at 4 °C with an equal volume of a solution containing 100 µg of the corresponding protein. Subsequently, the antiserum was used in the OPA as described above. Inhibition assays were performed at serum dilutions yielding 50-60% killing of the inoculum without the addition of the inhibitor. The percentage of inhibition of opsonophagocytic killing was compared to controls without inhibitor.

The results are shown in Figures 2 and 3.

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Claims

1. Medicament for the treatment or the prevention of a bacterial infection, characterized in that it contains at least one polypeptide selected from the group of SEQ ID NO: 1 to SEQ ID NO: 9, and contiguous fragments thereof, wherein said at least one polypeptide or contiguous fragment thereof induces opsonic antibodies in a patient in need thereof.

2. Medicament according to claim 1 wherein said polypeptide or contiguous fragment thereof is covalently bound to an immunocarrier, in particular a protein, a carbohydrate, and/or a glycoconjugate.

3. Medicament according to claim 1 or 2, characterized in that said bacterial infection is caused by *Enterococcus*.

4. Medicament according to any of claims 1 to 3, characterized in that it comprises at least one pharmaceutically acceptable adjuvant.

5. Medicament according to any of claims 1 to 4, characterized in that it is a vaccine.

6. Medicament according to claims 1 to 5, characterized in that it is a vaccine against an infection caused by a strain of *Enterococcus*, in particular *Enterococcus faecium or E. faecalis*.

7. At least one polypeptide selected from the group of SEQ ID NO: 1 to SEQ ID NO: 9, and contiguous fragments thereof for use in the treatment or prevention of a bacterial infection.

8. At least one polypeptide selected from the group of SEQ ID NO: 1 to SEQ ID NO: 9, and contiguous fragments thereof for use according to claim 7, wherein said polypeptide or contiguous fragment thereof is covalently bound to an immunocarrier.

9. At least one polypeptide selected from the group of SEQ ID NO: 1 to SEQ ID NO: 9, and contiguous fragments thereof for use according to claim 7 or 8, wherein the infection is caused by *Enterococcus faecium or E. faecalis.*

10. Use of at least one polypeptide selected from the group of SEQ ID NO: 1 to SEQ ID NO: 9, and contiguous fragments thereof for the preparation of antibodies.

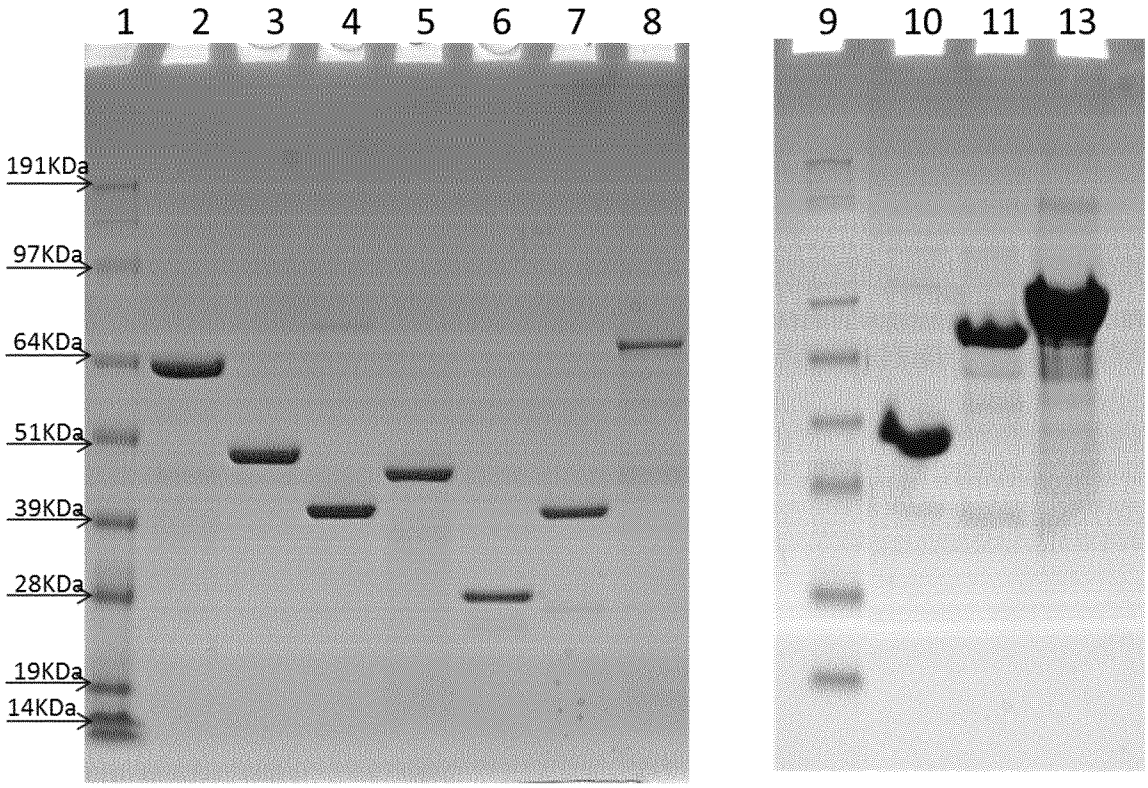
11. Use according to claim 10 whereby said polypeptide or contiguous fragment thereof is covalently bound to an immunocarrier.

