Proteomics-Based Identification of Anchorless Cell Wall Proteins as Vaccine Candidates against *Staphylococcus aureus*^{∇†}

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Staphylococcus aureus is an important human pathogen with increasing clinical impact due to the extensive spread of antibiotic-resistant strains. Therefore, development of a protective polyvalent vaccine is of great clinical interest. We employed an intravenous immunoglobulin (IVIG) preparation as a source of antibodies directed against anchorless *S. aureus* surface proteins for identification of novel vaccine candidates. In order to identify such proteins, subtractive proteome analysis (SUPRA) of *S. aureus* anchorless cell wall proteins was performed. Proteins reacting with IVIG but not with IVIG depleted of *S. aureus*-specific opsonizing antibodies were considered vaccine candidates. Nearly 40 proteins were identified by this preselection method using matrix-assisted laser desorption ionization—time of flight analysis. Three of these candidate proteins, enolase (Eno), oxoacyl reductase (Oxo), and hypothetical protein hp2160, were expressed as glutathione *S*-transferase fusion proteins, purified, and used for enrichment of corresponding immunoglobulin Gs from IVIG by affinity chromatography. Use of affinity-purified anti-Eno, anti-Oxo, and anti-hp2160 antibodies resulted in opsonization, phagocytosis, and killing of *S. aureus* by human neutrophils. High specific antibody titers were detected in mice immunized with recombinant antigens. In mice challenged with bioluminescent *S. aureus*, reduced staphylococcal spread was measured by in vivo imaging. The recovery of *S. aureus* CFU from organs of immunized mice was diminished 10- to 100-fold. Finally, mice immunized with hp2160 displayed statistically significant higher survival rates after lethal challenge with clinically relevant *S. aureus* strains. Taken together, our data suggest that anchorless cell wall proteins might be promising vaccine candidates and that SUPRA is a valuable tool for their identification.

Staphylococcus aureus is an opportunistic, nosocomial, community-acquired pathogen which causes several diseases ranging from minor skin infections to serious life-threatening infections like sepsis, endocarditis, pneumonia, and toxic shock syndrome (30). The rapid emergence of both hospital-associated methicillin (meticillin)-resistant *S. aureus* (MRSA) and community-acquired MRSA (CA-MRSA) is a major epidemiological problem worldwide (5, 25). A further threatening trend concerning *S. aureus* infections is the emergence of isolates with resistance to vancomycin, currently the antibiotic of choice against MRSA strains, and also to newly introduced drugs, such as daptomycin and linezolid (47). Hence, it is not surprising that interest in developing alternative approaches to prevent and treat staphylococcal infections has increased in recent years (34, 48).

The major effector mechanism of the human immune system against *S. aureus* infection is comprised of professional phagocytes, such as neutrophils, that ingest and eliminate bacteria (16). However, phagocytosis of *S. aureus* relies on the opsonization of bacteria by antibodies and complement (7). Recognition of opsonizing antibodies bound to the surface of *S. aureus* via Fcγ receptors of neutrophils is a prerequisite for

induction of the oxidative burst and therefore for killing of the phagocytosed bacteria (23) and induction of a long-term immune response (38). On the other hand, the presence of anti-staphylococcal antibodies does not guarantee protection against reinfection. The reason for this apparent discrepancy is still not well understood. However, it was reported recently that antibodies against certain staphylococcal antigens present in healthy donors were missing or underrepresented in patient sera, indicating that antibodies reacting to these antigens are more efficient for induction of phagocytosis and for subsequent elimination of *S. aureus* than other antibodies (10, 12). Because of this, identification of protective antigens is a crucial step for vaccine development.

Until now, most strategies for vaccination against *S. aureus*, active or passive, concentrated on single-component vaccines based on capsular polysaccharides or well-known virulence factors possessing the LPXTG motif, such as fibronectin binding protein (FnBP), collagen binding protein (CnBP), or clumping factor A (ClfA), as vaccination targets (14, 44). However, despite promising vaccination results obtained with animal models, so far most of the potential vaccines tested in clinical trials have failed to provide significant protection against *S. aureus* infection (48).

