Characterization of Two Metal Binding Lipoproteins as Vaccine Candidates for Enterococcal Infections

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Abstract

Background

Enterococcus faecium and faecalis are Gram-positive opportunistic pathogens that have become leading causes of nosocomial infections over the last decades. Especially multidrug resistant enterococci have become a challenging clinical problem worldwide. Therefore, new treatment options are needed and the identification of alternative targets for vaccine development has emerged as a feasible alternative to fight the infections caused by these pathogens.

Results

We extrapolate the transcriptomic data from a mice peritonitis infection model in E. faecalis to identify putative up-regulated surface proteins under infection conditions in E. faecium. After the bioinformatic analyses two metal binding lipoproteins were identified to have a high homology (>72%) between the two species, the manganese ABC transporter substrate-binding lipoprotein (PsaAfm) and the zinc ABC transporter substrate-binding lipoprotein (AdcAfm). These candidate lipoproteins were overexpressed in Escherichia coli and purified. The recombinant proteins were used to produce rabbit polyclonal antibodies that were able to induce specific opsonic antibodies that mediated killing of the homologous strain E. faecium E155 as well as clinical strains E. faecium E1162, Enterococcus faecalis 12030, type 2 and type 5. Mice were passively immunized with the antibodies raised against recombinant lipoproteins, showing significant reduction of colony counts in mice livers after the bacterial challenge and demonstrating the efficacy of these metal binding lipoproteins as promising vaccine candidates to treat infections caused by these enterococcal pathogens.
Conclusion

Overall, our results demonstrate that these two metal binding lipoproteins elicited specific, opsonic and protective antibodies, with an extensive cross-reactivity and serotype-independent coverage among these two important nosocomial pathogens. Pointing these two protein antigens as promising immunogens, that can be used as single components or as carrier proteins together with polysaccharide antigens in vaccine development against enterococcal infections.

Introduction

Enterococci are normal inhabitants of the gastrointestinal tract of animals and humans, but have been reported also as causative agent of infectious diseases in humans [1]. In recent years enterococci have emerged as important nosocomial pathogens due to their multiple antibiotic resistances, ranking *E. faecalis* and *E. faecium* as the third and fourth most commonly isolated species [2–5]. Until 1980s, the majority of enterococcal infections were caused by *E. faecalis*, but since the beginning of 1990s *E. faecium* has become as common as cause of nosocomial infections as *E. faecalis* [6,7]. This shift in enterococcal epidemiology may be due to the high levels of antibiotic resistance that *E. faecium* presents in contrast to *E. faecalis* [6]. Therefore, there is an urgent need to develop alternative therapies and preventive strategies against enterococcal infections [8,9]. Currently, vaccines and immunotherapies are among the most promising alternative approaches to fight these opportunistic pathogens, since they allow specific targeting, not affecting commensal flora, and therefore are associated with a low risk of development of bacterial resistance [10].

In Gram-positive bacteria, lipoproteins are involved in many important cellular processes within the subcellular region of the cell envelope between the plasma membrane and the outer layers of the cell (i.e. peptidoglycan and other layers of the cell wall). Molecules residing in the area represent approximately 2–3% of the bacterial proteome [11,12]. The most abundant functional group of lipoproteins are substrate binding proteins (SBPs) which deliver substrate-binding proteins to ATP-binding cassette (ABC) transporters, accounting for ~40% of the predicted lipoproteins. ABC transporters are classified into at least nine subfamilies according to the substrate transported [12]. Lipoproteins perform diverse functions including nutrient and substrate uptake, folding of excreted proteins, conjugation, antibiotic resistance and transport [11,13]. In Gram-positive bacteria, some lipoproteins have been demonstrated to play crucial roles in host-pathogen interactions such as adhesion, colonization and initiation of inflammatory processes by recruiting immune cells and activating toll-like receptor 2 [11–14]. To date, many lipoproteins from several bacterial pathogens, as well as the proteins and enzymes involved in their biosynthesis, have been studied and proposed as potential vaccine candidates and targets for drug development [13,14]. The rationale behind a lipoprotein-directed vaccine relies in the immunostimulatory activity, specific location and the potential implication in virulence that these protein-antigens possess [11,12].

Few studies have been conducted to determine the role of lipoproteins in enterococcal virulence. Rince and co-workers identified lipoprotein-encoding genes in the genome of the clinical isolate *E. faecalis* V583 and analyzed their putative function. Among the predicted lipoproteins, 43% accounted as components of the ABC transporters and 40% have been already demonstrated either to be involved in *E. faecalis* virulence or to share high homologies with lipoproteins implicated in virulence of other Gram-positive pathogens [11]. The prolipoprotein
diacylglycerol transferase (Lgt) and the \textit{E. faecalis} antigen A (efaA) lipoproteins have been described to be involved in stress response and virulence \cite{14,15}. Until now, the potential use of these proteins as vaccine candidates in enterococci has not been explored. Interestingly, Burnie and co-workers examined antibody responses in sera from patients infected by vancomycin-resistant \textit{E. faecium}, and demonstrated that one phage antibody, directed against amino acid sequences containing ABC transporters, was able to reduce colony counts in a mouse infection model \cite{16}. In the present study, the transcriptomic data obtained from a bacteremia mouse model with \textit{E. faecalis} was used to identify putative cell-wall related lipoproteins with high homologies in the vancomycin-resistant \textit{E. faecium} E155. The putative \textit{in vivo} up regulated and cell-wall related proteins were overexpressed in \textit{E. coli}, purified and immunologically confirmed to be potential protein vaccine candidates against enterococcal infections.

