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(54) Title: S. AUREUS ANTIGENE

(57) Abstract: Described are novel antigens from S. aureus selected from the group consisting of Seq.ID.Nos. 7 to 12, as well as their use in a pharmaceutical preparation, especially in a vaccine.
S. Aureus Antigene

The invention relates to a method for identification, isolation and production of novel antigens of Staphylococcus aureus suitable for use in a vaccine for a given type of animal or for humans.

Recent years have shown a dramatic increase in the number of antibiotic-resistant isolates of pathogenic bacteria. The need for new drugs in order to treat these infections is unmatched with the current situation as only a limited number of antibiotics received market approval in the last decades. Passive or active immunization might therefore provide a solution for this caveat. Specifically, proteins conserved among different isolates of a given species are well-suited for the development of defined vaccines as non-protein compounds like polysaccharides vary often among isolates, frequently even specify a serotype, and also fail to induce a long lasting immune response, except for the more costly conjugate vaccines.

Traditionally, single virulence factors have been used as candidate genes for vaccine development. More recently, whole-genome, bioinformatic-based approaches have been reported (Pizza et al., 2000; Wizemann et al., 2001). Proteins from a given pathogen predicted to be cell-surface localized or secreted have been expressed as recombinant proteins and empirically tested in animal models. Besides the fact that this approach is highly dependent on proper gene identification and annotation, its intrinsic shortcoming as a generally applicable method is that it is not known how many of these proteins i) are actually expressed in vivo during an infection and ii) are able to induce an immune response in humans. Both aspects are requirements for effective vaccines. A functional selection to identify proteins with these characteristics would therefore be advantageous. The human humoral immune system is able to raise a specific antibody profile upon encountering a given pathogenic microorganism. These antibodies can be seen as an inverse, immunological blueprint suitable to identify the corresponding proteins out of proteomic samples or expression libraries. A number of these identified proteins were shown to induce antibodies that posses
anti-staphylococcal activity (Burnie et al., 2000; Colque-Navarro et al., 2000; Etz et al., 2002).

Staphylococcus pathogens, especially Staphylococcus aureus, are opportunistic pathogens which can cause illnesses which range from minor infections to life threatening diseases. Of the large number of Staphylococci at least 3 are commonly associated with human disease: S. aureus, S. epidermidis and rarely S. saprophyticus (Crossley and Archer, 1997). S. aureus is also an important organism with respect to its severe pathogenic impacts on humans. Staphylococcal infections are imposing an increasing threat in hospitals world-wide. The appearance and disease causing capacity of Staphylococci are related to the wide-spread use of antibiotics which induced and continue to induce multi-drug resistance. For that reason medical treatment against Staphylococcal infections cannot rely only on antibiotics anymore. Therefore, a tactic change in the treatment of these diseases is desperately needed which aims to prevent infections. Inducing high affinity antibodies of the opsonic and neutralizing type by vaccination helps the innate immune system to eliminate bacteria and toxins.

A way of combating infections is preventing them by active immunisation. Vaccine development against S. aureus has been initiated by several research groups and national institutions world-wide, but there is no effective vaccine approved so far. It has been shown that an antibody deficiency state contributes to staphylococcal persistence, suggesting that anti-staphylococcal antibodies are important in host defence. Antibodies - added as passive immunisation or induced by active vaccination - directed towards surface components could both prevent bacterial adherence, neutralize toxins and promote phagocytosis. A vaccine based on fibronectin binding protein induces protective immunity against mastitis in cattle and suggest that this approach is likely to work in humans. Taking all this together it is suggestive that an effective vaccine should be composed of proteins or polypeptides, which are expressed by all strains and are able to induce high affinity, abundant antibodies against cell surface components of S. aureus. The antibodies should be IgG1 and/or IgG3 for opsonization, and any IgG subtype and IgA for
neutralisation of adherence and toxin action. A chemically defined vaccine must be definitely superior compared to a whole cell vaccine (attenuated or killed), since components of S. aureus which paralyze TH cells (superantigens) or inhibit opsonization (protein A) can be eliminated, and the individual proteins inducing protective antibodies can be selected. Identification of the relevant antigens help to generate effective passive immunisation (humanised monoclonal antibody therapy), which can replace human immunoglobulin administration with all its dangerous side-effects.

Examples for promising hyperimmune-reactive antigens from Staphylococcus aureus have been disclosed in PCT/EP02/00544.

It is therefore an object of the present invention to comply with these demands and to provide novel antigens from Staphylococcus aureus allowing the design of suitable vaccines to treat and prevent infections with S. aureus.

Therefore, the present invention provides antigens comprising the sequences according to Seq.ID.Nos 7 to 12, as well as immunogenic fragments thereof comprising more than 7 amino acid residues.

Preferred immunogenic S. aureus antigen fragments according to the present invention comprise amino acid residues 4 to 13 and 1 to 9 of Seq.ID No.7, 4 to 14, 7 to 30 and 27 to 34 of Seq.ID No.8, 7 to 32 and 3 to 27 of Seq.ID No 9, 4 to 13 and 1 to 8 of Seq.ID No.10, 10 to 77, 82 to 101, 104 to 124 and 28 to 46 of Seq.ID No.11; and 4 to 40, 43 to 54, 62 to 76 and 29 to 57 of Seq.ID No.12.

