Biofilm matrix exoproteins induce a protective immune response against *Staphylococcus aureus* biofilm infection

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Running title*:* *S. aureus* biofilm matrix exoproteins vaccine

ABSTRACT

The *Staphylococcus aureus* biofilm mode of growth is associated with several chronic infections that are very difficult to treat due to the recalcitrant nature of biofilms to clearance by antimicrobials. Accordingly, there is an increasing interest in preventing the formation of *S. aureus* biofilms and developing efficient anti-biofilm vaccines. Given the fact that during a biofilm-associated infection, the first primary interface between the host and the bacteria is the self-produced extracellular matrix, in this study, we have analysed the potential of extracellular proteins found in the biofilm matrix to induce a protective immune response against *S. aureus* infections. By using proteomic approaches we characterized the exoproteomes of exopolysaccharide-based and protein-based biofilm matrices produced by two clinical *S. aureus* strains. Remarkably, results showed that independently of the nature of the biofilm matrix, a common core of secreted proteins is contained in both types of exoproteomes. Intradermal administration of an exoproteome extract of an exopolysaccharide-dependent biofilm induced a humoral immune response and elicited the production of IL-10 and IL-17 in mice. Antibodies against such extract promoted opsonophagocytosis and killing of *S. aureus*. Immunization with the biofilm matrix exoproteome significantly reduced the number of bacterial cells inside a biofilm and on the surrounding tissue, using an *in vivo* model of mesh-associated biofilm infection. Furthermore, immunized mice also showed limited organ colonization by bacteria released from the matrix at the dispersive stage of the biofilm cycle. Altogether, these data illustrate the potential of biofilm matrix exoproteins as a promising candidate multivalent vaccine against *S. aureus* biofilm-associated infections.

INTRODUCTION

Staphylococcus aureus is one of the bacterial species most frequently associated with biofilm-mediated infections. It can be found as a commensal bacterium on the skin, nares and mucosa but in some situations, it can become the source of biofilm-related infections where bacteria grow into multicellular communities attached to a surface and embedded in a self produced extracellular matrix. *S. aureus* biofilms can occur on host tissues such as heart valves (endocarditis) and bone tissue (osteomyelitis) although they are more frequently related with medical devices (catheters, prostheses, portacaths). Implanted medical devices are easily coated with plasma and extracellular matrix proteins such as fibrinogen and fibronectin (1). *S. aureus* has the ability to bind to these components via specific receptors and thus, implants become colonized. After primary attachment to the polymeric surface, bacteria proliferate and accumulate in multilayered clusters surrounded by an extracellular matrix. The added level of bacterial resistance inside a biofilm makes these infections difficult to treat and, as a consequence, in most situations, the device must be surgically removed and replaced (2). Bacteria from the biofilm can also propagate through detachment of small or large clumps of cells, or by the release of individual cells allowing bacteria to colonize other surfaces or tissues far from the original infection site. Bloodstream infections originating from device-associated infections account for 11% of all health care–associated infections. An estimation of 250,000 catheter-related bloodstream infections occur in the United States per year, resulting in significant morbidity, mortality, and costs for health care delivery (3-5). *S. aureus* is frequently associated with such infections, and therefore a great effort is being made in order to prevent and/or obtain effective treatments against this bacterium. Given the fact that bacteria living in a biofilm express a different set of genes than the same free-living bacteria (6-10), the

process of antigen selection for the development of an efficient protection against *S. aureus* infections should also take into consideration those antigens expressed during the biofilm growth.

In this respect, a wide variety of extracellular compounds have been identified as mediators of staphylococcal biofilms such as poly-N-acetyl-glucosamine exopolysaccharide, PNAG (also named PIA), (11-16), extracellular DNA (eDNA) (17, 18), and different surface-associated proteins including the biofilm-associated protein (Bap), fibronectin-binding proteins (FnBPs), SasG and Protein A (19-23). Some of these biofilm mediators have been already proposed as vaccine antigens against *S. aureus* infections. Different studies have shown that administration of deacetylated PNAG conjugated with diphtheria toxin as a carrier protein induces an immunological response that protects against *S. aureus* infection (14, 24-26). Furthermore, a recent study of Cywes-Benttey *et al.* has shown that PNAG or a structural variant of PNAG is a conserved surface polysaccharide produced by many pathogenic bacteria, fungi and protozoal parasites and has demonstrated that passive immunization with antibodies to PNAG protects mice against both local and systemic infections caused by many of these pathogens (27). Protein A and FnBPs have also been evaluated for vaccine development. These antigens generate an immune response that confers partial protection against *S. aureus* challenge using systemic infection models (28-30). However, no evidence has been obtained of the efficiency of these molecules for the protection against biofilm-based infections.

In the last few years, several studies have demonstrated that biofilms harbor multiple cell types, resulting in heterogeneous populations that have followed different developmental pathways (31-33). In this regard, Brady et al. identified immunogenic cell-wall proteins expressed during a *S. aureus* biofilm infection and demonstrated

differing expression patterns for each antigen (34, 35). These authors reasoned that immunization with a monovalent vaccine would likely mean that only a fraction of the biofilm would be targeted and thus, the infection would persist (36, 37). Therefore, they used a quadrivalent vaccine, including four of the identified antigens (glucosaminidase, an ABC transporter lipoprotein, a conserved hypothetical protein, and a conserved lipoprotein), combined with antibiotic therapy and demonstrated a reduced *S. aureus* biofilm formation on infected tibias, using a chronic osteomyelitis model (37).

Taking into consideration that the biofilm matrix is the first primary interface between the host and bacteria during a biofilm-associated infection and the relevance of using a multivalent vaccine for the prevention of biofilm-type infections, in this study we aimed at investigating whether an extract containing all proteins secreted into the biofilm matrix might be a potential polyvalent vaccine candidate that protects against *S. aureus* biofilm related infections. Thus, we have first isolated and identified the exoproteins of both PNAG-dependent and independent biofilm matrices produced by a methicillin sensitive and also a methicillin resistant clinical strain. Notably, exoproteomes were uniform in that they contained a common set of proteins. Immunization with a biofilm matrix exoproteins extract effectively reduced biofilm formation in an *in vivo* model of mesh-associated biofilm infection, which significantly correlated with the production of immunoglobulins (IgG and IgM) antibodies with opsonic activity. Our results also suggested a role for IL-10 and IL-17 cytokines in biofilm matrix exoproteins-mediated protection. Finally we showed that administration of this multicomponent protein extract reduces organ colonization conducted by bacteria released via detachment from the biofilm.

Materials and methods

Ethics statement.

All animal studies were reviewed and approved by the ‘‘Comité de Ética, Experimentación Animal y Bioseguridad’’ of the Universidad Pública de Navarra (approved protocol PI-019/12). Work was carried out at the Instituto de Agrobiotecnología building (Idab) under the principles and guidelines described in the ‘‘European Directive 86/609/EEC’’ for the protection of animals used for experimental purposes.

Bacterial strains and culture conditions.

Staphylococci were cultured on tryptic soy agar or broth at 37°C supplemented with glucose (0.25%) or NaCl (3%) when indicated. Strains used in this study were included in table 1. *S. aureus* 15981, 132 and 12313 were isolated at the Microbiology Department of the Clínica Universidad de Navarra (Pamplona, Spain) (23, 38). *S. aureus* V329 is a Bap positive strain isolated from a bovine mastitis (19). *S. aureus* Newman::*bap* is a Newman derivative strain containing a chromosomal copy of the *bap* gene (39). ISP479r is a derivative of ISP479 with a functional *rsbU* gene. As a biofilm negative strain we used *S. aureus* Newman strain (ATCC 25905).

Biofilm formation and protein extracts purification.