**Materials and Methods**

**Bacterial strains and sera**

The bacterial strains and sera used for the present study are listed in the Table 1 \cite{17–21}. Polyclonal sera against the recombinant proteins were produced as follows: New Zealand white rabbits were immunized with two subcutaneous injections of 10 μg protein given 2 weeks apart; in the third week, three injections of 5 μg were given intravenously every other day. Finally, in the fifth week two injections of 5 μg were given intravenously with three days between doses. Three different sera were obtained from each rabbit: a pre-immune serum seven days prior to the first immunization (Day 0, NRS-protein), a test bleed serum 15 days after the fifth immunization (Day 45, Test-protein) and a terminal bleed serum collected five

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**Table 1. Bacterial strains and sera used for this study.**

| Strain or serum | Description* | Reference or source |
|-----------------|--------------|---------------------|
| **Strains**     |              |                     |
| \textit{E. faecium} E155 | ARE, VRE strain isolated from a patient in the USA (Chicago), CC17 | [17] |
| \textit{E. faecium} E1162 | ARE strain isolated from blood in the Netherlands, CC17 | [18] |
| \textit{E. faecalis} 12030 | isolated from a patient in the USA (Cleveland) | [19] |
| \textit{E. faecalis} type 2 | isolated from a patient in Japan (Sapporo) | [20] |
| \textit{E. faecalis} type 5 | isolated from a patient in Japan (Kobe) | [20] |
| \textit{E. coli} M15/pRep4 | M15 harboring pRep4 plasmid | (INVITROGEN) |
| \textit{E. coli} M15/pQE30AdcA\textsubscript{tm} | M15 harboring pRep4 and pQE30AdcA\textsubscript{tm} plasmids | This study |
| \textit{E. coli} M15/pQE30PsaA\textsubscript{tm} | M15 harboring pRep4 and pQE30PsaA\textsubscript{tm} plasmids | This study |
| **Sera**        |              |                     |
| NRS             | Pre-immune sera from rabbit | This study |
| Anti-SagA       | Rabbit serum raised against the recombinant SagA | [21] |
| NRS-AdcA\textsubscript{tm} | Pre-immune sera from rabbit immunized with AdcA\textsubscript{tm} collected at day 0 | This study |
| Test-AdcA\textsubscript{tm} | Rabbit serum raised against the recombinant AdcA\textsubscript{tm} collected at day 45 | This study |
| Anti-AdcA\textsubscript{tm} | Rabbit serum raised against the recombinant AdcA\textsubscript{tm} collected at day 63 | This study |
| NRS-PsaA\textsubscript{tm} | Pre-immune sera from rabbit immunized with PsaA\textsubscript{tm} collected at day 0 | This study |
| Test-PsaA\textsubscript{tm} | Rabbit serum raised against the recombinant PsaA\textsubscript{tm} collected at day 45 | This study |
| Anti-PsaA\textsubscript{tm} | Rabbit serum raised against the recombinant PsaA\textsubscript{tm} collected at day 63 | This study |

*AdcA\textsubscript{tm}, zinc ABC transporter substrate-binding lipoprotein from \textit{E. faecium}; ARE, ampicillin resistant enterococci; CC17, clonal lineage complex 17; PsaA\textsubscript{tm}, manganese ABC transporter substrate-binding lipoprotein from \textit{E. faecium}; SagA, major secreted antigen; VRE, vancomycin resistant enterococci.
days after the last immunization (Day 63, Anti-protein). All sera were heat inactivated at 56°C for 30 min and frozen at -20°C.

Extrapolation from the transcriptomic data from *E. faecalis* in *E. faecium*

The transcriptomic data were obtained from the experiments of Muller et al. [22], in which the differences in expression of 368 *E. faecalis* proteins was analyzed in a mouse peritonitis model, allowing the identification of a set of 211 up-regulated proteins under infection conditions. Among these up-regulated proteins we analyzed the 18 that corresponded to surface related proteins (e.g. membrane, cell wall associated, extracellular and lipoproteins). The extrapolation of these data in the closely related species *E. faecium* was made by the protein BLAST tool (http://blast.st-va.ncbi.nlm.nih.gov/Blast.cgi) comparing these 18 proteins against the sequences available for *E. faecium* species (taxid:1352).

General molecular methods

PCR was performed with Phusion highfidelity DNA polymerase (Finnzymes) and using as template the genomic DNA of the *E. faecium* E155. The primers used are listed in Table 2. PCR products and plasmids were purified using the NucleoSpin Gel and PCR Clean-up and NucleoSpin plasmid kit (Macherey-Nagel). Restriction enzymes and T4 DNA ligase were purchased from Promega and used as recommended by the manufacturer. Genomic DNA extraction and other standard techniques were carried out as described by Sambrook et al. [23].

Construction of *E. coli* strains M15/pQE30AdcA fm and M15/pQE30PsaA fm

The proteins were recombinantly expressed to raise antibodies against the different antigens. The respective genes (EFF33485 and EFF33471) were amplified without including the sequence corresponding to the signal peptide using primers listed in Table 2. The amplicons where then digested with the corresponding restriction enzymes and inserted downstream of the IPTG (Iso-propyl β-D-1-thiogalactopyranoside)-inducible promoter into the pQE30 expression vector (QIAexpressionist kit; Qiagen) to obtain an N-terminal His6-tagged recombinant protein. The resulting construct was electroporated into the *E. coli* M15pRep4, creating the different M15/pQE30protein strains (see Table 1). Recombinant proteins were overproduced and purified under denaturing conditions using the Protino Ni-NTA Agarose (Macherey-Nagel) resin, following the manufactures instructions. Finally, the purified recombinant proteins were desalted by diafiltration using the Amicon Ultra-15 Centrifugal Filter Units of 3KDa (Merck-Millipore).

Mass-spectrometry analyses

The identity of the recombinant proteins after affinity purification was performed by mass spectrometry. Overnight tryptic digestion of the obtained proteins was done as described

| Primer name         | 5´-3´ sequence + | Restriction site |
|---------------------|------------------|-----------------|
| AdcA fm-5-BamHI     |aggcGGATCCCTGAAATGATAAAGATGGAAAAT|BamH I|
| AdcA fm-3-PstI      |aggcCTGCAAGTTAATGAGCCATATTCTTGA|Pst I|
| PsaA fm-5-BamHI     |aggcGGATCCAAAGATACGTGGCGCTCGAAGCA|BamH I|
| PsaA fm-3-PstI      |aggcCTGCAAGTTTCTCAGAACCTGCGA|Pst I|

+ Bases in lowercase letters are not complementary to the target sequence. Underlined bases correspond to restriction sites.