12. A monoclonal antibody or fragment thereof which reacts specifically with at least one polypeptide selected from the group of SEQ ID NO: 1 to SEQ ID NO: 9, or contiguous fragments thereof, or said at least one polypeptide or contiguous fragments thereof when covalently bound to an immunocarrier.

13. The antibody or fragment thereof according to claim 12, characterized in that said antibody or fragment thereof is opsonic.

14. Medicament for the treatment or the prevention of a bacterial infection, characterized in that it contains at least one antibody or fragment thereof according to claim 12 or 13.

Figure 1


1
2
3
4
5
6
7
8
9
10
11
13
191KDa
97KDa
64KDa
51KDa
39KDa
28KDa
19KDa
14KDa

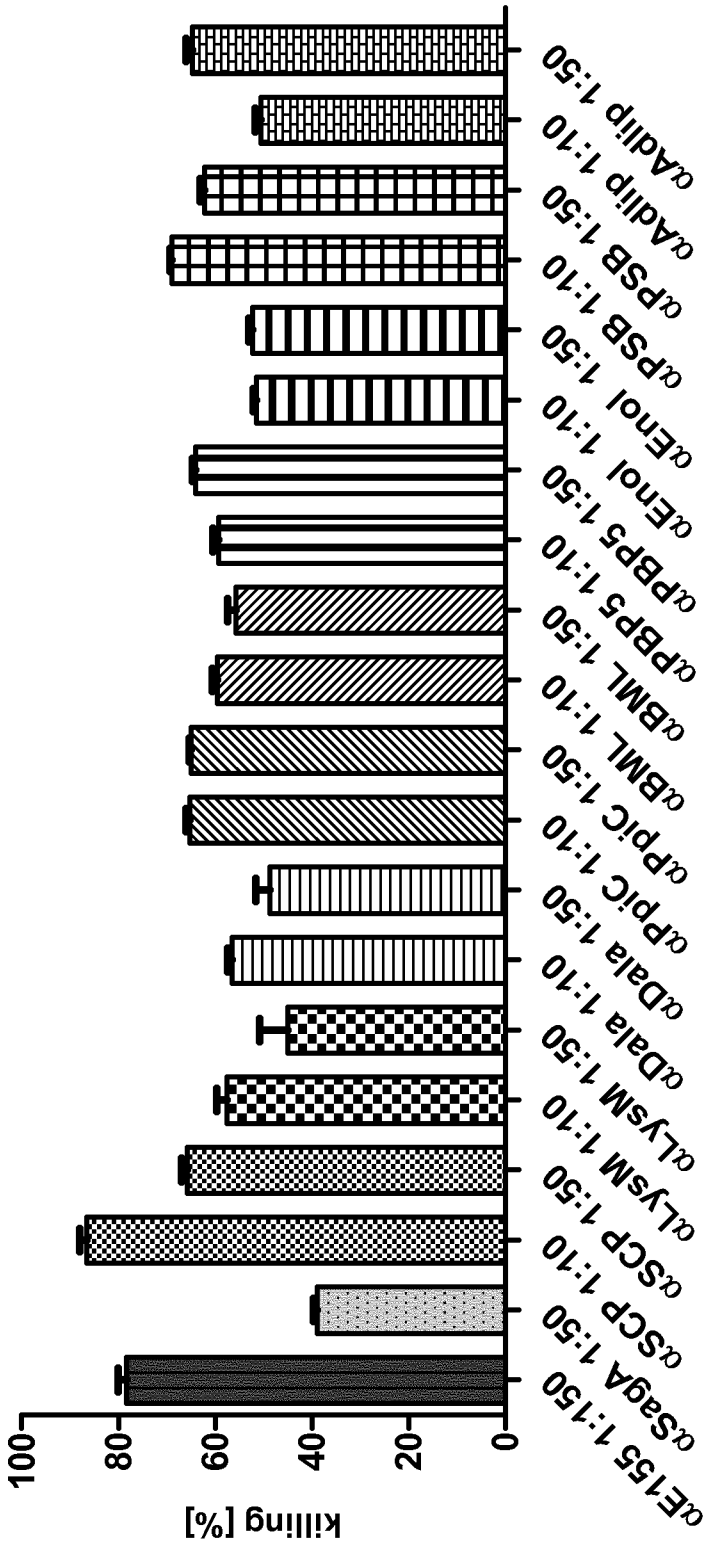

Opsonophagocytic Assay
E. faecium E155 / Protein Antibodies
Absorbed E1162
killing [%]
0
20
40
60
80
100
αE155 1:150
αSagA 1:50
αSCP 1:10
αSCP 1:50
αLysM 1:10
αLysM 1:50
αDala 1:10
αDala 1:50
αPpiC 1:10
αPpiC 1:50
αBML 1:10
αBML 1:50
αPBP5 1:10
αPBP5 1:50
αEnol 1:10
αEnol 1:50
αPSB 1:10
αPSB 1:50
αAdiip 1:10
αAdiip 1:50