According to another aspect, the present invention also relates to nucleic acids encoding the antigens or antigen fragments according to the present invention.

The present invention also relates to pharmaceutical preparations comprising one or more antigens or fragments according to the present invention or nucleic acid molecules encoding these polypeptides.
The present invention also relates to the use of an antigen or fragment or a nucleic acid according to the present invention for the manufacture of a pharmaceutical preparation, especially for the manufacture of a vaccine against staphylococcal infections or colonization in particular against S. aureus.

A preferred pharmaceutical preparation is a vaccine comprising an antigen or fragment according or a nucleic acid according to the present invention, preferably with a pharmaceutically acceptable carrier.

The pharmaceutical preparation may be administered in an effective amount, especially to achieve a suitable immunisation for prevention of diseases connected with S. aureus infections. A preferred use of a preparation according to the present invention is therefore the manufacturing of a medicament for treating or preventing staphylococcal infections or colonization in particular against S. aureus.

According to another aspect, the present invention also relates to a screening method assessing the consequences of functional inhibition of at least one antigen according to the present invention or a fragment thereof.

These antigens may be delivered e.g. by a screening method using expression libraries which mainly consists of three important parts, namely

1.: identifying hyperimmune serum sources containing specific antibodies against a given pathogen,
2.: screening of suitable expression libraries with a suitable antibody preparation wherein candidate antigens (or antigenic fragments of such antigens) are selected, and
3.: in a second screening round, wherein the hyperimmune serum-reactive antigens are identified by their ability to bind to a relevant portion of individual antibody preparations from individual sera in order to show that these antigens are practically relevant and not only hyperimmune serum-reactive, but also widely immunogenic (i.e. that a lot of individual sera react with a given antigen). With the present method it is possible to provide a set of antigens of a given pathogen.
A serum collection used for identifying the antigens according to the present invention should be tested against a panel of known antigenic compounds of a given pathogen, such as polysaccharide, lipid and proteinaceous components of the cell wall, cell membranes and cytoplasm, as well as secreted products.

Preferably, three distinct serum collections are used: 1. With very stable antibody repertoire: normal adults, clinically healthy people, who overcome previous encounters or currently carriers of e.g. a given pathogen without acute disease and symptoms,
2. With antibodies induced actually by the presence of the pathogenic organism: patients with acute disease with different manifestations (e.g. S. aureus sepsis or wound infection, etc.),
3. With no specific antibodies at all (as negative controls): 5-8 months old babies who lost the maternally transmitted immunoglobulins 5-6 months after birth. Sera have to react with multiple pathogen-specific antigens in order to consider hyperimmunity for S.aureus.

Also serum pools or plasma fractions or other pooled antibody containing body fluids are useable as "plasma pools".

Ribosome display is an established method for in vitro protein selection technology, which is applicable for each specific pathogen for the sake of the present invention (Schaffitzel et al, 1999). The antigen preparation for the first round of screening in the method according to the present invention may be derived from any source containing antibodies to a given pathogen. Preferably, if a plasma pool is used as a source for the antibody preparation, a human plasma pool is selected which comprises donors which had experienced or are experiencing an infection with the given pathogen. Although such a selection of plasma or plasma pools is in principle standard technology in for example the production of hyperimmunoglobulin preparations, it was surprising that such technologies have these effects as especially shown for the preferred embodiments of the present invention.
Preferably, a method for screening for the antigens according to the present invention comprises screening a ribosomal display library with the antibody preparation and identifying antigens which bind in said screening to antibodies in said antibody preparation. Such antigens may then be regarded extremely suited as hyperimmunogenic antigens.

It has been discovered that only 1-2% of the antibody repertoire of a patient having high titers against S.aureus are indeed antibodies directed against S.aureus. Moreover, over 70% of this specific 1% portion is directed against non-protein antigens, such as teichoic acid, so that only a total of 0.1% or less of the antibodies are directed to proteinaceous antigens.

The antibody preparation for screening for the antigens according to the present invention may be derived from patients with have suffered from an acute infection with S.aureus, especially from patients who show an antibody titer to the given pathogen above a certain minimum level, for example an antibody titer being higher than 80 percentile, preferably higher than 90 percentile, especially higher than 95 percentile of the human (patient or carrier) sera tested. Using such high titer individual antibody preparations in the second screening round allows a very selective identification of the present antigens.

The screening with the antibody preparations (which may also be the selected serum) allows a selective identification of the antigens. Therefore, preferably at least 10 individual antibody preparations (i.e. antibody preparations (e.g. sera) from at least 10 different individuals having suffered from an S.aureus infection) should be used in identifying these antigens in the second screening round. Of course, it is possible to use also less than 10 individual preparations, however, selectivity of the step may not be optimal with a low number of individual antibody preparations. On the other hand, if a given antigen (or an antigenic fragment thereof) is recognized in at least 10 individual antibody preparations, preferably at least 30, especially at least 50 individual antibody preparations, identification of the antigen is also selective enough for a proper identification. Hyperimmune serum-reactivity may of
course be tested with as many individual preparations as possible (e.g. with more than 100 or even with more than 1000).

The sera from which the individual antibody preparations for the screening are prepared (or which are used as antibody preparations), are selected by their titer against the specific pathogen (e.g. against a preparation of this pathogen, such as a lysate, cell wall components and recombinant proteins).