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Briefly, after SDS-PAGE and Coomassie blue staining, the protein-containing regions (bands) were excised, and washed twice with ultrapure water and once with acetonitrile/50 mM ammonium bicarbonate (1:1, v/v). Samples were stirred for 15 min and vacuum-dried for 30 min. In-gel digestion of the excised protein bands was carried out using 0.5 μg trypsin (Promega), incubating overnight at 37°C. Trypsin-cleaved samples were desalted and concentrated on a tipmicroC18 Omix (Agilent) before nano-liquid chromatography nanoLC-MS/MS analysis. The chromatography step was performed on a nano-LC system (Prominence, Shimadzu). Peptides were concentrated on a Zorbax 5x0.3mm C18 precolumn (Agilent) and separated onto a Zorbax 150x75μm C18 column (Agilent). Mobile phases consisted of 0.1% trifluoroacetic acid, 99.9% water (v/v) (A) and 0.1% trifluoroacetic acid, 20% water in 79.9% ACN (v/v/v) (B). The nanoflow rate was set at 300 nl/min, and the gradient profile was as follows: constant 7% B for 5 min, from 7 to 70% B in 183 min, from 70 to 100% B in 5 min, and return to 7% B. The 300 nl/min volume of the peptide solution was mixed with 1.2 μL/min volumes of solutions of 5mg/ml CHCA matrix prepared in a diluant solution of 50% ACN with 0.1% TFA. Twenty nine second fractions were spotted by an AccuSpot spotter (Shimadzu) on a stainless steel Opti-TOF 384 targets. MS experiments were performed on an AB SCIEX 5800 proteomics analyser equipped with TOF ion optics and OptiBeam on-axis laser irradiation with a 1000 Hz repetition rate. The resulting fragmentation patterns were used to determine the sequences of the peptides. Database searching was performed using the mascot 2.3.02 program (Matrix Science). A database corresponding to an updated compilation download from the NCBI database was used with _E. faecium_ as selected species (including 169,998 entries). The variable modifications allowed were as follows: C-Carbamidomethyl, K-acetylation, methionine oxidation, and dioxidation. Trypsin was selected as the enzyme, with three miscleavages also allowed. Mass accuracy was set to 200 p.p.m. and 0.6 Da for MS and MS/MS modes, respectively.

**Measurement of protein specific IgG titers in polyclonal anti-protein sera**

Total IgG concentration was determined for each polyclonal anti-protein sera (i.e. pre-immune serum, test-serum and anti-serum) with the Easy-Titer Rabbit IgG Assay kit (Thermo Scientific) according to the manufacturer’s instructions and adjusted to 1mg/mL. Serum specific IgG titers against AdcAfm and PsaAfm proteins were measured by ELISA as described previously [24]. In brief, Nunc-immuno Maxisorp MicroWell 96 well plates (Thermo Scientific) were coated with 0.20 μg of recombinant proteins AdcAfm or PsaAfm in 0.2M carbonate-bicarbonate coating buffer. Plates were incubated overnight at 4°C and washed three times with washing buffer (WB) (PBS containing 0.05% Tween 20). The plates were blocked with 3% bovine serum albumin (BSA, Applichem GmbH) in PBS at 37°C for 2 hours and washed as described above. Rabbit sera were prepared in twofold serial dilutions (from 1:5 to 1:31,720) in PBS supplemented with 3% of BSA, incubated 1 hour at 37°C and washed three times with WB. Alkaline-phosphatase-conjugated anti-rabbit IgG (Sigma-Aldrich) diluted 1:1,000 in PBS supplemented with 3% BSA was used as secondary antibody. After incubation for 1 hour at 37°C the wells were washed four times with WB. Finally, p-nitrophenyl phosphate (Sigma-Aldrich) was used as substrate (1mg/mL in 0.1M glycine, 1mM MgCl₂, 1mM ZnCl₂, pH 10.4). After 30min of incubation at room temperature, the absorbance was measured at 405nm on a microtiter plate reader (Multiskan Ascent, Thermo scientific). Each experiment was performed twice at different time-points, and wells were measured in triplicate. Polyclonal anti-protein sera IgG titers were calculated as follows: for each sample, a plot of OD value against the antibody dilution [Log₁₀(antibody dilution)] was used to calculate the intercept with the specified cutoff value of each test, and the extrapolated inverse value was used to generate the end point titer [25].
Opsonophagocytic assay (OPA)

An *in vitro* opsonophagocytic assay (OPA) was performed as described elsewhere [19,21]. Briefly, four components were prepared: (a) baby rabbit serum (Cedarlane Laboratories) absorbed with the target bacterial strain as a source of complement, (b) the different rabbit sera (see Table 1), (c) polymorphonuclear neutrophils (PMNs) freshly prepared from human blood collected from healthy adult volunteers, and (d) the bacterial strains grown to OD$_{650}$ = 0.4 in TSB. Equal volumes of bacterial suspension (2.5x $10^4$ μl$^{-1}$), PMNs (2.5x $10^4$ μl$^{-1}$), complement source (1.7% and 0.85% final concentration for *E. faecalis* and *E. faecium* respectively), and either the serum raised against the recombinant proteins or heat-inactivated pre-immune rabbit serum (NRS, as control) were combined and incubated on a rotor rack at 37°C for 90 minutes. After incubation, colony forming units (CFUs) surviving in the tubes with bacteria were quantified by agar culture of serial dilutions. Percentage of killing was assessed as described by Theilacker et al. [26] by comparing the colony counts at 90 min (t90) of a control not containing PMNs (PMNneg) to the colony counts of a tube that contained all four components of the assay using the following formula:

\[
\frac{\text{[(mean CFU PMNneg at t90) − (mean CFU at t90)]}}{\text{(mean CFU PMNneg at t90)}} \times 100
\]

Opsonophagocytic inhibition assay (OPIA)

For inhibition studies, rabbit serum raised against the recombinant proteins was diluted 1:50 and incubated for 60 min at 4°C with the purified recombinant proteins at a final concentration of 100μg/mL, 10μg/mL and 1μg/mL. BSA was used as control without inhibitory activity at a final concentration of 100μg/mL. Subsequently, the respective antibody was used in the OPA as described above. Inhibition assays were performed at serum dilutions yielding 55–65% killing of the inoculum without the addition of the inhibitor. The percentage of inhibition of opsonophagocytic killing was compared to controls without inhibitor.