Biofilm formation under flow conditions was performed using 60-ml microfermenters (Pasteur Institute, Laboratory of Fermentation) with a continuous 40 ml h^{-1} flow of medium and constant aeration with sterile compressed air (0.3 bar) (40). Submerged glass slides (spatulas) served as growth substratum. Approximately 10^8 bacteria from an overnight culture of each strain grown in the appropriate medium (*S. aureus* 15981

was grown in TSB-gluc and *S. aureus* 132 was grown either in TSB-gluc or TSB-NaCl) were used to inoculate the microfermenters that were then kept at 37°C for 24 h. The biofilm formed on the spatula was resuspended in 20 ml of PBS (phosphate-buffered saline) and vigorously homogenized by vortexing. The suspension was centrifuged at 4800 g for 30 min at 4ºC. Then, the supernatant was collected, centrifuged again at 4800 g for 30 min at 4ºC and filtered through a 0.45 µm filter (SARSTEDT). Matrix proteins were extracted with trichloroacetic acid 10%. After precipitation, proteins were dissolved in PBS for quantification with the Bicinchoninic Acid Protein Assay kit (SIGMA). The planktonic culture exoproteins extract (PLKE) was obtained as follows. An overnight culture of *S. aureus* 15981 was diluted 1:100 in an Erlenmeyer flask containing 50 ml of TSB-gluc medium and was incubated overnight at 37°C with shaking. The culture was centrifuged at 4800 g. Supernatant was collected and filtered through a 0.45 µm filter (SARSTEDT). Secreted proteins into the supernatant were precipitated by the addition of trichloroacetic acid 10%. Proteins extracts were dissolved in PBS for quantification with the Bicinchoninic Acid Protein Assay kit (SIGMA). Proteins were resolved using SDS-polyacrylamide gel electrophoresis and stained with Bio-Rad Silver Stain according to the manufacturer ´s recommendations. To obtain the bacterial heat extract, a *S. aureus* 15981 cell suspension containing 10^8 CFU was heat inactivated at 80°C for 1 h (41).

Protein identification.

The extracellular protein extract was subjected to tryptic digestion and analyzed as previously described (22). Briefly, the tryptic peptide mixtures were injected onto a strong cationic exchange microprecolumn with a flow rate of 30 µl/min as a first-dimension separation. Peptides were eluted from the column as fractions by injecting

salt of ammonium acetate of increasing concentrations. Ammonium salts were removed and peptides were analyzed in a continuous acetonitrile gradient on a C18 reversed-phase self-packing nanocolumn. Peptides were eluted (at flow rate of 300 nl/min) from the reversed-phase nanocolumn to a PicoTip emitter nano-spray needle (New Objective, Woburn, MA) for real-time ionization and peptide fragmentation on an Esquire HCT ion trap mass spectrometer (Bruker-Daltonics, Bremen, Germany). Every 1 s, the instrument cycled through acquisition of a full-scan mass spectrum and one MS/MS spectrum. A 4-Da window (precursor m/z ±2), an MS/MS fragmentation amplitude of 0.80 V, and a dynamic exclusion time of 0.30 min were used for peptide fragmentation. 2DnLC was automatically performed on an advanced microcolumn-switching device (Switchos; LC Packings) coupled to an auto-sampler (Famos; LC Packings) and a nano-gradient generator (Ultimate nano- HPLC; LC Packings). The software Hystar 2.3 was used to control the whole analytical process. MS/MS spectra were batch processed by using DataAnalysis 5.1 SR1 and MS BioTools 2.0 software packages and searched against the *S. aureus* protein databases using Mascot software (Matrix Science, London, United Kingdom). The criteria for confirming highly confident protein identification was set at obtaining a MASCOT total protein score ≥ 50 and at least one peptide e-value of ≤ 0.05.

RNA extraction.

For planktonic growth conditions, an overnight culture of *S. aureus* 15981 was diluted 1:100 in an Erlenmeyer flask containing 50 ml of TSBgluc medium and was incubated to OD_{600}=0.8 at 37°C with shaking. For biofilm growth conditions, microfermentors where inoculated as described above and incubated at 37ºC for 6 h. Biofilm-grown and planktonically grown cells were harvested. Total RNA from bacterial pellets was

196 extracted by using a TRIzol reagent method (42). Briefly, bacterial pellets were
197 resuspended into 400 µl of solution A (glucose 10%, Tris 12.5 mM, pH 7.6, EDTA 10
198 mM), mixed to 60 µl of 0.5M EDTA and transferred into Lysing Matrix B tubes
199 containing 500 µl of acid phenol (Ambion). Cells were mechanically lysed by using the
200 Fastprep apparatus (BIO101) at speed 6.0 during 45 s at 4 °C. After centrifugation the
201 aqueous phase was transferred to 2-ml tubes containing 1 ml of TRIzol and 100 µl of
202 chloroform. Tubes were centrifuged and the aqueous phase was transferred into a 2-ml
203 tube containing 200 µl of chloroform, mixed, and incubated for 5 min at room
204 temperature. Tubes were centrifuged and the aqueous phase containing the RNA was
205 precipitated by addition of 500 µl of isopropanol and incubation for 15 min at room
206 temperature. RNA concentrations were quantified, and RNA qualities were determined
207 by using Agilent RNA Nano LabChips (Agilent Technologies). RNAs were stored at
208 −80 °C until needed.
209
210 **cDNA labeling and DNA microarray hybridization.**
211 Ten µg RNAs were reverse transcribed using SuperScript II reverse transcriptase
212 (Invitrogen Life Technologies). cDNA was digested by DNase I (PIERCE) in 10X
213 DNAse I buffer (USB-Affymetrix) and the size of digestion products was analyzed in
214 the Agilent Bioanalyser 2100 using RNA Nano LabChips to ensure that the
215 fragmentation resulted in a majority of products in the range of 50 to 200 base-pairs.
216 The fragmented cDNA were then biotinylated using terminal deoxynucleotidyl
217 transferase (Promega) and the GeneChip DNA labeling reagent (Affymetrix) following
218 the manufacturer's recommendations. Biotinylated cDNA (5 microgram per array) were
219 hybridized to custom *S. aureus* tiling microarrays designed as described (43)
220 (ArrayExpress accession: A-AFFY-165) and incubated for 16 h according to the

Affymetrix protocol in a total volume of 200 µl per hybridization chamber. Following incubation, the arrays were washed and stained in the Fluidics station 450 (Affymetrix) using the protocol n°FS450_0005. Scanning of the arrays was then performed using the GeneChip scanner 3000 (Affymetrix). Intensity signals of each probe cells were computed by the GeneChip operating software (GCOS) and stored in cell intensity files (.CEL extension) before preprocessing and analysis. Microarray data were analyzed using limma package (44). Raw data are available under ArrayExpress accession: E-MEXP-3924.

Immunization studies.

CD1 mice were obtained from Charles River and maintained in the animal facility of the Instituto de Agrobiotecnología, Universidad Pública de Navarra. The biofilm matrix exoprotein extract used for immunization consisted of the exoproteins purified from the biofilm matrix produced by *S. aureus* 15981 strain and was referred as BME. Five-week-old female CD1 mice were injected intradermally with 10 µg of BME diluted in adjuvant (Sigma Adjuvant System®). The control group was treated with PBS and adjuvant. Two weeks later, the vaccinated group received a booster dose of 5 µg of BME, while the control group received PBS and adjuvant. Mice were bled via the retroorbital venous plexus on day 0 (pre-immune serum) and 21 days after the first vaccination (immune serum). Both serum samples were analyzed by ELISA and Western Blot for determination of antibody responses against the BME.

Detection of antibodies in the sera.

Serum IgG and IgM expression against BME were quantified by coating 96-well ELISA plates (Nunc Maxisorp, Millipore) with 100 µl/well of a 0.1 µg ml^{-1} BME in

carbonate buffer (0.5 M; pH 9.4). Plates were incubated at 4ºC overnight. After incubation, wells were then washed three times with PBS containing 0.1% Tween-20 (PBS-T; pH 7.4) and blocked with blocking buffer (5% nonfat dried milk powder in PBS-T) at room temperature for 1h. After washing three times with PBS-T, 100 µl of pre-immune (negative-control) and immune serum diluted 1:100 in PBS were added to each well and incubated at 37ºC for 2 h. After incubation, wells were washed three times with PBS-T and 100 µl of HRP-conjugated goat anti-mouse IgG and IgM (Thermo Scientific) were added to each well. The plates were incubated for 1 h at 37°C and then washed three times. One hundred µl of ABTS solution (diammonium 2,2'-azinobis[3-ethyl-2,3-dihydrobenzothiazole-6-sulphonate; Millipore) were added to each well and the absorbance at 405nm was determined on an ELISA reader. Results were reported as the OD_{405} of immune serum/OD_{405} of the control serum (T/C).