It turns out that the efficacy of a monovalent vaccine may be hampered by the functional redundancy of adhesion proteins (17) or the appearance of escape mutants (56). Recently, it has been shown that a multivalent vaccine consisting of four antigenic determinants provides protection against lethal challenge with *S. aureus* in mice, whereas single-component immunization was much less effective (55). Therefore, identification

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of novel targets for an effective *S. aureus* vaccine has repeatedly been recognized as a high priority by experts in this field (20, 28, 34, 40, 43, 48). Indeed, numerous staphylococcal surface proteins predicted to be promising antigenic targets have been identified so far using recently adopted technologies, like proteomics (19, 36, 57) or protein selection methods based on expression libraries (10, 13, 58, 59). Unfortunately, most studies have not provided functional proof that identified proteins are vaccine candidates.

Due to the fact that most of the previous experimental vaccine studies concentrated on candidates exhibiting the LPXTG sorting signal, we focused primarily on identification of noncovalently linked, cell wall-associated proteins, so-called anchorless cell wall (ACW) proteins. Proteins belonging to this class possess neither a conserved signal peptide nor an LPXTG motif and were recently recognized as novel virulence factors in gram-positive bacteria (9). Most of these ACW proteins are multifunctional; e.g., they are involved in different metabolic pathways and also in adhesion to extracellular matrix and invasion of host cells. Such proteins cannot be targeted by genome sequence screening due to the lack of conserved epitopes like LPXTG.

For identification of new potential vaccine targets among ACW proteins we used intravenous immunoglobulin (IVIG) preparations to avoid limitation of the antibody source (i.e., individual sera). IVIG is a pool of immunoglobulins (Igs) from healthy persons that contains a broad spectrum of opsonizing antibodies against various pathogens, including *S. aureus*. By employing two-dimensional gel electrophoresis (2-DE), subtractive immunoblotting, and mass spectrometry, we identified several ACW proteins as novel vaccine targets. This method for identification of potential vaccines was called subtractive proteome analysis (SUPRA). The protective activity of antibodies raised against some of the identified proteins was evaluated in vitro and in a murine sepsis model.

MATERIALS AND METHODS

Bacterial strains. *S. aureus* strain ATCC 29213, a methicillin-sensitive *S. aureus* strain derived from a wound infection, was obtained from the American Type Culture Collection (ATCC) and used for extraction of ACW proteins. For opsonophagocytosis assays green fluorescent protein (GFP)-expressing *S. aureus* strain ATCC 29213-GFP, generated in our laboratory, was used (51). The bioluminescent *S. aureus* Xen29 strain purchased from Xenogen Corp. (Alameda, CA) was employed for in vivo imaging studies. This strain was derived from *S. aureus* ATCC 12600, was previously isolated from pleural fluid, and was stably transformed with a luciferase gene cassette (*luxABCDE* from *Photobacterium luminescens* expressing the enzyme and substrate constitutively) (18, 24). The highly virulent community-associated MRSA strain USA 300 (11) and the protein A-deficient strain Wood 46 (NRS 105) were obtained from the Network on Antimicrobial Resistance in *Staphylococcus aureus* (www.narsa.net). For cloning and expression of recombinant proteins *Escherichia coli* strains TOP10, DH5 α , and BL21 (Invitrogen Corp., Karlsruhe, Germany) were used.

