Identification of SagA as a novel vaccine target for the prevention of Enterococcus faecium infections

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Infections caused by multiresistant Gram-positive bacteria represent a major health burden in the community as well as in hospitalized patients. Enterococci, especially Enterococcus faecium, are well-known pathogens of hospitalized patients and are frequently linked with resistance against multiple antibiotics, which compromises effective therapy. Rabbit immune serum raised against heat-killed E. faecium E155, a HIRECC clone, was used in an opsonophagocytic assay, an inhibition assay and a mouse bacteraemia model to identify targets of opsonic and protective antibodies. Serum against whole heat-killed bacteria was opsonic and recognized a protein of about 72 kDa that was abundantly secreted. This protein, identified as SagA by LC-ES-MS/MS, was expressed in Escherichia coli and purified. Rabbit serum raised against the purified protein showed opsonic killing activity that was inhibited by almost 100 % using 10 μg purified protein ml⁻¹. In a mouse bacteraemia model, a statistically significant reduction of the colony counts in blood was shown with immune rabbit serum compared with preimmune serum using the homologous and a heterologous vancomycin-resistant enterococci (VRE) strain. These results indicate that SagA could be used as a promising vaccine target to treat and/or prevent VRE bacteraemia.

INTRODUCTION

Enterococci are among the three most common nosocomial pathogens and, due to their multiple antibiotic resistance, they cause substantial morbidity and mortality, especially among intensive care and immunocompromised patients (Arias et al., 2010). While several new antibiotics have been introduced in the last decade, resistance to these new drugs is developing and spreading rapidly (Arias & Murray, 2009; Herrero et al., 2002). Life-threatening systemic diseases such as endocarditis caused by resistant strains may at times be untreatable (Schwartz et al., 2008); therefore, alternative treatment and prevention strategies are desperately needed to counter the rise of multiply resistant strains.

In an attempt to identify targets of opsonic antibodies in Enterococcus faecium, we decided to use rabbit sera raised against an E. faecium strain associated with hospital outbreaks of vancomycin-resistant enterococci (VRE) (Leavis et al., 2007). Related strains from the same clonal complex 17 have been isolated in hospitals and nursing homes worldwide (Top et al., 2008; Willems et al., 2005). These hospital outbreaks increase the length of stay by 2.2–3.5 days for patients with VRE bloodstream infection, and additional costs attributable to vancomycin resistance are on average between $1339 and $1968 per episode, depending on the statistical method used (Butler et al., 2010). In a larger VRE outbreak in our own hospital, we identified 25 patients with invasive VRE infection with an overall survival of 48 % after 30 days. Four of 13 deaths were considered to be directly related to the VRE infection (Theilacker et al., 2009a). These alarming facts underscore the importance of the development of alternative treatments and preventive strategies to eliminate these infections.

METHODS

Bacterial strains and sera. The bacterial strains used in this study are shown in Table 1. A New Zealand white rabbit was immunized with heat-killed E. faecium strain E155 (VRE) (Leavis et al., 2007). Bacteria were heat-killed at 65 °C for 1 h, and a final concentration of 1.4 × 10¹² c.f.u. per dose was injected intravenously three times a week for a total of 3 weeks. Another rabbit was immunized with purified protein (see below) by two subcutaneous injections of 10 μg protein given 2 weeks apart; in the third week, three injections of 5 μg were given intravenously.

SDS-PAGE and Western blot. Purified proteins were subjected to SDS-PAGE with 10 % acrylamide/bisacrylamide resolving gels (NuPAGE, Invitrogen) and stained with Coomassie brilliant blue (SimplyBlue SafeStain, Invitrogen) for protein detection (Sambrook & Russell, 2001) or transferred onto polyvinylidene difluoride (PVDF, Invitrogen) using electro transfer as described by the manufacturer (X Cell SureLock Electrophoresis, Invitrogen). Non-specific binding sites were blocked with 3 % skimmed milk in 1 × PBS. After three washing...
steps, the membrane was incubated with the primary antibody by shaking for 1 h at room temperature. The membrane was washed again and incubated with the alkaline phosphate-labelled secondary antibody for an additional hour at room temperature. After three washes, bound antibodies were detected with bromochloro-indolyl phosphate and nitro bluetetrazolium as described by Blake et al. (1984). The reaction was stopped with water. The SeeBlue Plus2 Prestained Standard (Invitrogen) was used as molecular mass standard.