Immune response was also determined by Western blot. For that, 5 µg of the BME or a planktonic culture exoproteins extract were resolved using SDS-polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane and incubated with blocking buffer. Then, the membrane was exposed to pre-immune (negative-control) and immune serum at 4ºC overnight. After washing five times with washing buffer (PBS-T 0.1%), the membrane was incubated with goat anti-mouse IgG and IgM (H+L) secondary antibody HRP conjugate and proteins were detected using SuperSignal® WestPico Chemiluminescent Substrate (Thermo Scientific).

Opsonophagocytic assays.

Opsonophagocytosis and killing assay has been previously described in (45). Briefly, 1 ml of a planktonic culture of strain *S. aureus* 132 grown overnight was pelleted for 5 min at 12,000 g at 4°C, washed twice with PBS, and subsequently diluted to an OD_{600}

of 0.5. Bacteria were pre-incubated with 1% or 10% of immune serum, pre-immune serum or PBS for 1 h at 4ºC. The opsonophagocytosis assay was performed with fresh blood obtained from human healthy volunteers. Fresh whole blood from three volunteers was collected and mixed in tubes containing the anticoagulant heparin and then aliquoted into 1.5-ml microcentrifuge tubes (0.5 ml/tube). After pre-incubation, 10 µl of bacterial suspensions were added to the 1.5-ml microcentrifuge tubes containing 0.5 ml of fresh blood. Tubes were incubated at room temperature with gentle rocking and, after 30 min, samples were serially diluted and plated onto TSA plates to determine the number of surviving CFU. On the other hand, to analyze the opsonophagocytosis and killing of bacteria that are being part of a biofilm, 0.5x0.5cm polypropylene meshes (Prolene®) were incubated with an 1:100 overnight dilution of a culture of the biofilm forming strain *S. aureus* 132 for 2 hours at 37ºC with shaking. Meshes were then washed with PBS and pre-incubated with 1% or 10% of immune serum, pre-immune serum or PBS for 1 h at 4ºC. After pre-incubation, meshes containing bacteria inside a biofilm were added to the 1.5-ml microcentrifuge tubes containing 0.5 ml of fresh blood. Tubes were incubated at room temperature with gentle rocking and, after 30 min, meshes were removed and gently washed and then placed in 1 ml of PBS and vigorously vortexed. Samples were serially diluted and plated onto TSA plates for enumeration of viable staphylococci. Four independent samples of each treatment were performed. The percent amount of bacterial killing was calculated as [1 - (no. of cfu recovered from the treated samples / no. of cfu recovered from the PBS control samples)] *100.

Cytokines production of splenocytes.

295 Groups of 5 CD1 mice were immunized as established before in the "Immunization
296 Studies" section. One week after the second immunization, mice were sacrificed and
297 their spleens were collected under aseptic conditions. Cells suspensions were prepared
298 by resuspending the spleens in RPMI 1640 medium supplemented with 10 % heat-
299 inactivated fetal bovine serum and 1 % Penicillin/Streptomycin and subsequent
300 trituration and filtration through a 70 μm nylon mesh. Red blood cells were lysed using
301 ACK lysing buffer. Splenocytes were counted and dispensed into 24-well plates at a
302 concentration of 2×10^5 cells/well. The cells were restimulated with either 1 μg of
303 BME or with PBS during 24, 48 and 96h. The supernatants were harvested and
304 analyzed for interleukins IL-10, IL-2, IL17A and gamma interferon production using
305 their respective ELISA kit (eBioscience) according to the manufacturer´s instructions.
306
307 **Vaccination/challenge protocol using an *in vivo* model of mesh-associated biofilm**
308 **infection.**
309 The vaccination protocol was performed as described in the "Immunization Studies"
310 section using BME, PLKE or 10^8 heat-killed bacteria emulsified in adjuvant for
311 immunization. Groups of 6 CD1 mice were used. One week after the second
312 immunization, a model of mesh-associated biofilm infection was performed as
313 previously described (46) with the following modifications. Prior to surgical procedure,
314 0.5x0.5cm polypropylene meshes (Prolene®) were incubated with 0.5 ml of a 1:100
315 overnight dilution of a culture of the biofilm forming strain *S. aureus* 132 for 1 hour
316 and 15 minutes at 37ºC with shaking. To calculate the initial inoculum, duplicate
317 meshes were placed in 1 ml of PBS and vigorously vortexed. Samples were serially
318 diluted and plated onto TSA plates for enumeration of viable staphylococci. Control
319 and vaccinated CD1 mice were anesthetized by intraperitoneal injection of a

ketamine/xylazine mixture. After abdominal epilation and antisepsis of the operative field, the animals were operated. An incision of 1.5 cm in the skin was performed with displacement of the subcutaneous space and opening the peritoneal cavity. Then, a mesh, coated with 10^4 CFU of *S. aureus* strain 132, was fixed at the abdominal wall with one anchor point. Finally, the peritoneal cavity was closed by suture with 6/0 Monosyn®. The animals were put in a warm environment and when awake, they were put back in their cages. After 5 days, all animals were sacrificed. Mesh and surrounding tissue were extracted and placed in 1 ml of PBS and vigorously vortexed. Samples were serially diluted and plated onto TSA plates for enumeration of viable staphylococci.

To analyze the additional protection against bacterial population that propagates through detachment from the biofilm, kidneys and liver from the operated animals or from animals challenged by an intravenous injection of a bacterial suspension containing 10^7 CFU of *S. aureus* Newman, were extracted after 5 days. Viable counts were performed on the homogenates by plating the samples on TSA.

RESULTS

Identification of the *S. aureus* biofilm matrix exoproteome.

In order to isolate and identify the exoproteins present within the biofilm matrix, the biofilm formed by the clinical strain *S. aureus* 15981 grown in TSB-gluc was isolated (38). This strain forms a PNAG dependent biofilm when grown under the conditions tested. Exoproteins present within the PNAG-mediated biofilm matrix of 3 independent samples were purified as described in the materials and methods section. Proteins from these extracts were precipitated and then separated by 1-D SDS-PAGE followed by trypsin digestion and identified by 2DnLC-MS/MS. Only proteins identified in at least two of the three samples were considered for further analysis. Thus, a total of 33 extracellular proteins were detected with a MASCOT score higher than 50 (Table 2). Importantly, the proteins identified have been recurrently detected in extracellular proteomes of various *S. aureus* isolates (34, 47-54). More notably, 28 out of the 33 proteins identified in our analysis have also been found in the biofilm exoproteome of *S. aureus* D30 strain, isolated from a persistent nasal carrier (Table 2) (50). These data reliably support the validity of the method used to identify exoproteins of the biofilm matrix.

Specifically, exoproteome analyses revealed the presence in the extracellular biofilm matrix of many proteins involved in pathogenesis such as toxins (leukocidin, EsaA and truncated beta-hemolysin) or immunomodulatory proteins (lipoprotein, immunodominant antigen B, immunodominant antigen A, Protein A, IgG-binding protein, secretory antigen precursor SsaA and SceD). The biofilm matrix also contained a markedly large number of proteins involved in carbohydrate metabolism, namely phosphoglycerate mutase, triosephosphate isomerase, enolase, glyceraldehyde-3-phosphate dehydrogenase, glucose-6-phosphate isomerase, alcohol dehydrogenase, L-

lactate dehydrogenase and fructose bisphosphate aldolase. Finally, albeit to a lesser extent, enzymes involved in cell-wall peptidoglycan synthesis (autolysin and N-acetylmuramoyl-L-alanine amidase), DNA metabolism and stress proteins (foldase protein, DNA binding protein II, nuclease and superoxide dismutase) were also encompassed in the biofilm exoproteome.