Preferably, some are selected with a total IgA titer above 4000 U, especially above 6000 U, and/or an IgG titer above 10 000 U, especially above 12 000 U (U = units, calculated from the OD$_{405\text{nm}}$ reading at a given dilution) when whole organism (total lysate or whole cells) is used as antigen in ELISA. Individual proteins with Ig titers of above 800-1000 U are specifically preferred for selecting the hyperimmune serum-reactive antigens according to the present invention only for total titer.

Accordingly, novel hyperimmune serum-reactive antigens from Staphylococcus aureus have been made available by the method according to the present invention.

The hyperimmune serum-reactive antigens obtained by the present invention may be immediately finished to a pharmaceutical preparation, preferably by addition of a pharmaceutically acceptable carrier and/or excipient, immediately after its production.

Preferably, the pharmaceutical preparation containing the present antigens is a vaccine for preventing or treating an infection with S.aureus.

The pharmaceutical preparation may contain any suitable auxiliary substances, such as buffer substances, stabilisers or further active ingredients, especially ingredients known in connection of vaccine production.

A preferable carrier/or excipient for the antigens according to the present invention is a immunostimulatory compound (as "immuniser") for further stimulating the immune response to the given hyperimmune serum-reactive antigen. Preferably the immun-
ostimulatory compound in the pharmaceutical preparation according to the present invention is selected from the group of polycationic substances, especially polycationic peptides, immunostimulatory deoxynucleotides, alum, Freund's complete adjuvans, Freund's incomplete adjuvans, neuroactive compounds, especially human growth hormone, or combinations thereof.

The polycationic compound(s) to be used according to the present invention may be any polycationic compound which shows the characteristic effects according to the WO 97/30721. Preferred polycationic compounds are selected from basic polypeptides, organic polycations, basic polyamino acids or mixtures thereof. These polyamino acids should have a chain length of at least 4 amino acid residues (see: Tuftsin as described in Goldman et al. (1983)). Especially preferred are substances like polylysine, polyarginine and polypeptides containing more than 20%, especially more than 50% of basic amino acids in a range of more than 8, especially more than 20, amino acid residues or mixtures thereof. Other preferred polycations and their pharmaceutical compositions are described in WO 97/30721 (e.g. polyethyleneimine) and WO 99/38528. Preferably these polypeptides contain between 20 and 500 amino acid residues, especially between 30 and 200 residues.

These polycationic compounds may be produced chemically or recombinantly or may be derived from natural sources.

Cationic (poly)peptides may also be anti-microbial with properties as reviewed in Ganz et al, 1999; Hancock, 1999. These (poly)peptides may be of prokaryotic or animal or plant origin or may be produced chemically or recombinantly (Andreu et al., 1998; Ganz et al., 1999; Simmaco et al., 1998). Peptides may also belong to the class of defensins (Ganz, 1999; Ganz et al., 1999). Sequences of such peptides can be, for example, be found in the Antimicrobial Sequences Database under the following internet address (also corresponding to Tossi et al, 2000):

http://www.bbcm.univ.trieste.it/~tossi/pag2.html

Such host defence peptides or defensives are also a preferred form of the polycationic polymer according to the present inven-
tion. Generally, a compound allowing as an end product activation (or down-regulation) of the adaptive immune system, preferably mediated by APCs (including dendritic cells) is used as polycationic polymer.

Especially preferred for use as polycationic substance in the present invention are cathelicidin derived antimicrobial peptides or derivatives thereof (WO 02/13857 A, incorporated herein by reference), especially antimicrobial peptides derived from mammal cathelicidin, preferably from human, bovine or mouse.

Polycationic compounds derived from natural sources include HIV-REV or HIV-TAT (derived cationic peptides, antennapedia peptides, chitosan or other derivatives of chitin) or other peptides derived from these peptides or proteins by biochemical or recombinant production. Other preferred polycationic compounds are cathelin or related or derived substances from cathelin. For example, mouse cathelin is a peptide which has the amino acid sequence NH$_2$-RLAGLLRKGGEKIGEKLKKGIGOKIKNFPQKLVPQPE-COOH. Related or derived cathelin substances contain the whole or parts of the cathelin sequence with at least 15-20 amino acid residues. Derivations may include the substitution or modification of the natural amino acids by amino acids which are not among the 20 standard amino acids. Moreover, further cationic residues may be introduced into such cathelin molecules. These cathelin molecules are preferred to be combined with the antigen. These cathelin molecules surprisingly have turned out to be also effective as an adjuvant for a antigen without the addition of further adjuvants. It is therefore possible to use such cathelin molecules as efficient adjuvants in vaccine formulations with or without further immunactivating substances.

Another preferred polycationic substance to be used according to the present invention is a synthetic peptide containing at least 2 KLK-motifs separated by a linker of 3 to 7 hydrophobic amino acids (WO 02/32451 A, incorporated herein by reference).

Immunostimulatory deoxynucleotides are e.g. neutral or artificial CpG containing DNA, short stretches of DNA derived from non-vertebrates or in form of short oligonucleotides (ODNs) con-
Containing non-methylated cytosine-guanine di-nucleotides (CpG) in a certain base context (e.g. Krieg et al., 1995) but also inosine or uridine containing ODNs (I-ODNs, U-ODNs) as described in WO 01/93905.