Amplification of genes and expression of proteins. Chromosomal DNA from E. faecium E155 was prepared using the MasterPure Gram-positive DNA Purification kit (Epicenter). The forward primer used for cloning of SagA was 5'-CACCAAAAA-GAGTTTAATATCAGCA; the reverse primer had the sequence 5'-TTACATGCTGACAGCAAAGT. The additional nucleotides CACC (underlined) were introduced for cloning into pET151/D-TOPO (Invitrogen). Proteins expressed using pET151/D-TOPO and purification was carried out according to the manufacturer’s instructions using Escherichia coli BL21 and the ProBond Purification System (Invitrogen).

Nano-LC-ES-MS/MS. Bands of interest were cut from a Coomassie-stained SDS-PAGE gel. Aliquots of the samples were reduced, alkylated and subjected to tryptic digestion. The resulting peptides were analysed by nano-LC-ES-MS/MS (GE Healthcare ETTAN-MDLC system configured in nano flow; column, Agilent Zorbax C18). Micromass Q-TOF elution was carried out using 0.05 % aqueous formic acid (solvent A) and 90 % acetonitrile (10 % of solvent B). Data-dependent acquisition was utilized, and peptides eluting from the nano-LC column were automatically fragmented in the Q-TOF by recognition of their forward primer used for cloning of SagA was 5’-CACCAAAAA-GAGTTTAATATCAGCA; the reverse primer had the sequence 5’-TTACATGCTGACAGCAAAGT. The additional nucleotides CACC (underlined) were introduced for cloning into pET151/D-TOPO (Invitrogen). Proteins expressed using pET151/D-TOPO and purification was carried out according to the manufacturer’s instructions using Escherichia coli BL21 and the ProBond Purification System (Invitrogen).

ELISA. ELISA experiments were performed by standard methods as described previously (Maira-Litran et al., 2002). In brief, microtitre plates (Immulon 4 HBXTM, Dynex Technologies) were coated with 100 μl recombinant SagA protein (10 μg ml⁻¹ in 0.05 M Na₂CO₃ buffer, pH 9.0) and incubated overnight at 4 °C. Washing steps were performed with 1 × PBS containing 0.05 % Tween 20. Plates were blocked with 3 % skimmed milk in 1 × PBS/0.02 % sodium azide at 37 °C for 2 h. Dilutions of each antiserum (from 1:25 to 1:12 800) were added to antigen-coated wells and incubated for a further 90 min at 37 °C. A goat anti-rabbit IgG alkaline phosphatase conjugate (Sigma) diluted 1:1000 was used as secondary antibody, and p-nitrophenyl phosphate was used as a substrate (Sigma). After 60 min incubation at 37 °C, the absorbance was measured at 405 nm. Wells in which the first antibody was omitted were used as negative controls.

Opsonophagocytic assay (OPA) and opsonophagocytic inhibition assay (OPIA). OPAs and OPIAs were performed as described previously (Theilacker et al., 2009b). In brief, white blood cells (WBCs) were prepared from fresh human blood collected from healthy adult volunteers. With trypan blue staining to differentiate dead from live leukocytes, the final WBC count was adjusted to 2.5 × 10⁶ WBC ml⁻¹. Baby rabbit serum (Cedarlane Laboratories), diluted 1:15 in RPMI plus 15 % fetal bovine serum (FBS) and absorbed with the target strain, was used as complement source. Bacteria cultured on TSA plates were resuspended in TSB to OD₆₅₀ 0.1 and then grown to OD₆₅₀ 0.4. After washing the culture once, a final 1:100 dilution was made in RPMI plus 15 % FBS. Rabbit sera were heat-inactivated and diluted from 1:10 to 1:1000 in RPMI plus 15 % FBS. The actual phagocytic killing assay was performed by mixing 100 μl (each) of the WBC, target bacteria, dilutions of test sera and the complement source. The reaction mixture was incubated on a rotor rack at 37 °C for 90 min; samples were taken at time zero and after 90 min. A 10-fold dilution was made in TSB and samples were plated onto TSA plates. Tubes lacking any serum and tubes with normal rabbit serum (NRS) were used as controls. The percentage of opsonophagocytic killing was calculated by determining the ratio of the c.f.u. surviving in the tubes with bacteria, leukocytes, complement and antibody, to the c.f.u. surviving in the tubes with all these components but lacking leukocytes. For inhibition studies, rabbit serum was diluted 1:10 and incubated for 60 min at 4 °C with an equal volume of a solution containing 0.8–100 μg SagA ml⁻¹. Subsequently, the antiserum was used in the OPA as described above. Inhibition assays were performed at serum dilutions yielding 70–80 % killing of the inoculum without the addition of the inhibitor. The percentage of inhibition of opsonophagocytic killing was compared to controls without inhibitor.