With the aim of extending the biofilm matrix exoproteome analysis to other *S. aureus* strains we used strain 132, which is an MRSA clinical isolate able to alternate between a PNAG-independent biofilm matrix mediated by the Fibronectin Binding Proteins (FnBPs) and an exopolysaccharidic PNAG-mediated biofilm depending of whether it is cultured under TSB-gluc or TSB-NaCl growing conditions, respectively (23). *S. aureus* 132 was incubated in microfermentors under these two different conditions that allowed the formation of the two biofilm matrices and subsequently, these were isolated for matrix exoproteins identification. Analysis of the PNAG-mediated biofilm matrix revealed the presence of 24 proteins, 17 of which (71%) had been previously identified in the *S. aureus* 15981 strain exoproteome. On the other hand, analysis of the FnBPs-mediated biofilm matrix led to the identification of 19 proteins, being nearly half of them also present in the exoproteome of the PNAG-mediated matrix, and the other half in the *S. aureus* 15981 strain exoproteome. When we considered the biofilm matrices formed by *S. aureus* 132 under these two experimental conditions as a unit, results showed that almost 80% of the matrix exoproteins were included into the biofilm matrix exoproteome of the *S. aureus* 15981 strain.

In conclusion, we identified the PNAG-dependent and FnBPs-dependent biofilm matrix exoproteomes of a methicillin resistant *S. aureus* isolate and also the PNAG-dependent exoproteome of a methicillin sensitive clinical strain. The results indicated that independently of the nature of the biofilm matrix, a common core of secreted proteins

386 is contained in both types of exoproteomes. The biofilm matrix exoprotein extract used
387 for the rest of the study consisted of the 33 exoproteins identified in the biofilm matrix
388 produced by *S. aureus* 15981 strain and was referred as BME.
389
390 **Transcriptional analysis of genes coding for biofilm matrix exoproteins.**
391 Previous studies with several bacteria have shown that gene expression and protein
392 production differ when bacteria are grown under biofilm conditions in comparison with
393 planktonic growth (6-9). Therefore, we proceeded to investigate whether genes coding
394 for the biofilm matrix exoproteins identified with the proteomic analysis were
395 differentially expressed in biofilm conditions with respect to planktonic growth.
396 Transcriptome analyses revealed that *S. aureus* 15981 cells grown under biofilm
397 conditions expressed a markedly different repertoire of genes in comparison to their
398 planktonic counterparts. In total, we observed that 626 genes were differentially
399 expressed under biofilm growing conditions. From these, 276 genes were expressed in
400 higher amounts in biofilm cells, whilst 350 genes were down-regulated under biofilm
401 conditions. Then, we focused on expression levels of the genes coding for the BME
402 previously identified and found that expression of more than half of the identified
403 proteins (58%) was up-regulated under biofilm growing conditions (Table 2).
404 Importantly, genes encoding for 39% of matrix exoproteins were not differentially
405 expressed under biofilm conditions, indicating that the *S. aureus* biofilm matrix
406 encompasses not only proteins that are specific of the biofilm mode of growth, but also
407 a set of proteins that *S. aureus* expresses at the same level during planktonic growth.
408
409 **Biofilm extracellular proteins induce a humoral immune response in mice.**

In order to investigate whether this multivalent extract might be able to induce a protective immune response against *S. aureus*, we firstly evaluated the antibody response in mice immunized with BME. For that, groups of 8 mice were immunized with BME. Blood and sera samples were obtained at day 0 and 21 post immunization and serum IgG and IgM levels were determined by ELISA. Results showed that immunoglobulin levels were significantly higher in sera from mice immunized with BME than in sera from control mice (Figure 1A).

Next, BME were separated in a SDS-PAGE gel (Fig. 1B) and interrogated with a pool of sera obtained either from immunized or control mice. Results showed that the majority of the biofilm matrix exoproteins were recognized by sera from immunized mice while only a slight cross-reaction, probably caused by the presence of Protein A, was observed when sera pool from control mice was used (Figure 1B). Also, because BME contains a group of proteins that are equally expressed under biofilm or planktonic growth conditions, we tested immune and control sera against an extract containing extracellular proteins secreted by *S. aureus* cells grown planktonically (PLKE). As expected, immune serum recognized part of the proteins present in the planktonic extract (Figure 1C).

Finally, with the aim of analyzing if antibodies raised against the BME extract recognized the biofilm formed by different *S. aureus* strains we isolated biofilm matrix exoproteins from biofilms formed by several *S. aureus* strains and these were interrogated with immune and control sera. In particular, we tested *S. aureus* 132 strain (PNAG and FnBPs mediated biofilms), V329 and Newman::Bap strains (Bap dependent biofilms) and ISP479 and 12313 strains (PNAG mediated biofilms). As it is shown in figure 1D, immune sera against the BME extract recognized many proteins present in all extracts analyzed.

435 Taken together, these data showed that BME was able to induce a humoral immune
436 response and that many of the proteins present in the extract contributed to this
437 immunogenicity. Also, antibodies generated against BME were capable of targeting a
438 broad range of biofilm matrices, suggesting that this multivalent extract might be
439 effective against a large number of relevant biofilm producing strains.
440
441 **Antibodies against BME induce opsonophagocytic killing of *S. aureus*.**
442 The presence of IgG and IgM in the immune serum can be correlated with high opsonic
443 activity (55). Thus, our next objective was to evaluate whether hyperimmune serum
444 obtained against BME promoted opsonophagocytic killing of *S. aureus*. *S. aureus* 132
445 strain grown under planktonic or biofilm conditions was pre-incubated with preimmune
446 serum, 1% or 10% of BME specific sera or PBS as control. After incubation, bacteria
447 were mixed with whole blood for 30 min (45). Staphylococcal killing was monitored
448 by spreading sample aliquots on TSA agar medium followed by colony formation and
449 enumeration. Results showed that antibodies against BME significantly induced
450 opsonophagocytic killing of both planktonic and sessile *S. aureus* cells (Figure 2).
451 Additionally, data showed that killing of biofilm *S. aureus* cells was slightly higher
452 than killing of planktonic cells.
453
454 **BME induces the production of IL-10 and IL-17 in *ex-vivo* stimulated splenocytes.**
455 We next sought to characterize the cellular response stimulated by BME. For that,
456 cytokines production was examined after *ex-vivo* splenocyte stimulation with BME as
457 described in the materials and methods section. Supernatants of stimulated cells were
458 analyzed for the production of IFN-γ and IL-2 (prototypes Th1 cytokines), IL-10
459 (prototype Th2 cytokines) and Th17-associated cytokine IL-17. When production of

460 IL-17 was analyzed over time, a 10 fold increase was observed at the early time of 24
461 hours post-stimulation, when supernatants of splenocytes coming from mice
462 immunized with the BME extract where compared to supernatants of control mice
463 splenocytes. This difference increased to 50 fold at 96 hours post-stimulation (Figure
464 3). It is important to note that levels of IL-17 over time were barely detectable in
465 supernatants of control mice splenocytes (Figure 3). With respect to cytokine IL-10, an
466 approximately 2.5 fold increase was observed at 24 hours post-stimulation that was
467 maintained over time ($P<0.05$) (Figure 3). Lastly, mice immunization with BME led to
468 neither stimulation of cytokine IL-2 production nor induction of IFN-γ (Figure 3).
469 Taken together, these results showed that immunization with BME induced a cellular
470 response characterized by production of cytokines IL-17 and IL-10.
471
472 **Immunization with BME reduced biofilm formation in a mesh-biofilm model.**
473 We next hypothesized whether immunization with BME might reduce the number of
474 bacterial cells inside a biofilm formed *in-vivo*. To analyze this hypothesis, we
475 compared the efficiency of BME in a mesh-biofilm model with the protective effect of
476 an extract containing the secreted proteins of *S. aureus* 15981 grown planktonically
477 (PLKE) and also, of a heat extract obtained from *S. aureus* 15981 (HE). Mice were
478 immunized at an interval of two weeks with 10 μg and 5 μg of the BME, PLKE, 10^8
479 heat-killed bacteria (HE) or with adjuvant alone. After immunization, sera from
480 immunized mice were extracted and were interrogated against the BME proteins.
481 Results showed that sera from mice immunized with PLKE and HE recognized fewer
482 proteins of the BME extract than sera from BME immunized mice (Figure 4B).
483 Seven days after the second immunization, polypropylene meshes coated with 10^4 CFU
484 of the biofilm forming strain *S. aureus* 132 were implanted in the intraperitoneal cavity