Neuroactive compounds, e.g. combined with polycationic substances are described in WO 01/24822.

The present antigens from Staphylococcus aureus may be used in the manufacture of a pharmaceutical preparation, especially for the manufacture of a vaccine against Staphylococcus aureus infections.

An immunogenic or hyperimmune fragment is defined as a fragment of the identified antigen which is for itself antigenic or may be made antigenic when provided as a hapten. Therefore, also antigen or antigenic fragments showing one or (for longer fragments) only a few amino acid exchanges (preferably one or two) are enabled with the present invention, provided that the antigenic capacities of such fragments with amino acid exchanges are not severely deteriorated on the exchange(s) i.e. suited for eliciting an appropriate immune response in a individual vaccinated with this antigen and identified by individual antibody preparations from individual sera (e.g. according to the rules disclosed in Tordot et al., 2000 (Eur. J. Immunol. 30 (2000), 3411-3421.

Preferred examples of fragments of a the antigens according to the present invention are selected from the group consisting of peptides comprising the amino acid sequences of column "predicted immunogenic aa", "Location of identified immunogenic region" and "Serum reactivity with relevant region" of Table 2 and fragments comprising at least 6, preferably more than 7, more preferred more than 8, especially more than 10 aa of said sequences . All these fragments individually and each independently form a preferred selected aspect of the present invention.

According to another aspect, the present invention relates to a vaccine comprising such an antigen or a fragment thereof as identified above for Staphylococcus aureus. Such a vaccine may
comprise one or more antigens against S. aureus. Optionally, such S. aureus antigens may also be combined with antigens against other pathogens in a combination vaccine. Preferably this vaccine further comprises an immunostimulatory substance, preferably selected from the group comprising polycationic polymers, especially polycationic peptides, immunostimulatory deoxy-nucleotides (ODNs), neuroactive compounds, especially human growth hormone, alum, Freund's complete or incomplete adjuvans or combinations thereof. Such a vaccine may also comprise the antigen displayed on a surface display protein platform on the surface of a genetically engineered micro-organism such as E. coli.

According to another aspect, the present invention relates to specific preparations comprising antibodies raised against at least one of the Staphylococcus aureus antigens or Staphylococcus aureus antigen fragments according to the present invention. These antibodies are preferably monoclonal antibodies.

A preferred method for producing an antibody preparation according to the present invention is characterized by the following steps:

- initiating an immune response in a non human animal by administering an antigen according to the present invention or a fragment thereof, to said animal,

- removing the spleen or spleen cells from said animal,

- producing hybridoma cells of said spleen or spleen cells,

- selecting and cloning hybridoma cells specific for said antigen and

- producing the antibody preparation by cultivation of said cloned hybridoma cells and optionally further purification steps.

Preferably, this said removing the spleen or spleen cells in this method is connected with killing said animal.

Another preferred method for producing an antibody preparation
according to the present invention comprises the following steps:

- initiating an immune response in a non human animal by adminis-
tering an antigen according to the present invention or a frag-
ment thereof, to said animal,

- removing an antibody containing body fluid from said animal,

and

- producing the antibody preparation by subjecting said antibody
containing body fluid to further purification steps.

Monoclonal antibodies and fragments thereof can be chimerized or
humanized (Graziano et al. 1995) to enable repeated administra-
tion. Alternatively, human monoclonal antibodies and fragments
thereof can be obtained from phage-display libraries (McGuinness
et al., 1996) or from transgenic animals (Brüggemann et al.,
1996).

Monoclonal or polyclonal antibody preparations may be used for
the manufacture of a medicament for treating or preventing dis-
eases due to staphylococcal infection. Moreover, they may be
used for the diagnostic and imaging purposes.

The invention is further described in the following examples and
in the figures, but should not be restricted thereto.

Figure 1 shows the pre-selection of sera based on anti-staphylo-
coccal antibody titers measured by ELISA; RT-PCR with specific
primers for the indicated novel putative ORFs and the IsaA-gene
as control; lanes indicated with '+' indicate RT-PCR reaction;
'-' are controls without reverse transcription, followed by PCR.

EXAMPLES

Discovery of novel Staphylococcus aureus antigens

Example 1: Preparation of antibodies from human serum
The antibodies produced against staphylococci by the human immune system and present in human sera are indicative of the in vivo expression of the antigenic proteins and their immunogenicity. These molecules are essential for the identification of individual antigens in the approach as the present invention which is based on the interaction of the specific anti-staphylococcal antibodies and the corresponding S. aureus peptides or proteins. To gain access to relevant antibody repertoires, human sera were collected from
I. patients with acute S. aureus infections, such as bacteriemia, sepsis, infections of intravascular and percutan catheters and devices, wound infections, and superficial and deep soft tissue infection. S. aureus was shown to be the causative agent by medical microbiological tests.
II. A collection of serum samples from uninfected adults was also included in the present analysis, since staphylococcal infections are common, and antibodies are present as a consequence of natural immunization from previous encounters with Staphylococci from skin and soft tissue infections (furunculus, wound infection, periodontitis etc.).