Isolation of ACW proteins. *S. aureus* ATCC 29213 was inoculated into LB broth to obtain a starting optical density at 600 nm (OD₆₀₀) of 0.05. Bacteria were cultured until the early exponential growth phase was reached. ACW proteins were extracted from bacterial pellets using the method of Antelmann et al. (1), with modifications. The pellets were extensively washed, resuspended in 1.5 M LiCl, 25 mM Tris-HCl (pH 7.2) containing protease inhibitors (Roche Diagnostics, Mannheim, Germany), and incubated on ice for 30 min. After centrifugation, the supernatants were pooled and precipitated with 10% (wt/vol) trichloroacetic acid (Sigma, Seelze, Germany) overnight (ON) at 4°C. The precipitate was washed three times with ice-cold ethanol and dried under vacuum. Extracted proteins were dissolved in 8 M urea for 2-DE.

SDS-PAGE. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was performed with 10% polyacrylamide gels using the method of Schägger and von Jagow (49) and a Criterion cell (Bio-Rad, Munich, Germany). Gels were stained either with Coomassie brilliant blue R250 dissolved in 25% ethanol and 8% acetic acid or with silver using the method described by Shevchenko et al. (52).

Depletion of *S. aureus*-specific IgGs from IVIG. *S. aureus* ATCC 29213 from an ON culture was pelleted, washed in phosphate-buffered saline (PBS) (pH 7.3), and resuspended in an IVIG preparation (Octagam [Octapharma, Langenfeld, Germany] or Venimmun N [Aventis-Behring, Marburg, Germany]). The suspension was rotated slowly ON at 4°C. Bacteria were removed by centrifugation, and the supernatant was passed through a 0.2- μ m membrane filter.

SUPRA. 2-DE was performed according to the method described by Bernardo et al. (4) using the Multiphor II system (GE Healthcare, Munich, Germany) according to the instructions of the manufacturer. Proteins dissolved in 8 M urea were separated on 18-cm immobilized pH gradient (IPG) strips using nonlinear pH ranges of 3 to 10 and 4 to 7 (GE Healthcare). Isoelectric focusing was performed using 500 μ g protein for Coomassie blue-stained gels and Western blots and 100 μ g for silver-stained gels and Western blots. The proteins were separated on 12.5% Tris-glycine-SDS gels (25 cm by 20 cm by 1.0 mm) using the Ettan Dalt II system (GE Healthcare).

For immunoblotting, proteins separated by 2-DE were transferred to nitrocellulose membranes using a Trans-Blot cell (Bio-Rad) according to the instructions of the manufacturer. Membranes were probed ON at 4°C either with IVIG (Octagam or Venimmun N) at a dilution of 1:500 or with IVIG depleted of *S. aureus*-specific IgGs (dSaIVIG) using an equivalent antibody concentration. Specific detection of immune complexes was performed using anti-human IgG-horseradish peroxidase antibody at a 1:2,500 dilution. Each membrane was probed twice. After treatment with dSaIVIG the membrane was stripped at 50°C using stripping buffer containing 62.5 mM Tris-HCl (pH 6.8), 2% SDS, and 100 mM β -mercaptoethanol and incubated with IVIG. To assess the reproducibility of spot patterns, three separate experiments were performed.

Signals of interest were matched with corresponding spots on the Coomassie brilliant blue R250-stained preparative gel, excised, and digested with trypsin, and this was followed by matrix-assisted laser desorption/ionization—time of flight (MALDI-TOF) mass spectrometry performed using the procedure described by Bernardo et al. (4). Probability-based scoring (probability based on implementation of the Mowse algorithm for assessing peptide and protein matches [41]) was performed by using $-10 \cdot \log(P)$, where P is the probability that the observed peptide match is a random event. Scores greater than 56 were considered significant ($P < 0.05$).