485 of immunized and control mice. After five days, all animals were sacrificed and meshes
486 were extracted. When the abdominal cavity of mice was opened, abdominal wall
487 adhesions were observed in all animals. Meshes removed from non-vaccinated mice
488 (control) were more difficult to extract from the abdominal cavity than meshes from
489 vaccinated mice. Also, as it is shown in figure 4C, meshes from control mice were
490 surrounded by purulent and necrotic tissue, whilst a healthier and a more vascularized
491 tissue surrounded the meshes coming from immunized mice. When the number of
492 bacteria on meshes was determined, results showed that immunization with BME
493 significantly reduced the number of bacteria attached to the polypropylene meshes
494 ($P \leq 0.05$) (Figure 4A). In contrast, immunization with PLKE or HE showed a slight but
495 not statistically significant reduction of the number of bacteria in the mesh-biofilm
496 model (Figure 4A).
497 Finally, we decided to investigate whether BME vaccinated mice were additionally
498 protected against bacterial population that propagates via detachment from the biofilm.
499 To do so, mesh-surrounding tissue, kidneys and liver from BME immunized mice were
500 extracted and bacterial colonization was determined. In contrast to the non-vaccinated
501 group (control), mice immunized with BME presented a significantly reduced number
502 of bacteria in liver and mesh-surrounding tissue ($P \leq 0.05$) (Figure 4D). Although there
503 was also a slight reduction in kidney colonization in immunized mice, differences
504 between control and vaccinated mice were not statistically significant ($P=0.06$) (Figure
505 4D).
506 Reduction in organ colonization in immunized mice might be the consequence of not
507 only reduction of biofilm formation capacity inside the animal and thus, a reduction in
508 the number of released bacteria from the biofilm, but also the efficacy of the immune
509 response against organ colonization by released bacteria. In order to analyze this

possibility, we tested whether vaccination with the exoproteins extract might protect against a systemic infection and subsequent organ colonization caused by *S. aureus*. For this, mice were immunized as above and were challenged with a retroorbital injection containing 10^7 cfu/mice of *S. aureus*. Five days after the infection, animals were killed and kidneys and livers were removed. No bacteria were found in the liver of either vaccinated or control mice. Contrary, visual examination of kidneys from non-vaccinated mice showed the presence of abscesses all around the surface of the organs. Much fewer abscesses were detected on kidneys from immunized mice (Figure 5). Enumeration of *S. aureus* cells from the organs showed that kidneys of immunized mice were significantly less colonized than kidneys of control mice ($P<0.01$) (Figure 5).

From all these results we inferred that immunization with BME significantly reduced biofilm formation in an *in vivo* model of mesh-associated biofilm infection and also moderated organ colonization conducted by bacteria that were released via detachment from the biofilm.

DISCUSSION

In the last years, *S. aureus* has emerged as one of the most critical nosocomial pathogens. Success of *S. aureus* as a pathogen is the result of different abilities such as the capacity to invade a wide variety of cell types, to secrete a diversity of proteins and toxins and to persist in the host remaining resistant to clearance by the immune system or antibiotics through a biofilm mode of growth. Numerous approaches have been adopted in order to identify staphylococcal surface and cell wall associated proteins as antigenic candidates for a vaccine against *S. aureus* infections (34, 49, 51, 53, 56-60). However, few works have been focused on the selection of antigens that could also protect against biofilm-associated bacteria (14, 24-26). This is particularly important because *S. aureus* biofilms play a major role in persistent infections formed on the surface of implanted medical devices and in deep tissues. In this study we have demonstrated that a multicomponent extract containing biofilm matrix exoproteins is able to elicit a protective immune response against *S. aureus* biofilm-mediated infections.

According to Harro et al (36), the selection of appropriate antigens effective in preventing the establishment of a biofilm related infection should meet the following criteria: (i) they must be expressed *in vivo* throughout the infection cycle in a large number of genetically unrelated strains; (ii) they must target the entire microbial population of the biofilm; and (iii) they must also induce a protective immune response against planktonic bacteria.

Numerous evidence have demonstrated that *S. aureus* is able to produce polysaccharidic and proteinaceous biofilm matrices (11-16). Therefore potential antigens against *S. aureus* biofilm infections should be expressed by strains that form either type of biofilm matrix. Our results showed that BME extracted from

551 exopolysaccharidic matrices of two unrelated clinical strains (*S. aureus* 15981 and 132)
552 comprised a high number of proteins in common. Moreover, all proteins except one
553 present in the BME isolated from a proteinaceous matrix produced by *S. aureus* 132
554 were also contained in PNAG-dependent matrices (Table 2). Also, it is important to
555 note that 85% of exoproteins encompassed in the BME of *S. aureus* 15981 are identical
556 to the first *S. aureus* biofilm exoproteome identified and produced by the nasal carrier
557 strain *S. aureus* D30 (50). Accordingly, here we showed that antibodies raised against
558 an extract coming from a PNAG-dependent biofilm formed by strain 15981 recognized
559 many proteins from biofilms of different nature produced by different *S. aureus* strains
560 (Figure 1D). These data might explain why immunization with a BME extract obtained
561 from strain 15981 was effective to protect against a challenge with the clinical relevant
562 MRSA strain *S. aureus* 132 (Figure 4) and with *S. aureus* Newman strain (Figure 5).
563 Because individual cells within biofilms can display different protein expression
564 patterns depending on nutrient availability, respiratory conditions or environmental
565 stresses, Harro et al (36) proposed that vaccines that only aim at one specific antigen
566 would likely eliminate the section of the biofilm in which the antigen is expressed,
567 whereas, other biofilm areas that do not express the vaccinated antigen will probably
568 persist. Hence, BME extract comprising most exoproteins of the biofilm matrix may
569 ensure that not only different areas of the biofilm but also various cell types present
570 within the biofilm are targeted. It is important to note that vaccination with other
571 multicomponent extracts such as a heat-killed or a PLKE extract, which have been
572 shown to provide protection against *S. aureus* infections (41, 61, 62), were less efficient
573 than BME to reduce the number of bacteria inside a biofilm, using a mesh associated
574 biofilm infection model. The reason behind the low efficiency of heat-killed and PLKE
575 extracts might be that they probably do not enclose biofilm specific antigens as the

BME extract (Figure 4). Additional experimentation will be required to arrive at a detailed picture of the localization of BME proteins into the biofilm structure.

Biofilm formation is a dynamic process that occurs through sequential steps in which the initial attachment of planktonic bacteria to a surface is followed by their subsequent proliferation and accumulation in multilayer cell clusters where bacteria are enclosed in a self-produced polymeric matrix. As biofilm ages, bacterial cells escape from the matrix and return to a planktonic existence, being able to reach other locations in the host. This step represents a potentially important mechanism for the dissemination of bacteria during infection. Our proteomic, transcriptomic and immunological analysis showed that BME extract contains antigens that *S. aureus* produces under both planktonic and biofilm growing conditions (Table 2). As a consequence, sera from BME immunized mice recognized several proteins in the exoproteome extract of planktonic bacteria (Fig. 1C). Accordingly, mice immunized with this extract not only showed a reduction in the number of bacteria inside a *S. aureus* biofilm but also moderated tissue and organ colonization by bacteria that were released through detachment from the biofilm. Nevertheless, clearance of the infection would likely require an added antimicrobial treatment as it has been already proposed by Brady et al (37).