The sera were characterized for S. aureus antibodies by a series of ELISA assays. Several staphylococcal antigens have been used to prove that the titer measured was not a result of the sum of cross-reactive antibodies. For that purpose not only whole cell S. aureus (protein A deficient) extracts (grown under different conditions) or whole bacteria were used in the ELISA assays, but also individual cell wall components, such as lipoteichoic acid and peptidoglycan isolated from S. aureus. More importantly, a recombinant protein collection was established representing known staphylococcal cell surface proteins for the better characterization of the present human sera collections.

Recently it was reported that not only IgG, but also IgA serum antibodies can be recognized by the FcRIII receptors of PMNs and promote opsonization (Shibuya et al., 2000). The primary role of IgA antibodies is neutralization, mainly at the mucosal surface. The level of serum IgA reflects the quality, quantity and specificity of the dimeric secretory IgA. For that reason the serum collection was not only analyzed for anti-staphylococcal IgG,
but also for IgA levels. In the ELISA assays highly specific secondary reagents were used to detect antibodies from the high affinity types, such as IgG and IgA, and avoided IgM. Production of IgM antibodies occurs during the primary adaptive humoral response, and results in low affinity antibodies, while IgG and IgA antibodies had already undergone affinity maturation, and are more valuable in fighting or preventing disease.

**Experimental procedures**

Enzyme linked immune assay (ELISA). ELISA plates were coated with 2-10 μg/ml of the different antigens in coating buffer (sodium carbonate pH 9.2). Serial dilutions of sera (100-100,000) were made in TBS-BSA. Highly specific (cross-adsorbed) HRP (Horse Radish Peroxidase)-labeled anti-human IgG or anti-human IgA secondary antibodies (Southern Biotech) were used according to the manufacturers' recommendations (~2,000x). Antigen-antibody complexes were quantified by measuring the conversion of the substrate (ABTS) to colored product based on OD405nm readings in an automated ELISA reader (Wallace Victor 1420). The titers were compared at given dilution where the dilution response was linear (Table 1). The ~100 sera were ranked based on the reactivity against multiple staphylococcal components, and the highest ones (above 90 percentile) were selected for further analysis in antigen identification. Importantly, the anti-staphylococcal antibodies from sera of clinically healthy individuals proved to be very stable, giving the same high ELISA titers against all the staphylococcal antigens measured after 3, 6 and 9 months (data not shown). In contrast, anti-S. aureus antibodies in patients decrease, then disappear after a couple of weeks following the infection (Coloque-Navarro et al, 1998). However, antibodies from patients are very important, since these are direct proof of the in vivo expression of the bacterial antigens tested in or ELISAs or identified as immunogenic during the screens according to the present invention.

This comprehensive approach followed during antibody characterization is unique, and led to unambiguous identification of anti-staphylococcal hyperimmune sera.
Purification of antibodies for genomic screening. Five sera from both the patient and the noninfected group were selected based on the overall anti-staphylococcal titers. Antibodies against E. coli proteins were removed by either incubating the heat inactivated sera with whole cell E. coli (DH5alpha, transformed with pHIE11, grown under the same condition as used for bacterial display) or with E. coli lysate affinity chromatography for ribosome display. Highly enriched preparations of IgG from the pooled, depleted sera were generated by protein G affinity chromatography, according to the manufacturer's instructions (UltraLink Immobilized Protein G, Pierce). IgA antibodies were purified also by affinity chromatography using biotin-labeled anti-human IgA (Southern Biotech) immobilized on Streptavidin-agarose (GIBCO BRL). The efficiency of depletion and purification was checked by SDS-PAGE, Western blotting, ELISA, and protein concentration measurements. For proteomics, the depletion the IgG and IgA preparation was not necessary, since the secondary reagent ensured the specificity.

Generation of highly random, frame-selected, small-fragment, genomic DNA libraries of Staphylococcus aureus was performed as previously described in PCT/EP/00544 or WO01/96554.

Example 2: Identification of highly immunogenic peptide sequences from genomic fragments from S. aureus using ribosome display and human serum

Experimental procedures

Ribosome display screening: 2.4 ng of the genomic library from S. aureus LSA250/1 in pMAL4.1 (described in WO01/96554) was PCR amplified with oligos ICC277 and ICC202 in order to be used for ribosome display. Oligos ICC277 (CGAACCTACGACTCTATAGGGGACCCACAACGGTGGTCCCCTAGTAATAATTTTGTTTAACTTCCAAAGGAGATAATCCATCGACAACCTTGCGCCGCTCCC) and ICC202 (GGCCCGACGCTGAGTAGCAAACCGCGCTGTTGTTGTTGACTGG) hybridize 5' and 3' of the Fse I-Not I insertion site of plasmid pMAL4.1, respectively. ICC277 introduces a T7 phage RNA polymerase promoter, a palindromic sequence resulting in a stem-loop structure on the RNA level, a ribosome binding site (RBS) and the transla-
tion start of gene 10 of the T7 phage including the ATG start
codon. Oligo ICC202 hybridizes at nucleotide position 668 of the
β-lactamase open reading frame and also introduces a stem-loop
structure at the 3' end of the resulting RNA. PCR was performed
with the High fidelity PCR kit (Roche Diagnostic) for 25 cycles
at 50°C hybridization temperature and otherwise standard condi-
tions.

The resulting PCR library was used in 4 consecutive rounds of
selection and amplification by ribosome display similar as de-
scribed previously (Hanes et al., 1997) but with modifications
as described below.