Table 1. Bacterial strains and sera used in the present study

<table>
<thead>
<tr>
<th>Strain or serum</th>
<th>Description</th>
<th>Reference or source</th>
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<tbody>
<tr>
<td>Strains</td>
<td></td>
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<tr>
<td>E. faecium E155</td>
<td>VRE strain from a patient in the USA (Chicago), clonal complex 17</td>
<td>Leavis et al. (2007)</td>
</tr>
<tr>
<td>E. faecium VRE 757875</td>
<td>VRE strain from a patient in the USA (Boston)</td>
<td>Hucbner et al. (1999)</td>
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<tr>
<td>Sera</td>
<td></td>
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<tr>
<td>zWB</td>
<td>Rabbit serum raised against heat-killed E. faecium E155</td>
<td>This study</td>
</tr>
<tr>
<td>zSagA</td>
<td>Rabbit serum raised against purified recombinant SagA from E. faecium E155</td>
<td>This study</td>
</tr>
<tr>
<td>NRS</td>
<td>Preimmune serum from rabbit subsequently immunized with SagA</td>
<td>This study</td>
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Fig. 1. Opsonic killing of rabbit serum against E. faecium E155. Serum raised against whole bacterial cells (zWB) was used in different dilutions using the homologous strain E. faecium E155 as target strain. Data are mean ± SEM.
Animal model. A mouse bacteraemia model was used as described elsewhere (Theilacker et al., 2006). In brief, 6–8-week-old female BALB/c mice (eight mice per group) received 200 µl NRS or serum raised against the purified protein. Animals were challenged with *E. faecium* E155 after 24 h (1.18 × 10^{10} c.f.u. per mouse) and received a second dose of 200 µl rabbit serum 2 h after inoculation. Mice were sacrificed 8 h after infection and colony counts in the blood were determined. The animal welfare committee of the University of Freiburg (Regierungspräsidium Freiburg Az 35/9185.81/G-07/15) approved all animal experiments.

RESULTS AND DISCUSSION

Rabbit serum raised against heat-killed *E. faecium* E155 was opsonic against the homologous strain (i.e. 77.8 % killing at a serum dilution of 1:100, Fig. 1). SDS-PAGE and Western blot using sera against heat-killed *E. faecium* E155 showed a strong band at around 72 kDa (Fig. 2); an identical band was present in five other *E. faecium* strains tested (data not shown). Nano-LC-ES-MS/MS analysis of the excised Coomassie blue-stained gel band yielded sequence data that were compared to the NCBI and MASCOT databases. The highest homology (score 1253) was obtained with a 55 kDa protein from *E. faecium* DO (MSDB accession no. Gi 69245436). The corresponding gene was subsequently amplified from *E. faecium* E155 by PCR, and the expressed protein was purified on nickel columns under denaturing conditions. The purified protein was shown to be reactive with the rabbit sera raised against whole *E. faecium* E155 (Fig. 3).

A rabbit was immunized with the purified protein, and 2 weeks after the last injection, titres were checked by ELISA (data not shown) and OPA (Fig. 4). An opsonophagocytic inhibition assay was performed to confirm specificity, showing that 100 µg purified protein inhibited killing by almost 100 %, while lower amounts inhibited killing in a dose-dependent fashion (Fig. 5). Protective efficacy of antibodies against SagA was shown in a mouse bacteraemia model, with the homologous and one heterologous VRE strain showing a statistically significant reduction in colony counts from blood when mice received immune rabbit serum compared with mice that received pre-immune serum (Fig. 6).

Nosocomial infections due to enterococci are an increasing problem in hospitals and, at least for *Enterococcus faecalis*, several alternative treatment and preventive strategies have been proposed (Koch et al., 2004; Theilacker et al., 2004). Our group has previously shown that enterococcal lipoteichoic acid (Huebner et al., 1999, 2000; Theilacker et al.,

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**Fig. 2.** Western blot with serum raised against heat-killed whole bacteria of *E. faecium* strain E155 (zWB). Lanes: 1, *E. faecium* E155 cell extracts; 2, *E. faecium* E155 culture supernatant.