With respect to the immune response mounted after mice immunization with BME, results showed an increase in the production of total immunoglobulins. The primary antibodies function in the protection against *S. aureus* infections is neutralization and opsonization of bacteria for phagocytosis. Although reduction in the number of biofilm bacteria on PS-meshes in the opsonophagocyitc experiment could be due to both neutralization and the opsonic activity of antibodies, we did not observe a significant direct effect of BME-antibodies on *S. aureus* biofilms *in vitro*, in the absence of

601 immune system components (Supplementary Figure 1). Hence, BME-antibodies seem
602 to protect against *S. aureus* infections likely through an increase in opsonization.
603 Importantly, these opsonic antibodies may help in the phagocytosis of bacteria inside a
604 biofilm that otherwise would be inaccessible due to the extracellular matrix coating.
605 Although antibodies unquestionably play an important role in the protection against *S.*
606 *aureus* infections, they may not be decisive for vaccine protective efficacy since
607 animals and humans have enough circulating antibodies to *S. aureus* (56, 63, 64).
608 Certain indications show a partial role of these antibodies in protecting humans against
609 staphylococcal infections (65). However, patients with defects in humoral immunity are
610 not particularly prone to *S. aureus* infections (66). In this respect, a cellular response
611 mediated by interleukin IL-17 is being considered critical for immunity against this
612 pathogen. It has been shown that vaccination with heat killed *S. aureus* provides
613 protection in systemic infection via staphylococcal lipoproteins that stimulate Th17/IL-
614 17 (67). Also, IL-17 induction has been shown to be determinant in the clearance of
615 IsdB-immunized mice (68). In biofilm-related infections, IL-17 cytokine production
616 increases during the development of the infection, indicating that infected mice mount a
617 robust Th17 response (69, 70). Bacteria in biofilm are embedded in an extracellular
618 matrix and are largely protected from phagocytosis by neutrophils and macrophages.
619 The release of inflammatory cytokines by Th17 cells provokes the recruitment and
620 activation of neutrophils and might aid to devitalize the biofilm surface helping to
621 bacterial clearance. In the case of BME extract immunization, it did not only induce a
622 humoral response but also stimulated the production of IL-17 that might help to clear
623 bacteria in the biofilm. In order to elucidate the role of the induction of IL-17 by BME
624 administration in the efficiency of this multicomponent extract, we have performed a
625 preliminary experiment in which IL-17 cytokine was neutralized by administration of

626 an antibody against IL-17. BME-immunized mice that had been administered the
627 neutralizing antibody to IL-17 showed non-significant reduction in the number of
628 bacteria recovered from biofilm-infected meshes when compared with control BME-
629 immunized mice (Supplementary Figure 2). These preliminary results suggest a
630 putative role of IL-17 cytokine in the immune response against a *S. aureus* biofilm
631 related infection. BME immunized mice presented also significantly higher levels of
632 IL-10 compared to non-vaccinated mice. IL-10 cytokine has been shown to protect the
633 host from staphylococcal enterotoxin, endotoxin and septic shock (71-73).
634 Furthermore, administration of an anti-IL-10 monoclonal antibody to mice inhibits the
635 clearance of *S. aureus,* suggesting that IL-10 might play a beneficial role in host
636 resistance to *S. aureus* systemic infections (74, 75). Further studies are needed to
637 explore the role of IL-10 induction by BME administration in the clearance of *S. aureus*
638 biofilm-related infections.
639 In summary, the work presented here shows that an extract containing biofilm matrix
640 exoproteins induces a protective immune response against a *S. aureus* biofilm related
641 infection and thus reduces colonization and persistence. This is likely because this
642 multicomponent vaccine ties together cell-mediate immunity and a humoral response
643 where opsonic antibodies play a supportive role to eradicate the biofilm infection. In
644 future work, it would be interesting to determine the contribution of each antigen
645 present in the BME extract to its immunogenicity in order to define a particular antigen
646 combination that provides efficient protection against *S. aureus* biofilm infections.
647

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926 **Table 1. Bacterial strains**
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928

Strains	Relevant characteristic(s)	Reference or source
S. aureus 15981	MSSA clinical strain. Biofilm positive; PNAG-dependent biofilm matrix	(38)
S. aureus 132	MRSA clinical strain. Biofilm positive, able to alternate between a protein-dependent biofilm matrix (grown in TSB-gluc) and a PNAG-dependent biofilm matrix (grown in TSB-NaCl)	(23)
S. aureus ISP479c	MSSA clinical strain. Biofilm positive; PNAG-dependent biofilm matrix	(76)
S. aureus 12313	MSSA clinical strain. Biofilm positive; PNAG-dependent biofilm matrix	(23)
S. aureus V329	Bovine subclinical mastitis isolate. Biofilm positive; protein-dependent biofilm matrix	(19)
S. aureus Newman	Strain used in systemic infection models	(77)

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931 **Table 2. Biofilm matrix exoproteomes**

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GenBank accession no.		Putative Protein	*S. aureus* 15981	*S. aureus* 132-PNAG	*S. aureus* 132-FnBPs	Theoric PI	Theoric Mw(10^{-3})	Total score[a]	Coverage %	RatioExp[b]
Exoproteins up-regulated under biofilm conditions										
gi15927581[c]	SA1813	Leukocidin	x			9.43	40.43	140.5	4.57	32.8
gi15926283[c]	SA0562	Alcohol dehydrogenase Adh1	x	x		5.34	36.05	113.51	10.12	16.6
gi15923805[c]	SA0746	Nuclease	x	x	x	9.27	25.12	1306.41	25.44	14
gi15926008[c]	SA0295	Lipoprotein	x		x	9.49	33.35	321.51	17.15	7.8
gi15927994[c]	SA2204	Phosphoglyceromutase GpmA	x		x	5.23	26.68	191.13	7.02	7.7
gi15928224[c]	SA2431	Immunodominant antigen B IsaB	x		x	9.67	19.37	1995,27	21.14	7.6
g15925815[c]	SA0107	Protein A	x			5.54	56.44	289.57	13.78	6.9
gi15927579	SA1811	Truncated beta-hemolysin Hlb	x		x	7.68	31.26	463.98	6.57	5.9
gi15926570	SA0841	MAP hypothetical protein	x	x	x	9.28	15.84	2018.96	18.06	5.7
gi15925596[c]	SA2399	Fructose-1,6-bisphosphate aldolase	x	x		4.88	33.04	376.91	10.14	4.1
gi15926551[c]	SA0823	Glucose-6-phosphate isomerase Pgi	x	x		4.83	49.82	69.43	8.8	3
gi15926634	SA0900	Cysteine protease precursor SspB	x		x	5.68	44.52	1662.70	24.68	2.9
gi15926265	SA0544	Hypothetical protein		x	x	5.12	29.39	143.28	5.2	2.8
gi15927415[c]	SA1659	Foldase protein PrsA	x	x		9.01	38.64	105.90	3.44	2.8
gi15926291[c]	SA0570	Hypothetical protein	x		x	9.17	18.59	557.99	23.81	2.7
gi15927996[c]	SA2206	IgG-binding protein SBI	x		x	9.38	50.07	172.37	5.87	2.6
gi15927419[c]	SA1663	Hypothetical protein	x	x		4.33	13.31	227.81	34.21	2.6
gi15925985[c]	SA0272	Type VII secretion protein EsaA	x			6.24	114.78	99.62	1.19	2.5
gi15926635[c]	SA0901	Serine protease SspA	x			5.00	36.97	421.27	12.69	2.3
gi15926639[c]	SA0905	Autolysin Atl	x		x	9.60	136.75	3160.15	24.68	2
Exoproteins non-differentially expressed										
gi15926452[c]	SA0730	Phosphoglycerate mutase Pgm	x			4.74	56.42	495.22	16.23	1.8
gi15926451[c]	SA0729	Triosephosphate isomerase TpiA	x	x	x	4.80	27.29	225.47	24.51	1.8
gi15926453[c]	SA0731	Enolase Eno	x	x		4.55	47.12	468.17	7.83	1.7
gi15923272[c]	SA0271	Hypothetical protein	x			4.61	11.04	2443.79	74.23	1.6
gi15926190[c]	SA0471	Cystein synthase CysK		x		5.37	32.97	243.53	7.74	1.6
gi15926073[c]	SA0359	Putative secreted protease inhibitor		x		5.70	21.27	82.70	6.32	1.5
gi15928230[c]	SA2437	N-acetylmuramoyl-L-alanine amidase	x			5.96	69.25	80.54	2.91	1.4
gi15928076[c]	SA2285	Cell wall surface protein SasG		x		5.35	178.53	73.21	1.93	1.4
gi15926396	SA0674	Sulfatase	x	x	x	9.04	74.4	1308.21	4.64	1.3
gi15927054[c]	SA1305	DNA-binding protein II	x		x	9.52	9.63	676.57	52.22	1.2
gi15926091	SA0375	Inositol-monophosphate dehydrogenase		x		4.49	55.81	52.29	2.25	1.2