One round of ribosome display contained the following steps: In
vitro transcription of 2 µg PCR product with the RiboMax kit
(Promega) resulted in ca. 50 µg A. In vitro translation was per-
formed for 9 minutes at 37°C in 22 µl volume with 4.4 µl Premix
Z (250 mM TRIS-acetate pH 7.5, 1.75 mM of each amino acid, 10 mM
ATP, 2.5 mM GTP, 5 mM cAMP, 150 mM acetylphosphate, 2.5 mg/ml E.
coli trNA, 0.1 mg/ml folinic acid, 7.5 % PEG 8000, 200 mM po-
tassium glutamate, 13.8 mM Mg(Ac)2, 8 µl S30 extract (x mg/ml)
and about 2 µg in vitro transcribed RNA from the pool. S30 ex-
tract was prepared as described (Chen et al, 1983). Next, the
sample was transferred to an ice-cold tube containing 35.2 µl 10
% milk-WBT (TRIS-acetate pH 7.5, 150 mM NaCl, 50 mM Mg(Ac)2, 0.1
% Tween-20, 10 % milk powder) and 52.8 µl WBT (as before plus
2.5 mg/ml heparin). Subsequently, immuno precipitation was per-
formed by addition of 10 µg purified IgGs, incubation for 90
minutes on ice, followed by addition of 30 µl MAGmol Protein G
beads (Miltenyi Biotec, 90 minutes on ice). The sample was ap-
plicated to a pre-equilibrated µ column (Miltenyi Biotec) and
washed 5 times with ice-cold WBT buffer. Next 20 µl EB20 elution
buffer (50 mM TRIS-acetate, 150 mM NaCl, 20 mM EDTA, 50 µg/ml S.
cerevisiae RNA) was applied to the column, incubated for 5
minutes at 4°C. Elution was completed by adding 2 × 50 µl EB20.
The mRNA from the elution sample was purified with the High pure
RNA isolation kit (Roche Diagnostics). Subsequent reverse tran-
scription was performed with Superscript II reverse tran-
scriptase kit (Roche Diagnostics) according to the instruction
of the manufacturer with 60 pmol oligo ICC202 for 1 hour at 50°C
in 50 µl volume. 5 µl of this mix was used for the following PCR reaction with primers ICC202 and ICC277 as described above.

Four rounds of ribosome display were performed and the resulting selected PCR pool subsequently cloned into plasmid pHIE11 (described above) by cleavage with restriction endonucleases NotI and FseI.

Evaluation of selected clones by sequencing and peptide-ELISA analysis:
Selected clones were grown over night at 37°C in 3 ml LB medium supplemented with 50 µg/ml Kanamycin to prepare plasmid DNA using standard procedures. Sequencing was performed at MWG (Germany) or at the Institute of Genomic Research (TIGR; Rockville, MD, U.S.A.). Peptides corresponding to the inserts were synthesized and coated in 10 mM NaHCO₃ pH 9.3 at a concentration of 10 µg/ml (50 µl) onto 96-well microtiter plates (Nunc). After blocking with 1% BSA in PBS at 37°C, 1:200 and 1:1000 dilutions of the indicated sera were diluted in 1% BSA/PBS and applied to the wells. After washing with PBS/0.1 % Tween-20, biotin-labeled anti-human IgG secondary antibodies (SBA) were added and these were detected by subsequent adding horseradish-peroxidase-coupled streptavidin according to standard procedures.

Results

The 250-bp genomic library (LSA250/1) as described previously (Etz et al., 2002) was used for screening. Purified IgGs from uninfected adults but with high titer against S. aureus as described above were used for selection of antigenic peptides.

Four rounds of ribosome display selection and amplification were performed according to Experimental procedures; finished by cloning and sequencing the resulting PCR pool.

Sequence analyses of a large number of randomly picked clones (1100) led to the identification of the gene and the corresponding peptide or protein sequence that was specifically recognized by the high titer serum used for screening. The frequency with which a specific clone was selected reflects at least in part
the abundance and/or affinity of the specific antibodies in the serum used for selection and recognizing the epitope presented by this clone. Remarkably, for a distinct fraction of clones their encoded nucleotide sequence did not match to any known or annotated ORF. Instead, they match in the opposite orientation to previously annotated ORFs. For one clone group, corresponding clones were picked 86 times, indicating their highly immunogenic property. Table 2 shows the ORF name, the Seq.ID No. and the number of times it was identified by the inventive screen.

Figure 1 shows RT-PCR results ((A) RT-PCR with specific primers for the indicated novel putative ORFs and the IsaaA-gene as control. Lanes indicated with '+' indicate RT-PCR reaction; '-' are controls without reverse transcription, followed by PCR. See also Table 3).

Table 4 shows peptide ELISA with serum from patients and healthy individuals with peptide derived from SAN8.

For one immuno-selected putative opposite-strand ORF, a peptide corresponding to the identified immunogenic region was synthesized and tested in peptide-ELISA for their reactivity towards the sera pool they were identified with and also a number of additional sera from patients who suffered from an infection by S. aureus. Figure 1 shows the reactivity of the peptide derived from SAN8. It is not only hyperimmune reactive against the high titer sera pool used for screening (indicated with *) but also towards a number of individual patient's sera.