Animal model

A mouse bacteremia model was performed to evaluate the passive protection conferred by antibodies raised against the recombinant proteins as describe elsewhere [27,28] with some modifications. In brief, Five female Balb-C mice 6 to 8 weeks-old (Charles River) received intravenously (i.v.) 200 μL of NRS, serum raised against the recombinant proteins or serum raised against recombinant protein SagA as a positive control, 48 and 24h before the challenge. Bacterial inoculum of *E. faecium* E155 (6.3 x 10$^8$ c.f.u. per mouse) was injected via the tail vein. 24 hours after challenge, mice were humanely euthanized by CO$_2$ asphyxiation and colony counts in liver were determined by homogenizing and plating of serial dilutions. Animals were closely monitored for morbidity during the course of the experiment (i.e. at least every 4 hours).

Statistical Analysis

The software program GraphPad PRISM version 5.00 was used for the statistical analyses. The percentage of organisms killed using immune sera in the opsonophagocytic assay was expressed as geometrical mean ± the standard error of the means. Significance of the bacterial counts in the animal experiment was determined by analysis of variance for multi-group comparisons using log-transformed data, and a Kruskal-Wallis test. A P value of < 0.05 was considered significant.
Ethics Statement

All animal experiments were performed in compliance with the German animal protection law (TierSchG). The mice were housed and handled in accordance with good animal practice as defined by FELASA and the national animal welfare body GV-SOLAS. The animal welfare committees of the University of Freiburg (Regierungspräsidium Freiburg Az 35/9185.81/G-11/118) approved all animal experiments.

Results

Identification and selection of *E. faecium* proteins candidates for immunological studies by extrapolation of the transcriptomic data in *E. faecalis*

To identify putative up-regulated surface proteins under infection conditions with *E. faecium*, the transcriptomic data from the mice peritonitis infection model in the closely related species *E. faecalis* was used. Among the 368 proteins studied by the transcriptomic approach in *E. faecalis*, 18 resulted to be up-regulated and surface related (e.g. extracellular, lipoproteins, membrane and cell wall associated). We considered that these identified proteins are potential vaccine candidates against infections produced by this species. However, in order to determine which of the identified proteins could be good candidates and be cross-reactive against infections caused by either *E. faecalis* or *E. faecium*, these proteins were blasted against the *E. faecium* sequences available in the NCBI database (taxid:1352). The blast analysis showed that eight of the proteins have significant homologies (Identity > 50% and Query Coverage > 80%) in both species (see Table 3). Among the eight proteins, two showed the highest homology and have been previously described as vaccine candidate in other Gram-positive pathogens. Therefore our studies focused on these two putative surface associated proteins: the 35.6 kDa protein manganese ABC transporter substrate-binding lipoprotein PsaAfm and the 57.4 kDa zinc ABC transporter substrate-binding lipoprotein AdcAfm.

The target proteins induce opsonic antibodies

The genes encoding the two genes EFF33471 and EFF33485, corresponding to the candidate proteins, were amplified by high fidelity PCR without including the sequence corresponding to their signal peptides. These genes were cloned into the pQE30 expression vector and transformed into *E. coli* M15. The recombinant proteins were then purified under denaturing conditions. The identity of the proteins was confirmed by LC-MS/MS and their purity was assessed by SDS-PAGE (data not shown). For the production of polyclonal antibodies, New Zealand white rabbits were immunized with purified proteins and exsanguinated two weeks after the last injection. To confirm that antibodies were generated after immunization against the recombinant proteins, specific IgG titers were measured for each serum at different time points (day 0, 45 and 63) of the immunization procedure. The IgG titers between the terminal bleeding and the pre-immune sera increased 4,000x and 2,000x for the AdcAfm and PsaAfm, respectively (see Fig 1).

The obtained serum raised against the two different proteins was tested in an opsonophagocytic assay (OPA) against the homologous strain *E. faecium* E155 showing that all the proteins were able to induce opsonic antibodies (see Fig 2). To confirm that the opsonic killing observed was mediated by the polyclonal antibodies in the OPA, different concentrations of sera were used to titer out their opsonic activity. The maximum opsonic killing activities of the antibodies at 1:10 dilution were 63% and 55% for the anti-AdcAfm and the anti-PsaAfm sera, respectively.
A reduction of killing was observed in a dose dependent fashion using increasingly higher dilutions of sera (see Fig 2).

The opsonic antibodies are specifically directed against the corresponding recombinant protein

In order to verify that the antibodies are directed against the corresponding recombinant protein, opsonophagocytic inhibition assays (OPIA) were carried out by pre-incubating the sera with the corresponding recombinant protein in three different concentrations 100, 10 and 1 μg/mL for 1 hour at 4°C. The resulting mixture (anti-protein sera / recombinant protein) was used in an OPA against the E. faecium E155 strain. A reduction of more than 70% of the opsonic killing was observed in the presence of the highest concentration of recombinant protein tested whereas the control BSA did not show any inhibitory activity at the same concentration. Also, inhibition of opsonic killing decreased in a dose-dependent fashion for both anti-protein sera (see Fig 3).