Cloning of GST fusion proteins. Recombinant enolase (rEno), recombinant oxoacyl reductase (rOxo), and recombinant hypothetical protein hp2160 (rhp2160) were cloned as N-terminal glutathione S-transferase (GST) fusion proteins using the Gateway Technology (Invitrogen) according to the instructions of the manufacturer. The open reading frames of the genes were amplified from *S. aureus* ATCC 29213 chromosomal DNA by PCR using primers 5'-CA CCATGCCAATTATTACAGATG-3' and 5'-TTATTTATCTAAGTTATAGA ATGATTTG-3', primers 5'-CACCATGAAAATGACTAAGAGTGCT-3' and 5'-TACATGTACATTCCACCATTACATG-3', and primers 5'-CACCTTGA TTAGAAACCGTGTATG-3' and 5'-TTAGTTATTTTGTGTACATCCTC ATC-3' for the Eno, Oxo, and hp2160 genes, respectively. The reaction products were cloned into pENTR/D-TOPO and transformed into *E. coli* TOP10 (Invitrogen). By using LR recombination (31) the genes of interest were transferred from the entry clone into the pDEST15 destination vector, generating a vector coding for the N-terminal GST fusion protein. After transformation of the destination vector into *E. coli* DH5 α , positive clones were identified by restriction analysis and transformed into *E. coli* BL21 for expression.

Expression and purification of staphylococcal antigens. *E. coli* BL21 cultures harboring the recombinant plasmids were grown in LB broth containing ampicillin at 30°C until the OD₆₀₀ was ~0.6. Protein expression was induced with 0.5 mM isopropyl- β -D-thiogalactopyranoside (IPTG) for 4 h at 27°C. Bacterial cells were harvested, resuspended in 1 $\times$ PBS containing protease inhibitors, and lysed mechanically using a French press (Thermo Scientific). The bacterial debris was removed by centrifugation at 17,000 $\times$ g and 4°C. The expression of soluble proteins was assessed by SDS-PAGE. The recombinant proteins were purified by affinity chromatography using glutathione Sepharose prepacked GStap FF columns and the ÄKTApurifier liquid chromatography system (GE Healthcare) according to the manufacturer's instructions.

Affinity absorption of specific IgGs from IVIG. For enrichment of specific IgGs, 5 to 10 mg of a purified recombinant protein (rEno, rOxo, or rhp2160) was covalently linked to N-hydroxysuccinimide-activated Sepharose according to the manufacturer's instructions (GE Healthcare). Absorption of IgGs was per-

formed ON at 4°C by slow recirculation of IVIG previously transferred into PBS (pH 7.3) using a HiPrep 26/10 desalting column (GE Healthcare).

Specifically bound IgGs were eluted from the column by pH shifting using 0.1 M glycine-HCl (pH 2.7) with the ÄKTApurifier liquid chromatography system. The pH of the eluted fractions was neutralized by collecting them in a sufficient amount of 1 M Tris-HCl (pH 9). IgG-containing fractions were pooled and, after buffer exchange into PBS (pH 7.3), concentrated using Centricon Plus-70 centrifugal filter units (Millipore, Schwalbach, Germany).

Surface localization of antigens. To assess the localization of the antigens on the bacterial surface, protein A-deficient *S. aureus* strain NRS105 was inoculated into LB broth and grown at 37°C to an OD₆₀₀ of 0.3. Bacteria were harvested and washed, and the concentration was adjusted to 1×10^9 CFU/ml in PBS containing 5% bovine serum albumin (BSA). IVIG, dSaIVIG, or enriched specific IgGs (anti-Eno, anti-Oxo, and anti-hp2160) were added to 1×10^8 CFU/ml at a concentration of 5 µg/ml as the primary antibody, incubated for 30 min at room temperature, and washed to remove free antibodies. A 1:100 dilution of a phycoerythrin-conjugated goat anti-human Fcγ F(ab)₂ fragment (dianova, Hamburg, Germany) was used as the secondary antibody, and the bacteria were incubated for 30 min at room temperature in the dark. After removal of free antibodies, samples were diluted 1:5 in PBS-0.5% BSA and analyzed by flow cytometry using the FACSCalibur immunocytometry system and CELLQuestPro software (BD Biosciences, Heidelberg, Germany).