**Characterization of newly identified putative ORFs**
All clone groups locate in the opposite orientation to annotated genes, indicating the presence of putative anti-sense transcripts, which encode immuno-dominant peptides. Analysis of the surrounding areas revealed possible open reading frames with sizes varying between 16 and 146 amino acid in length taken into account the closest start (incl. TTG codons) and stop codon in frame with the selected peptide. BLAST analysis of these putative ORFs against the non-redundant database did not reveal any
considerable similarity to any annotated sequence.

To confirm the actual in vivo expression of these putative short ORFs, total mRNA samples from *S. aureus* grown in BHI medium were used for mRNA expression analysis. First, RT-PCR analysis with primers specific for corresponding short ORFs were performed and confirmed the mRNA presence for four putative ORFs that were tested. Second, northern analysis with strand-specific single stranded probes revealed the existence of even longer mRNA transcripts than the calculated length corresponding to these short ORFs (table 3).

Moreover, the evidence provided here for the existence of anti-sense transcripts to known genes could indicate a regulatory function on the RNA level, as it was shown that anti-sense transcripts very efficiently turn down the expression of the corresponding genes (Ji et al, 1999). Interestingly, some of the anti-sense ORFs lie opposite to genes involved in virulence.

**Example 3: Applications**

A) An effective vaccine offers great potential for patients facing elective surgery in general, and those receiving endovascular devices, in particular. Patients suffering from chronic diseases with decreased immune responses or undergoing continuous ambulatory peritoneal dialysis are likely to benefit from a vaccine with *S. aureus* by immunogenic serum-reactive antigens according to the present invention. Identification of the relevant antigens will help to generate effective passive immunization (humanized monoclonal antibody therapy), which can replace human immunoglobulin administration with all its dangerous side-effects. Therefore an effective vaccine offers great potential for patients facing elective surgery in general, and those receiving endovascular devices, in particular. *S. aureus* can cause many different diseases:

- Sepsis, bacteriæmia
- Haemodialysed patients - bacteriemia, sepsis
• Peritoneal dialyses patients - peritonitis
• Patients with endovascular devices (heart surgery, etc) - endocarditis, bacteriemia, sepsis
• Orthopedic patients with prosthetic devices - septic arthritis
• Preventive vaccination of general population

B) Passive and active vaccination, both with special attention to T-cells with the latter one: It is an aim to induce a strong T helper response during vaccination to achieve efficient humoral response and also immunological memory. Up till now, there is no direct evidence that T-cells play an important role in clearing S. aureus infections, however, it was not adequately addressed, so far. An effective humoral response against proteinaceous antigens must involve T help, and is essential for developing memory. Naïve CD4+ cells can differentiated into Th1 or Th2 cells. Since, innate immunological responses (cytokines) will influence this decision, the involvement of T-cells might be different during an acute, serious infection relative to immunization of healthy individuals with subunit vaccines, not containing components which impair the immune response during the natural course of the infection. The consequences of inducing Th1 or Th2 responses are profound. Th1 cells lead to cell-mediated immunity, whereas Th2 cells provide humoral immunity.

C) Preventive and therapeutic vaccines

Preventive: active vaccination/passive immunization of people in high risk groups, before infection

Therapeutic: passive vaccination of the already sick.

Active vaccination to remove nasal carriage

Specific example for an application

Elimination of MRSA carriage and prevention of colonization of the medical staff

Carriage rates of S. aureus in the nares of people outside of the hospitals varies from 10 to 40%. Hospital patients and per-
sonnel have higher carriage rates. The rates are especially high in patients undergoing hemodialysis and in diabetics, drug addicts and patients with a variety of dermatologic conditions. Patients at highest risk for MRSA infection are those in large tertiary-care hospitals, particularly the elderly and immunocompromised, those in intensive care units, burn patients, those with surgical wounds, and patients with intravenous catheters.

The ELISA data show that there is a pronounced IgA response to *S. aureus*, which is not obvious or known from the literature. Since the predominant mucosal immune response is the production of IgA with neutralizing activity, it is clear that the staphylococcal epitopes and antigens identified with the highly pure IgA preparations lead to an efficient mucosal vaccine.
Table 1: ELISA titers of sera from non-infected individuals against multiple staphylococcal proteins.

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TABLE 2: IMMUNOGENIC PROTEINS IDENTIFIED BY RIBOSOME DISPLAY.

Ribosome display screen with LSA250/1 library and IC sera. Prediction of antigenic sequences longer than 5 amino acids was performed with the programme ANTIgenic (Kolaskar and Tongaonkar, 1990).

<table>
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<tr>
<th>S. aureus antigenic protein</th>
<th>Putative function (by homology)</th>
<th>predicted immunogenic aa**</th>
<th># of selected clones per ORF and screen</th>
<th>Location of identified immunogenic region</th>
<th>Serum reactivity with relevant region (positive/total)</th>
<th>Seq ID no: (DNA +Prot)</th>
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Table 3:

Transcriptional analysis and Genomic properties of putative ORFs.

In vivo transcriptional analysis of putative novel ORFs was determined by RT-PCR and Northern blot analysis. RNA was extracted from S. aureus COL.

RT-PCR: Northern: +,-: positive or negative in RT-PCR or Northern analysis, respectively. N.d.: not determined. ORFs with prefix 'P' indicate location on the plasmid of S. aureus COL isolate. Orientation indicates the location on the plus (+) or minus (-) strand of the genome or plasmid, respectively.