Table 3. Surface related proteins in E. faecalis up-regulated in the mice peritonitis model and corresponding BLAST homologies in E. faecium sequences.

| Accession N° | Name | Mean fold induction[22] | Accession N° | Name | Query cover | Identities | E value |
|--------------|------|-------------------------|--------------|------|-------------|------------|--------|
| EF0095       | Lipoprotein§ | 2.7                     | EFF33494     | Esp  | 36%         | 46%        | 5E-04  |
| EF0163       | Lipoprotein§ | 6.7                     | WP_002334751 | Hypothetical protein | 96% | 52% | 2E-52 |
| EF0164       | Lipoprotein§ | 6.7                     | WP_002390048 | Hypothetical protein | 58% | 22% | 9E-11 |
| EF0176       | ABC sugar transport sistem | 3.4 | EFF34523 | Basic membrane lipoprotein | 100% | 55% | 4E-135 |
| EF0177       | ABC sugar transport sistem | 3.4 | EFF34523 | Basic membrane lipoprotein | 100% | 60% | 4E-143 |
| EF0361       | Chitinase§ | 46.3                     | WP_002291042 | Chitinase | 98% | 55% | 1E-130 |
| EF0362       | Chitin binding protein | 46.3 | EFF34249 | Extracellular protein | 86% | 72% | 9E-89 |
| EF0577       | Lipoprotein | 22.1                     | EFF33471     | PsaAfm | 100% | 99% | 0 |
| EF1345       | Sugar ABC transporter | 15.4 | EFF35767.1 | Maltodextrin-binding protein | 92% | 26% | 1E-27 |
| EF1546       | LysM domain protein | 3.7 | EFF34140 | LysM domain protein | 100% | 39% | 3E-23 |
| EF1677       | Lipoprotein | 9.9                     | WP_002327802 | ABC transporter sugar-binding protein | 21% | 42% | 7E-04 |
| EF1817       | Serine protease | 7.7 | WP_002372955 | Extracellular metalloprotease family protein | 90% | 31% | 3E-32 |
| EF1818       | Gelatinase | 7.7 | EFF35763.1 | Phospholipid synthase | 2% | 53% | 4.1 |
| EF2713       | Cell wall surface anchor family | 8.5 | EFF34453 | Collagen adhesin | 22% | 37% | 1E-03 |
| EF3193       | LrgB family protein | 4.1 | EFF33766 | LrgB family protein | 96% | 36% | 5E-38 |
| EF3194       | LrgA family protein | 4.1 | EFF33767 | LrgA family protein | 76% | 21% | 2E-06 |
| EF3206       | Adhesion lipoprotein | 3.1 | EFF33485 | AdcAfm | 100% | 72% | 0 |
| EF3256       | Pheromone cAD1 Lipoprotein | 8.8 | ELB00929.1 | FMN-binding protein | 100% | 70% | 1E-142 |

* Standard Protein BLAST against E. faecium sequences in the NCBI (http://blast.st-va.ncbi.nlm.nih.gov/Blast.cgi)

§Putative.

PsaAfm, manganese ABC transporter substrate-binding lipoprotein; AdcAfm, zinc ABC transporter substrate-binding lipoprotein.

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Specific and opsonic antibodies are cross-reactive against different *E. faecium* and *E. faecalis* isolates

To determine if the antibodies directed against the recombinant proteins were able to opsonize other enterococcal strains, serum dilutions between 1:10 and 1:75 were tested in OPAs against...
The two anti-protein sera were able to opsonize effectively all strains, exhibiting killing above 50% (see Fig 4). A lower opsonophagocytic killing activity was observed for the homologous strain \( E. faecium \) E155 as well as for \( E. faecalis \) strains type 2 and type 5 in comparison with strains \( E. faecalis \) 12030 and \( E. faecium \) E1162.
Antibodies directed against the different recombinant proteins promote clearance of bacteria in mice livers

Mice were passively immunized twice within 48h before bacterial infection to determine if antibodies raised against the recombinant proteins confer protection to mice against bacteremia. Sera raised against the two recombinant proteins significantly reduced \textit{E. faecium} E155 colony counts in the livers. The protection conferred by these anti-protein sera seem to be better than the protection conferred by the antibodies raised against the previously reported protein SagA [21]. Immunization with sera raised against protein AdcAfm resulted in lower viable counts (\(P \leq 0.01\)) compared to serum raised against PsaAfm (\(P \leq 0.05\)) and SagA (\(P > 0.05\)) (see Fig 5).

Discussion

Most Gram-positive pathogens possess factors such as anti-phagocytic polysaccharide capsules, surface-associated proteins or toxins that have been studied as potential targets for vaccine development [30]. Most approaches for the discovery of these novel vaccine targets have been done through dissection of the pathogen using biochemical, immunological and microbiological methods [31]. Nevertheless, the arrival of the genome era has dramatically improved the identification of novel vaccine candidates by large-scale high-throughput genomic, transcriptomic, and proteomic analyses [32]. In this work, the identification of some protein vaccine candidates in enterococci has been accomplished by serological proteome analysis (also known as immunomics), i.e. the secreted antigen (SagA), two protein epitopes present on ABC-transporters in \textit{E. faecium}, and the Collagen Adhesin in \textit{E. faecalis} [21,33,34]. We have recently reported the identification of four additional peptidoglycan associated protein as

![Fig 5. Passive Immunization with the antibodies raised against the recombinant proteins promotes clearance of \textit{E. faecium} in mouse liver in comparison with normal rabbit serum. 24 hours after the bacterial challenge mice were sacrificed and livers were removed to assess viable counts. Each point represents the bacterial counts from a single mouse. Bars indicate the median CFU/100 mg of kidney for the group. P value was \(< 0.05\) (\(*P \leq 0.05\), \(**P \leq 0.01\)) for comparison between the animals immunized with the antibodies raised against the recombinant proteins and control animals immunized with NRS determined by analysis of variance for multi-group comparisons using on log-transformed data, and Kruskal-Wallis test.](https://doi.org/10.1371/journal.pone.0136625.g005)
vaccine candidates (i.e. a low affinity penicillin-binding protein 5, a peptidoglycan-binding protein LysM, a D-alanyl-D-alanine carboxypeptidase and the peptidyl-prolyl cis-trans isomerase) by proteomic analysis [35]. Transcriptomic analyses have been useful tools for the identification of protein antigens involved in pathogenesis allowing the discovery of novel mechanisms of pathogenicity, function of certain proteins and novel therapeutic targets [32,36]. However, to our knowledge, this is the first transcriptomic approach used for the identification and study of putative surface-related proteins as novel vaccine candidates against enterococcal infections.