gi15927699[c]	SA1927	Fructose-bisphosphate aldolase FbaA	x	x		5.01	30.84	776.38	19.23	1.1
gi15926449[c]	SA0727	Glyceraldehyde-3-phosphate dehydrogenase	x	x	x	4.89	36.28	604.09	19.94	1.1
gi15926679[c]	SA0944	Pyruvate dehydrogenase E1 PdhB		x		4.65	35.24	74.69	10.46	1.1
gi15928148[c]	SA2356	Immunodominant antigen A IsaA	x	x	x	6.11	24.2	424.96	23.18	1
g15927884	SA2097	Hypothetical protein			x	5.77	17.4	65.96	9.2	1
gi15925944[c]	SA0232	L-lactate dehydrogenase LctE	x	x	x	4.95	29.45	98.7	11.04	-1
gi15926229	SA0509	Chaperone protein HchA		x		4.90	32.17	95.91	5.14	-1.1
gi15927133[c]	SA1382	Superoxide dismutase SodA	x	x	x	5.08	22.71	3457.35	31.65	-1.1
gi15927879[c]	SA2093	Secretory antigen precursor SsaA homolog	x	x		8.96	29.33	324.85	22.85	-1.9
Exoproteins down-regulated under biofilm conditions										
gi15927670	SA1898	Similar to SceD precursor	x	x		5.52	24.07	57.82	7.79	-4.2

a MASCOT score obtained by2D-LC-MS/MS analysis

b Ratio of gene expression levels between biofilm and planktonic growth conditions

c Also found in *S. aureus* D30 biofilm exoproteome by (50).

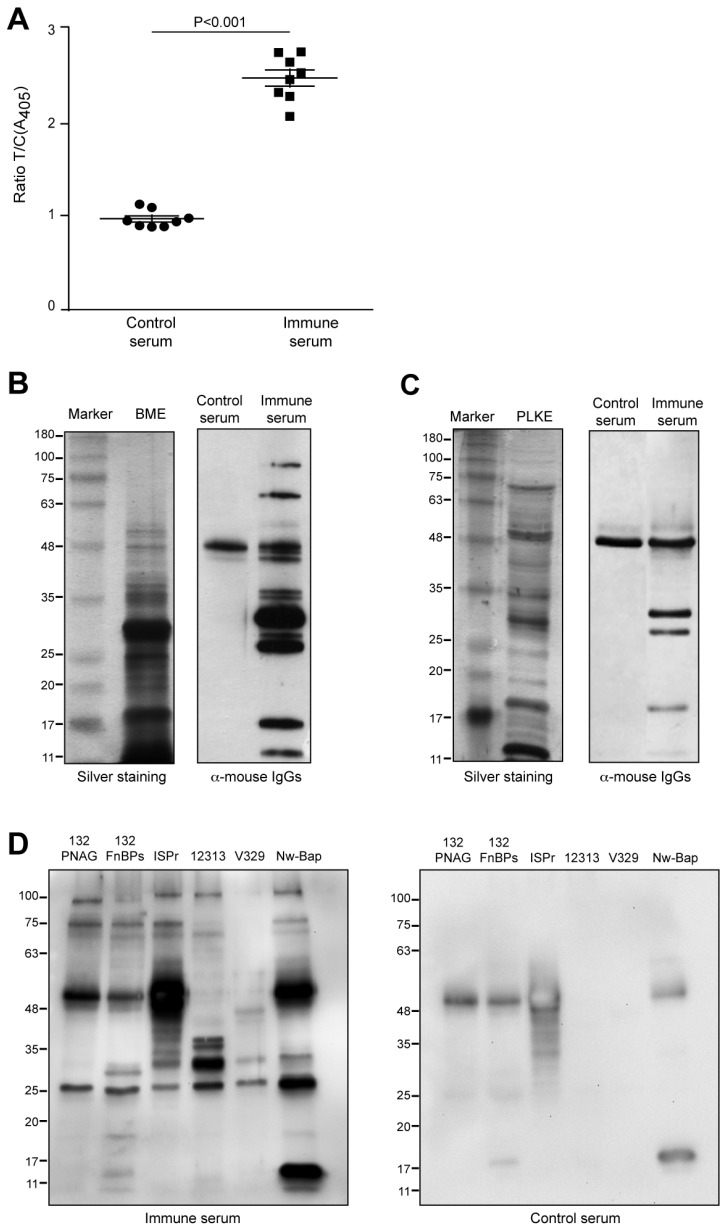

Figure 1: Immunogenicity of the BME extract in mice. Mice (n=8) were immunized twice at an interval of 2 weeks with 10 and 5 µg of the BME extract (treated serum) or with the adjuvant alone (control serum). Sera were collected at times 0 and 1 week after the last immunization. A) IgG titers in response to mice immunization were determined by ELISA. Results were reported as the OD405 of immune serum (treated)/OD405 of the control serum (control) (T/C). The biofilm matrix exoprotein extract (BME) (B) and a protein extract (PLKE) coming from the supernatant of a planktonic culture (C) were separated on a SDS gel and silver stained. Proteins were transferred to a nitrocellulose membrane by western-blotting and probed with immune or control serum and detected with goat anti-mouse IgG and IgM (H+L) secondary antibody HRP conjugated. (D) Western Blot analysis of matrix exoproteins extracts of biofilms formed by different *S. aureus* strains, probed with immune and control serum. *S. aureus* ISP479r (ISPr); *S. aureus* 12313; *S. aureus* V329; *S. aureus* Newman_Bap (Nw-Bap)

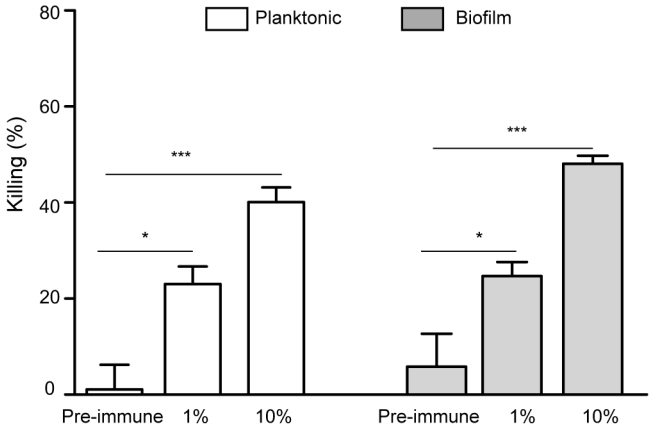

Figure 2: Opsonization with immune serum against BME enhances killing of *S. aureus*. Bacteria grown in planktonic form (white) or attached to polypropylene meshes (grey) were tested for their ability to survive in human blood after preincubation with sterile PBS, preimmune serum, 1% or 10% of immune serum. Surviving bacteria were measured by viable counting. Results are expressed as % of killing calculated as [1 - (no. of cfu recovered from treated samples / no. of cfu recovered from PBS control samples)] *100. Multiple comparisons were performed by one-way analysis of variance combined with the Bonferroni multiple comparison test. GraphPad Instat, version 5).