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<th>ORF orientation</th>
<th>ORF length (AA)</th>
<th>clone match from - to (nt)</th>
<th>opposite to ORF</th>
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- **Healthy IC Serum**
- **Patient Serum**
References

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Brüggemann et al. (1996), Immunol. Today 17, 391-397


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Hancock (1999), Drugs 57, 469-473


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Shibuya et al. (2000), Nature Immunology 1:441-446.

Simmaco et al. (1998), Biopolymers 47, 435-450

Tossi et al. (2000), Biopolymers 55, 1-30


Vytvytska et al. (2002), Proteomics 2, 580-590.

Claims:

1. A Staphylococcus aureus (S. aureus) antigen selected from the group consisting of Seq.ID Nos. 7 to 12 and immunogenic fragments thereof having at least 6 amino acid residues.

2. Immunogenic S. aureus antigen fragment comprising amino acid residues 4 to 13 and 1 to 9 of Seq.ID No.7, 4 to 14, 7 to 30 and 27 to 34 of Seq.ID No.8, 7 to 32 and 3 to 27 of Seq.ID No 9, 4 to 13 and 1 to 8 of Seq.ID No.10, 10 to 77, 82 to 101, 104 to 124 and 28 to 46 of Seq.ID No.11; and 4 to 40, 43 to 54, 62 to 76 and 29 to 57 of Seq.ID No.12.

3. Nucleic acid coding for an antigen or fragment according to claim 1.

4. Pharmaceutical preparation comprising an antigen or a fragment according to claim 1 or 2 or a nucleic acid molecule according to claim 3.

5. Use of an antigen or fragment according to claim 1 or 2 or a nucleic acid according to claim 3 for the manufacture of a pharmaceutical preparation, especially for the manufacture of a vaccine against staphylococcal infections or colonization in particular against S. aureus.

6. Vaccine comprising an antigen or fragment according to claim 1 or 2 or a nucleic acid according to claim 3.

7. Vaccine according to claim 6, characterized in that it further comprises an immunostimulatory substance, preferably selected from the group comprising polycationic polymers, especially polycationic peptides, immunostimulatory deoxynucleotides (ODNs), neuroactive compounds, especially human growth hormone, alum, Freund's complete or incomplete adjuvans or combinations thereof.

8. Preparation comprising antibodies against at least one antigen or a fragment thereof, as defined in claim 1 or 2.

9. Preparation according to claim 8, characterized in that said
antibodies are monoclonal antibodies.

10. Method for producing a preparation according to claim 8, characterized by the following steps:

• initiating an immune response in a non human animal by administering an antigen or a fragment thereof, as defined in claim 1 or 2, to said animal,

• removing the spleen or spleen cells from said animal,

• producing hybridoma cells of said spleen or spleen cells,

• selecting and cloning hybridoma cells specific for said antigen and

• producing the antibody preparation by cultivation of said cloned hybridoma cells and optionally further purification steps.

11. Method according to claim 10, characterized in that said removing the spleen or spleen cells is connected with killing said animal.

12. Method for producing a preparation according to claim 8, characterized by the following steps:

• initiating an immune response in a non human animal by administering an antigen or a fragment thereof, as defined in claim 1 or 2, to said animal,

• removing an antibody containing body fluid from said animal, and

• producing the antibody preparation by subjecting said antibody containing body fluid to further purification steps.

13. Use of a preparation according to claim 8 or 9 for the manufacture of a medicament for treating or preventing staphylococcal infections or colonization in particular against S. aureus.

14. A screening method assessing the consequences of functional inhibition of at least one antigen or a fragment thereof, as
defined in claim 1 or 2.
Fig. 1
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Trp Phe Asn Tyr Tyr Phe Ser Phe Asn Ile Ile Tyr Ser Ile Tyr Arg
20     25     30

Leu Ile Cys Ser Cys Val Tyr Leu Ile Ile Cys Leu Val Leu Ile Asn
35     40     45

Leu Ile Asn Cys Leu Ile Phe Lys Leu Ile Asp Leu Ile Asn Ser Ser
50     55     60

Thr Leu Arg His Phe Ser Ser Asp Phe Ile Ser Arg Cys Leu Phe Phe
85     90

Tyr Tyr Ile Asn Leu Asn Ser Ile Asp Ile Ile Leu Cys Tyr Cys Arg
100    105    110

Ser Leu Val Tyr Phe Cys Ile Cys Val Ser Leu Phe Asn Thr Phe Asn
115    120    125

Ser Leu Leu Ser Val Leu Ile
130    135

Met Leu Ile Met Pro Thr Leu Leu Phe Ser Leu Tyr Ala Cys Leu Thr
1      5     10     15
Ala Phe Val Thr Ala Ser Leu Thr Ala Ile Leu Ala Ser Ser Ile Ser
20

Ser Tyr Val Ala Pro Ala Leu Glu Lys Thr Pro Glu Ser Val Lys Arg
35

Ile Lys Leu Thr Tyr Ser Ala Asp Ala Gly Thr Arg Ile Ser Ile Lys
50

Ser Ser Leu Asp Cys Met Leu Phe Asp Leu Arg Ser Phe Ile Asn Met
65

His