The two candidate proteins evaluated in this study belong to the family of metal binding lipoproteins (MBL) that act as substrate binding proteins in the ABC transport systems. Some of these MBL have been evaluated as potential vaccine candidates and/or important virulence factors in other Gram-positive pathogens [15,37–44]. The pneumococcal surface antigen A (PsaA) from *S. pneumoniae*, which shares 78% of sequence homology with the PsaAfm evaluated in this study, has been widely described as MBL and as an adhesion protein that plays an important role in pneumococcal attachment to the host cell and virulence [40]. PsaA is immunogenic and has been very well studied as a vaccine component against pneumococcal infections [40]. On the other hand, the lipoprotein AdcAfm evaluated in this study shares 64% of sequence homology with the lipoprotein AdcA described in *S. pneumoniae*. This protein has been demonstrated to play an essential role as zinc transporter, which is required for proper cell division and for *S. pneumoniae* survival during infection, although its potential as vaccine candidate has not been explored yet [39].

The anti-AdcAfm and anti-PsaAfm sera effectively mediated *in vitro* opsonic killing in the parental strain *E. faecium* E155 as well as other clinically relevant enterococcal strains, i.e. *E. faecium* E1162 and *E. faecalis* type 2 and type 5 strains, exhibiting killing in a range from 50 to 98 percent. The broad cross-reactivity of the sera showed by antibodies raised against these MBL may effectively overcome the serotype-dependent coverage of polysaccharide-based vaccines.

Surprisingly, we observed lower bacterial killing in OPAs with the homologous strain in comparison with *E. faecium* E1162 and a much more pronounced effect with *E. faecalis* strain 12030, where the serum concentrations used were seven times lower. As previously explained for other cell wall associated protein vaccine candidates, these differences in opsonophagocytic killing activity may be attributed to hindrance of the target protein by different cell surface determinants, variability in gene expression, protein degradation, and other factors [35,45,46]. Moreover, the presence of capsular polysaccharides in the prototype CPS-C and CPS-D *E. faecalis* strains (Type 2 and Type 5) may mask the protein target, explaining the differences observed in the opsonophagocytic activity between these strains.

Further analysis of the sera showed that antibodies specifically recognized the proteins AdcAfm and PsaAfm, when opsonophagocytic activity elicited by the anti-protein sera was inhibited by pre-absorption with the target protein. Consistent with opsonophagocytic results, which usually correlate well with *in vivo* immune response and indicates bacteria’s ability to survive in human blood and cause infection [47], prophylactic treatment of mice with the antibodies against AdcAfm and PsaAfm significantly reduced the CFU numbers in the livers. These results demonstrate that passive transfer of the sera confers significant protection against bacteremia in mice after i.v. challenge and suggests that active immunization with these antigens may be feasible. While the levels of protection of mice by passive immunization were statistically significant, there was only about 1 log difference between colony counts in immunized mice versus controls. Therefore, additional experiments using different time-points as well as alternative infection models (e.g. rat endocarditis model) need to be done to strengthen the results. Under the experimental conditions explored in the present study, the two MBL
proteins efficiently induced antibodies in rabbits and protected mice against enterococcal infections.

**Conclusion**

In summary, we have demonstrated here that i) using transcriptomic data obtained from an *in vivo* model was a successful approach for the identification of novel surface-related proteins that serve as targets for vaccine development (ii) targeting proteins with high homology between closely-related species, i.e. *E. faecalis* and *E. faecium*, is a good strategy for the identification of novel protein vaccine candidates with a broad coverage among the Enterococcus genus (iii) the proteins PsaAfm and AdcAfm were able to induce specific, opsonic and protective antibodies with a broad cross-reactivity and serotype-independent coverage among the two clinically most important enterococcal species. However, cross-reactive protection studies among other Gram-positive bacteria should be considered as potentially useful for the identification of broadly active vaccine antigens since proteins PsaAfm and AdcAfm shared high homologies with MBL in streptococci, staphylococci and bacillus.

**Author Contributions**

Conceived and designed the experiments: FR-S AB AH JH. Performed the experiments: FR-S DL AB-V BB. Analyzed the data: FR-S DL CM AB AH JH. Wrote the paper: FR-S DL AH JH.

**References**

1. Aarestrup FM, Hasman H, Jensen LB, Moreno M, Herrero IA, Dominguez L, et al. (2002) Antimicrobial Resistance among Enterococci from Pigs in Three European Countries. Appl Environ Microbiol 68: 4127–4129. doi: 10.1128/AEM.68.8.4127–4129.2002 PMID: 12147518

2. Murray BE (2000) Vancomycin-resistant enterococcal infections. N Engl J Med 342: 710–721. doi: 10.1056/NEJM200003093421007 PMID: 10706902

3. Zarb P, Coignard B, Griskeviciene J, Muller A, Vankerckhoven V, Weist K, et al. (2012) The European Centre for Disease Prevention and Control (ECDC) pilot point prevalence survey of healthcare-associated infections and antimicrobial use. Euro Surveill 17.

4. Sievert DM, Ricks P, Edwards JR, Schneider A, Patel J, Srivinasa A, et al. (2013) Antimicrobial-resistant pathogens associated with healthcare-associated infections: summary of data reported to the National Healthcare Safety Network at the Centers for Disease Control and Prevention, 2009–2010. Infect Control Hosp Epidemiol 34: 1–14. doi: 10.1086/668770 PMID: 23221186

5. Werner G, Coque TM, Hammarum AM, Hope R, Hryniewicz W, Johnson A, et al. (2008) Emergence and spread of vancomycin resistance among enterococci in Europe. Euro Surveill 13.