**Fig. 3.** Western blot with purified recombinant SagA protein. Lanes: 1, molecular mass marker; 2, partially purified SagA; 3, purified SagA. Primary antibodies were serum raised against heat-killed *E. faecium* E155 (zWB).
and a novel capsular polysaccharide (Theilacker et al., 2011) are targets of opsonic antibodies, while Singh et al. (2010) have demonstrated that the Ace protein is also a suitable vaccine candidate and may be used for active or passive immunotherapy against *E. faecalis* infections. An *E. faecium* surface protein Esp has been shown to be involved in biofilm formation (Heikens et al., 2007) and persistence in the bloodstream, although this protein did not elicit opsonic or protective antibodies (Sava et al., 2010).

No appropriate vaccine targets have yet been identified in *E. faecium*, although this pathogen has been implicated in an increasing percentage of infections (Arias & Murray, 2009; Arias et al., 2010). Furthermore, the higher rate of antibiotic resistance in *E. faecium* and the association of specific clones of these bacteria with hospital outbreaks prompted us to search for appropriate targets for immunotherapy regimens. To accomplish this goal, we chose a previously studied *E. faecium* strain belonging to the clonal complex 17, which has been shown to be associated with hospital outbreaks worldwide (Willems et al., 2005).

The target identified through the experiments detailed above was initially described and characterized by Teng et al. (2003) as SagA, a major secreted *E. faecium* antigen cross-reactive with sera from patients with *E. faecium* endocarditis. These authors demonstrated that recombinant SagA bound to a number of extracellular matrix proteins (i.e. fibrinogen, collagen type I and IV, fibronectin and laminin). The SagA protein was shown to contain three domains and was essential for *E. faecium* growth. Analysis of the 70 *enterococcal* sequence data deposited at the IMG website showed that strains from all *enterococcal* species possessed genes with significant homology to *sagA*: three *Enterococcus casseliflavus* (identity 78%), 37 *E. faecalis* (identity between 46 and 100%), 27 *E. faecium* (identity between 88 and 100%), one *Enterococcus gallinarum* (identity 53%) and one *Enterococcus italicus* (identity 44%). Proteins with similar function have been described in other species [e.g. *Streptococcus mutans* (Chia et al., 2001) and *Listeria* (Bubert et al., 1992; Schubert et al., 2000)] as being involved in cell wall metabolism and virulence (Anantharaman & Aravind, 2003; Rigden et al., 2003). The homologous gene in *E. faecalis* is regulated by a two-component signal transduction pathway and has been
shown previously to be involved in stress resistance, biofilm formation and binding to collagen type I and fibronectin (Le Breton et al., 2003; Breton et al., 2002; Mohamed et al., 2006). However, the cell-wall-digesting amidase activity described in other species (Rigden et al., 2003) could not be confirmed in the \textit{E. faecium} protein by Teng et al. (2003). No indication has been presented so far that any of these antigens are targets of opsonic or protective antibodies and may thereby be used as vaccine candidates. Two other prominent protein bands present in the culture supernatant of \textit{E. faecium} E155 were purified, identified and recombiantantly expressed as described above (peptidase W3B, Gi 69247666, and 6pGD, Gi 69247351; data not shown). However, none of these proteins was a target of opsonic antibodies.

Our results confirm the findings of Teng et al. (2003) that \textit{E. faecium} SagA protein is secreted into the culture medium. In addition, our data demonstrate that SagA induces opsonic antibodies in rabbits immunized with recombinant SagA protein. This serum efficiently killed the homologous strain at a serum dilution of 1 : 10 in an OPA, and killing activity was almost completely inhibited by the purified antigen, confirming that the SagA protein was indeed the target of the opsonic antibodies. Two applications of rabbit serum raised against SagA statistically reduced colony counts in the blood of mice against the homologous strain and also an unrelated VRE strain, indicating that this antigen is a promising vaccine target for VRE bacteraemia.

To our knowledge, this is the first report identifying a promising vaccine target against nosocomial \textit{E. faecium} strains. This antigen may therefore be used as a single component or as a conjugate together with the above-mentioned polysaccharide antigens to prevent and combat the spread of multiresistant VRE.

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