Figure 2

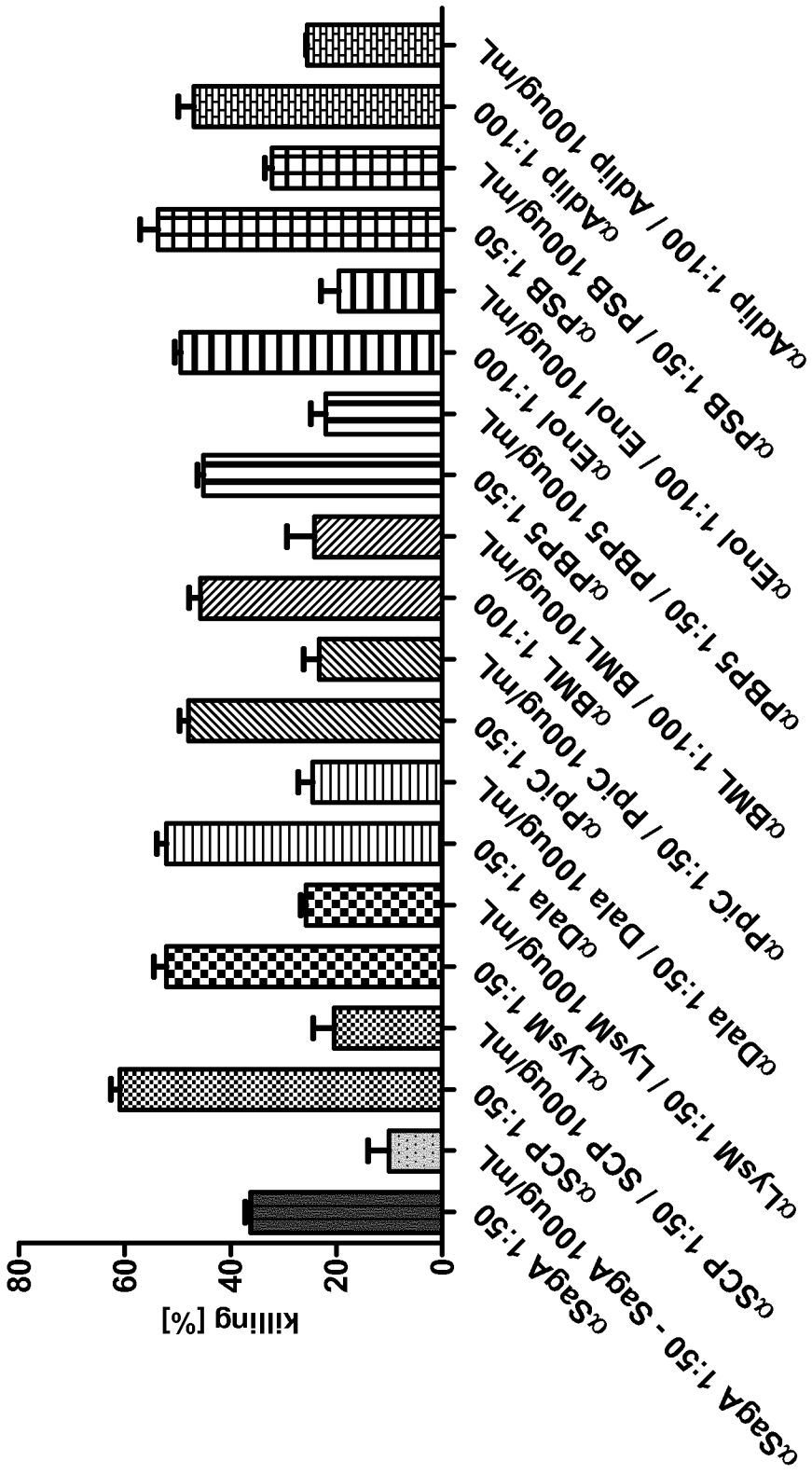

Opsonophagocytic Inhibition Assay
E. faecium E155 / Protein Antibodies
Killing [%]
0
20
40
60
80
αSagA 1:50 - SagA 100ug/mL
αSagA 1:50
αSCP 1:50
αSCP 1:50 / SCP 100ug/mL
αLysM 1:50
αLysM 1:50 / LysM 100ug/mL
αDala 1:50
αDala 1:50 / Dala 100ug/mL
αPpiC 1:50
αPpiC 1:50 / PpiC 100ug/mL
αBML 1:100
αBML 1:100 / BML 100ug/mL
αPBP5 1:50
αPBP5 1:50 / PBP5 100ug/mL
αEnol 1:100
αEnol 1:100 / Enol 100ug/mL
αPSB 1:50
αPSB 1:50 / PSB 100ug/mL
αAdiip 1:100
αAdiip 1:100 / Adiip 100ug/mL

Figure 3