6. Willems R, Top J, Schaik W Van, Leavis H (2012) Restricted gene flow among hospital subpopulations of Enterococcus faecium. MBio 3: e00151–e00112. doi: 10.1128/mBio.00151-12.Editor PMID: 22807567

7. Arias CA, Murray BE (2012) The rise of the Enterococcus: beyond vancomycin resistance. Nat Rev Microbiol 10: 266–278. doi: 10.1038/nrmicro2761 PMID: 22421879

8. Fisher K, Phillips C (2009) The ecology, epidemiology and virulence of Enterococcus. Microbiology 155: 1749–1757. doi: 10.1099/mic.0.026385–0 PMID: 19383684

9. Sava IG, Heikens E, Huebner J (2010) Pathogenesis and immunity in enterococcal infections. Clin Microbiol Infect 16: 533–540. doi: 10.1111/j.1469-0691.2010.03213.x PMID: 20569284

10. Fernebro J (2011) Fighting bacterial infections-future treatment options. Drug Resist Updat 14: 125–139. doi: 10.1016/j.drup.2011.02.001 PMID: 21367651

11. Reuffeuille F, Leneveu C, Sylvie C, Auffray Y, Rincé A, Chevalier S, et al. (2011) Lipoproteins of Enterococcus faecalis: bioinformatic identification, expression analysis and relation to virulence. Microbiology 157: 3001–3013. doi: 10.1099/mic.0.053314–0 PMID: 21903750

12. Hutchings MI, Palmer T, Harrington DJ, Sutcliffe IC (2009) Lipoprotein biogenesis in Gram-positive bacteria: knowing when to hold ‘em, knowing when to fold ‘em. Trends Microbiol 17: 13–21. doi: 10.1016/j.tim.2008.10.001 PMID: 19059780
Kovacs-Simon A, Titball RW, Michell SL (2011) Lipoproteins of bacterial pathogens. Infect Immun 79: 548–561. doi: 10.1128/IAI.00682-10 PMID: 20974828

Reffuveille F, Serror P, Chevalier S, Budin-Verneuil A, Ladjouzi R, Bernay B, et al. (2012) The prolipoprotein diacylglyceryl transferase (Lgt) of Enterococcus faecalis contributes to virulence. Microbiology 158: 816–825. doi: 10.1099/mic.0.055319-0 PMID: 22135097

Singh KV, Coque TM, Weinstock GM, Murray BE (1998) In vivo testing of an Enterococcus faecalis efaA mutant and use of efaA homologs for species identification. FEMS Immunol Med Microbiol 21: 323–331. doi: 10.1111/j.1574-695X.1998.tb01180.x PMID: 9753005

Burnie J, Carter T, Rigg G, Hodgetts S, Donohoe M, Matthews R (2002) Identification of ABC transporters in vancomycin-resistant Enterococcus faecium as potential targets for antibody therapy. FEMS Immunol Med Microbiol 33: 179–189. doi: 10.1111/j.1574-695X.2002.tb00589.x PMID: 12110480

Leavis HL, Willems RJL, van Wamel WJB, Schuren FH, Caspers MPM, Bonten M (2007) Insertion sequence-driven diversification creates a globally dispersed emerging multiresistant subspecies of E. faecium. PLoS Pathog 3: e7. doi: 10.1371/journal.ppat.0030007 PMID: 17257059

Van den Bogaard AE (1997) Antimicrobial resistance—relation to human and animal exposure to antibiotics. J Antimicrob Chemother 40: 453–454. PMID: 9338504

Huebner J, Wang Y, Krueger WA, Madoff LC, Martirosian G, Boisot S, et al. (1999) Isolation and chemical characterization of a capsular polysaccharide antigen shared by clinical isolates of Enterococcus faecalis and vancomycin-resistant Enterococcus faecium. Infect Immun 67: 1213–1219. PMID: 10024563

Maekawa S, Yoshioka M, Kumamoto Y (1992) Proposal of a New Scheme for the Serological Typing of Enterococcus faecalis Strains. Microbiol Immunol 36: 671–681. doi: 10.1111/j.1348-0421.1992.tb02070.x PMID: 1406370

Kropec A, Sava IG, Vonend C, Sakinc T, Grohmann E, Huebner J (2011) Identification of SagA as a novel vaccine target for the prevention of Enterococcus faecium infections. Microbiology 157: 3429–3434. doi: 10.1099/mic.0.02207-0 PMID: 21903755

Mulier C, Cacciari M, Sauvageot N, Sanguinetti M, Rattei T, Eder T, et al. (2015) The Intraperitoneal Transcriptome of the Opportunistic Pathogen Enterococcus faecalis in Mice. PLoS One 10: e0126143. doi: 10.1371/journal.pone.0126143 PMID: 25978463

Sambrook J, Russell DW (2001) Molecular Cloning: A Laboratory Manual, Volume 1. 3rd ed. Cold Spring Harbor, NY: CSHL Press. 2344 p.

Laverde D, Wobser D, Romero-Saavedra F, Hogendorf W, van der Marel G, Berthold M, et al. (2014) Synthetic Teichoic Acid Conjugate Vaccine against Nosocomial Gram-Positive Bacteria. PLoS One 9: e110953. doi: 10.1371/journal.pone.0110953 PMID: 25333799

Chen Q, Cannons JL, Paton JC, Akiba H, Schwartzberg PL, Snapper CM (2008) A novel ICOS-independent, but CD28- and SAP-dependent, pathway of T cell-dependent, polysaccharide-specific humoral immunity in response to intact Streptococcus pneumoniae versus pneumococcal conjugate vaccine. J Immunol 181: 8258–8266. doi: 10.4049/jimmunol.181.12.8258 PMID: 19050242

Theilacker C, Sanchez-Carballo P, Toma I, Fabretti F, Sava I, Kropec A, et al. (2009) Glycolipids are involved in biofilm accumulation and prolonged bacteraemia in Enterococcus faecalis. Mol Microbiol 71: 1055–1069. doi: 10.1111/j.1365-2958.2009.06587.x PMID: 19170884