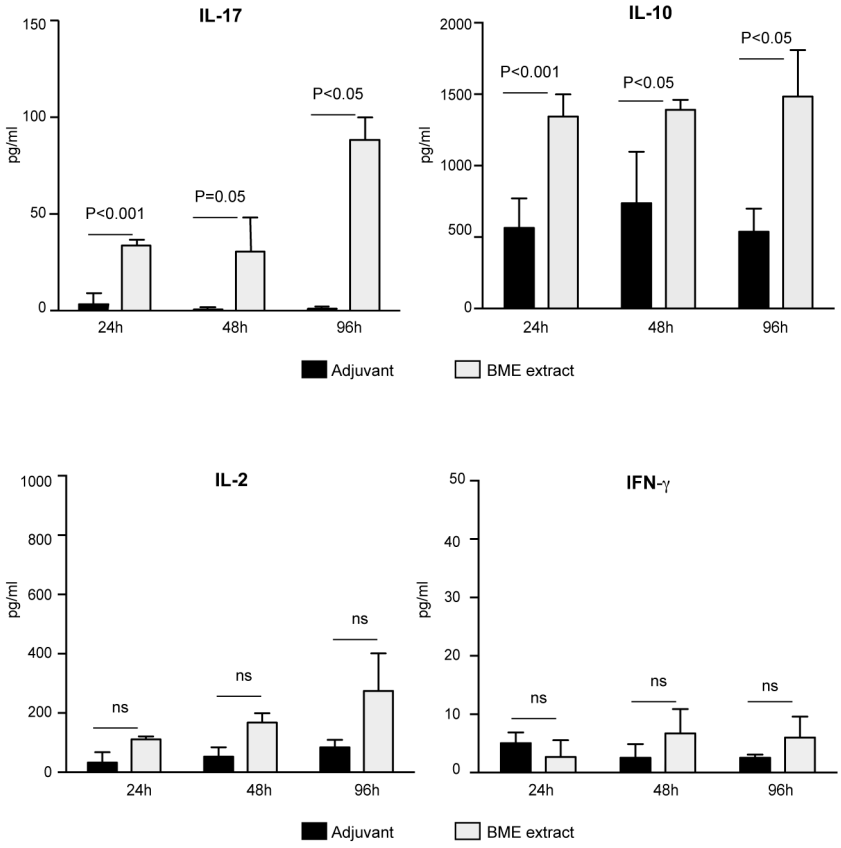

Figure 3: BME-induced production of cytokines in splenocytes. Mice were immunized twice at an interval of 2 weeks with 10 and 5 µg of the BME extract (treated) or with adjuvant alone (control). Three weeks postimmunization, splenocytes were harvested and restimulated for 24h, 48h and 96h with 1 µg of the BME extract. Cell supernatants were harvested and analyzed for IL-2, IL-10, IL17 and IFN-γ production using respective ELISA kits. Results are expressed as pg/ml of each cytokine and are representative of three independent samples. Statistical analysis was carried out using the unpaired Student t test.

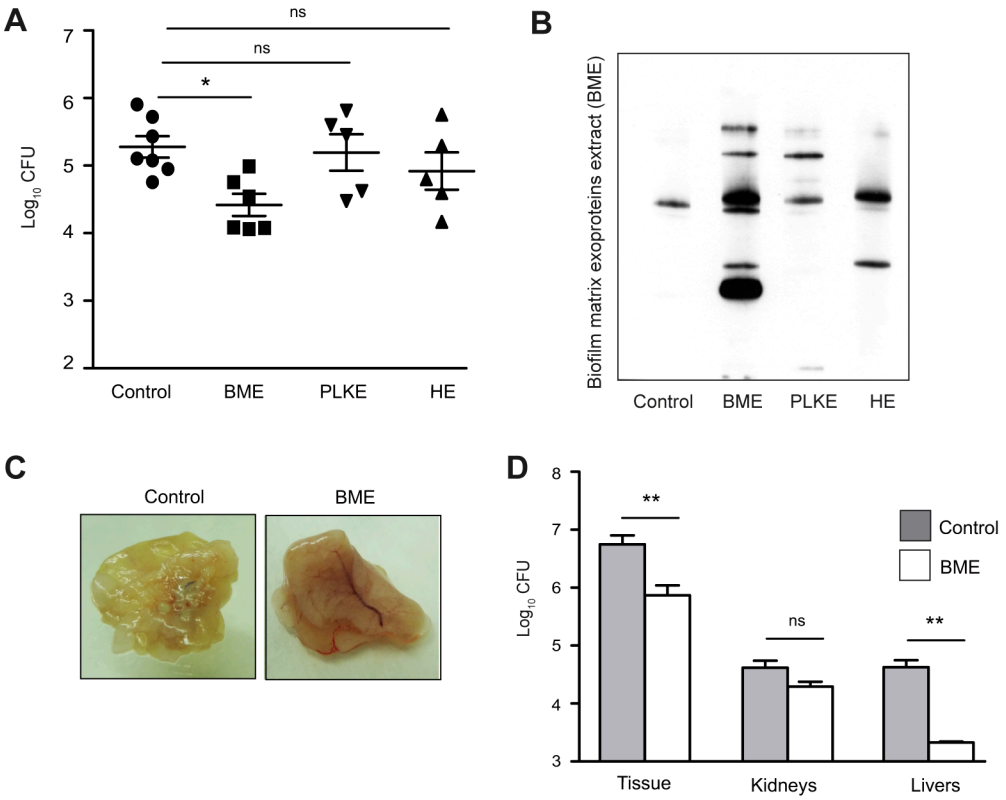

Figure 4: BME extract protects against a biofilm related infection. A) Mice were immunized twice at an interval of 2 weeks with 10 and 5 µg of the BME, PLKE, 10^8 heat-killed bacteria (HE) or with adjuvant alone (control). Polypropylene meshes coated with 10^4 CFU of *S. aureus* strain 132 were fixated at the abdominal wall. After 5 days, animals were sacrificed and meshes were extracted and placed in 1 ml of PBS. Samples were serially diluted and plated onto TSA plates for enumeration of viable staphylococci. Results are representative of six independent mice. Multiple comparisons were performed by one-way analysis of variance combined with the Bonferroni multiple comparison test. (GraphPad Instat, version 5). B) BME proteins were transferred to a nitrocellulose membrane by western-blotting and probed with sera purified from mice immunized with BME, PLKE or HE. C) Images of biofilm infected meshes after 5 days of infection. D) Vaccination with the BME extract also reduces colonization by bacteria that are released from the biofilm. Liver, kidneys and mesh-surrounding tissue from vaccinated and control mice were extracted after five days of insertion of contaminated meshes. Viable staphylococci in the organs and tissue were determined by plate counting.

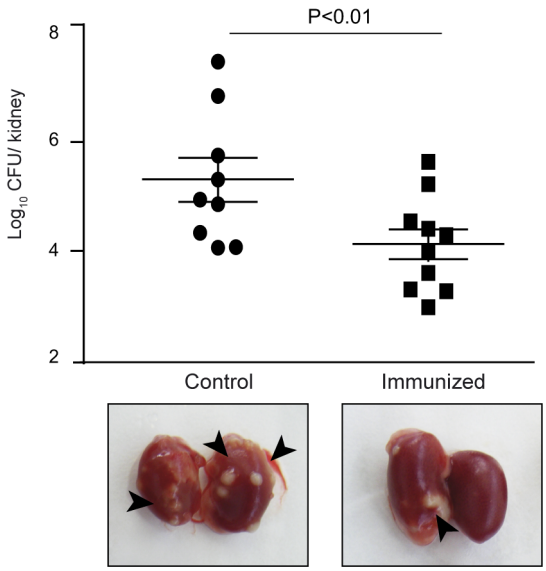

Figure 5: Immunization with the BME extract generates a significant protective immunity against *S. aureus* infection. Vaccinated and control mice were infected with a retrorbital injection containing 10^7 CFU of *S. aureus* Newman. Viable counts were performed on kidney homogenates by plating the samples on TSA. At the bottom, images of abscesses (black arrows) formed in kidneys from control and vaccinated mice are shown