Huebner J, Quaas A, Krueger WA, Goldmann DA, Pier GB (2000) Prophylactic and therapeutic efficacy of antibodies to a capsular polysaccharide shared among vancomycin-sensitive and-resistant enterococci. Infect Immun 68: 4631–4636. doi: 10.1128/IAI.68.8.4631–4636.2000 PMID: 10899866

Theilacker C, Kaczynski Z, Kropec A, Sava I, Ye L, Bychowska A, et al. (2011) Serodiversity of opsonic antibodies against Enterococcus faecalis—glycans of the cell wall revisited. PLoS One 6: e17839. doi: 10.1371/journal.pone.0110953 PMID: 21437253

Hufnagel M, Hancock LE LE, Koch S, Theilacker C, Gilmore MS, Huebner J (2004) Serological and genetic diversity of capsular polysaccharides in Enterococcus faecalis. J Clin Microbiol 42: 2548–2557. doi: 10.1128/JCM.42.6.2548–2557.2004 PMID: 15184433

Koch S, Hufnagel M, Theilacker C, Huebner J (2004) Enterococcal infections: host response, therapeutic, and prophylactic possibilities. Vaccine 22: 822–830. doi: 10.1016/j.vaccine.2003.11.027 PMID: 15040934

Rappuoli R (2001) Reverse vaccinology, a genome-based approach to vaccine development. Vaccine 19: 2688–2691. doi: 10.1016/S0264-410X(00)00554-5 PMID: 11257410

Rinaudo CD, Telford JL, Rappuoli R, Seib KL (2009) Vaccinology in the genome era. J Clin Invest 119: 2515–2525. doi: 10.1172/JCI38330 PMID: 19729849
33. Burnie J, Carter T, Rigg G, Hodgetts S, Donohoe M, Matthews R (2002) Identification of ABC transporters in vancomycin-resistant Enterococcus faecium as potential targets for antibody therapy. FEMS Immunol Med Microbiol 33: 179–189. doi: 10.1111/j.1574-695X.2002.tb00589.x PMID: 12110480

34. Singh K V, Nallapareddy SR, Sillanpää J, Murray BE (2010) Importance of the collagen adhesin ace in pathogenesis and protection against Enterococcus faecalis experimental endocarditis. PLoS Pathog 6: e1000716. doi: 10.1371/journal.ppat.1000716 PMID: 20072611

35. Romero-Saavedra F, Laverde D, Webser D, Michaux C, Budin-Verneuil A, Bernay B, et al. (2014) Identification of peptidoglycan-associated proteins as vaccine candidates for enterococcal infections. PLoS One 9: e111880. doi: 10.1371/journal.pone.0111880 PMID: 25369230

36. Betts JC (n.d.) Transcriptomics and proteomics: tools for the identification of novel drug targets and vaccine candidates for tuberculosis. IUBMB Life 53: 239–242. doi: 10.1080/15216540212651 PMID: 12121002

37. Whaley MJ, Sampson JS, Johnson SE, Rajam G, Stinson-Parks A, Holder P, et al. (2010) Concomitant administration of recombinant PsaA and PCV7 reduces Streptococcus pneumoniae serotype 19A colonization in a murine model. Vaccine 28: 3071–3075. doi: 10.1016/j.vaccine.2010.02.086 PMID: 20206671

38. Brown JS, Ogunniyi AD, Woodrow MC, Holden DW, Paton JC (2001) Immunization with components of two iron uptake ABC transporters protects mice against systemic Streptococcus pneumoniae infection. Infect Immun 69: 6702–6706. doi: 10.1128/IAI.69.11.6702–6706.2001 PMID: 11598041

39. Bayle L, Chimalapati S, Schoehn G, Brown J, Vernet T, Dumort C (2011) Zinc uptake by Streptococcus pneumoniae depends on both AdcA and AdcAll and is essential for normal bacterial morphology and virulence. Mol Microbiol 82: 904–916. doi: 10.1111/j.1365-2958.2011.07862.x PMID: 22023106

40. Rajam G, Anderton JM, Carlone GM, Sampson JS, Ades EW (2008) Pneumococcal surface adhesin A (PsaA): a review. Crit Rev Microbiol 34: 131–142. doi: 10.1080/10408410802275352 PMID: 18728990

41. Garmory HS, Titball RW (2004) ATP-binding cassette transporters are targets for the development of antibacterial vaccines and therapies. Infect Immun 72: 6757–6763. doi: 10.1128/IAI.72.12.6757–6763.2004 PMID: 15557595

42. Sampson JS, Furlow Z, Whitney AM, Williams D, Facklam R, Carlone GM (1997) Limited diversity of Streptococcus pneumoniae psaA among pneumococcal vaccine serotypes. Infect Immun 65: 1967–1971. PMID: 9125591

43. Talkington DF, Brown BG, Thrpe JA, Koenig A, Russell H (1996) Protection of mice against fatal pneumococcal challenge by immunization with pneumococcal surface adhesin A (PsaA). Microb Pathog 21: 17–22. doi: 10.1006/mpat.1996.0038 PMID: 8827703

44. Lowe AM, Lambert PA, Smith AW (1995) Cloning of an Enterococcus faecalis endocarditis antigen: homology with adhesins from some oral streptococci. Infect Immun 63: 703–706. PMID: 7822045

45. Maione D, Margarit I, Rinaudo CD, Masignani V, Mora M, Scarseli M, et al. (2005) Identification of a universal Group B streptococcus vaccine by multiple genome screen. Science 309: 148–150. doi: 10.1126/science.1109869 PMID: 15994562

46. Rodríguez-Ortega MJ, Norais N, Bensi G, Liberati S, Capo S, Mora M, et al. (2006) Characterization and identification of vaccine candidate proteins through analysis of the group A Streptococcus surface proteome. Nat Biotechnol 24: 191–197. doi: 10.1038/nbt1179 PMID: 16415855

47. Hufnagel M, Koch S, Kropec A, Huebner J (2003) Opsonophagocytic assay as a potentially useful tool for assessing safety of enterococcal preparations. Int J Food Microbiol 88: 263–267. PMID: 14596999