In vitro opsonophagocytosis and bactericidal assay. Human polymorphonuclear cells (PMNs) were isolated by dextran sedimentation and Ficoll-Hypaque gradient centrifugation using 50 ml of heparinized blood from a healthy donor and standard protocols. Extracted human PMNs were resuspended in PBS containing 10 mM glucose (pH 7.3), and this was followed by determination of the number of cells by trypan blue counting. For the opsonophagocytosis assay *S. aureus* ATCC 29213-GFP was inoculated into LB broth and grown at 37°C to an OD₆₀₀ of 0.3. Bacteria were harvested and washed, and the concentration was adjusted to 1×10^9 CFU/ml. IVIG and dSaIVIG were added at a concentration of 500 µg/ml, specific human IgGs (anti-Eno, anti-Oxo, and anti-hp2160), enriched from IVIG, were added at a concentration of 50 µg/ml, and 2.5×10^6 human PMNs and bacteria at a multiplicity of infection (MOI) of 10 were incubated for 60 s at 37°C with slow rotation, pelleted by centrifugation for 60 s at $400 \times g$ at 37°C, and then incubated for 60 s at 37°C in order to synchronize phagocytosis. Pellets were resuspended, and the phagocytosis was stopped by differential centrifugation for 5 min at $150 \times g$ at 4°C three times. Immediately after centrifugation (t3) an aliquot was removed and stored at a 1:5 dilution in PBS-0.5% BSA (pH 7.3) at 4°C, whereas incubation was continued for a further 30 min at 37°C. Finally, three aliquots of each sample were analyzed by flow cytometry. Bacterial uptake in the presence of 2.5% heat-inactivated human serum (HI serum) was used as a reference.

For the bactericidal assay, IgGs, 2.5×10^6 human PMNs, and 2.5×10^6 bacteria (MOI, 1) were used. PMNs, bacteria, and human IgGs were treated as described above. Immediately after differential centrifugation (t3), as well as after another 30 min (t33), 60 min (t63), and 90 min (t93), samples were removed, diluted 1:10 in 0.1% Triton X-100 dissolved in sterile H₂O, and incubated on ice for 5 min to lyse the PMNs. Dilutions of three aliquots were spread with a spiral plater (Eddy Jet; IUL Instruments, Königswinter, Germany) on Mueller-Hinton agar plates, and the numbers of CFU were determined by using the instructions of the manufacturer. To calculate the percentage of surviving bacteria, the number of CFU present at t33, t63, or t93 was divided by the number of CFU present at t3 and the result was multiplied by 100.

Immunization. Female C57BL/6 and BALB/c mice (6 to 8 weeks old) were purchased from Charles River Laboratories (Sulzfeld, Germany). Mice were injected intraperitoneally with a 1:1 emulsion (total volume, 200 µl) containing 100 µg recombinant protein (rEno, rOxo, or rhp2160) and complete Freund's adjuvant (Sigma) on day 0, and this was followed by subcutaneous administration of three booster doses using an emulsion containing 50 µg antigen and incomplete Freund's adjuvant (1:1) on days 14, 28, and 42. Mice immunized with an emulsion containing BSA and adjuvant served as controls. To determine the specific antibody titer, blood samples were taken 1 week after each booster injection.

Detection of antibody levels. An enzyme-linked immunosorbent assay was used to determine levels of total IgGs and individual IgG subclasses directed against the Eno, Oxo, and hp2160 proteins in sera of immunized mice. Ninety-six-well polystyrene Maxisorb plates (Nunc, Wiesbaden, Germany) were coated ON at 4°C with 5 µg/ml of recombinant antigen (rEno, rOxo, or rhp2160) or BSA in PBS (pH 7.3). The wells were washed three times with PBS containing 0.05% Tween 20 and blocked using StartingBlock T20-PBS (Pierce, Bonn, Germany) for 30 min at room temperature. Triplicate samples of 10-fold serial dilutions of serum in blocking buffer were incubated for 2 h at room temperature. After

washing, bound antibodies were detected either with peroxidase-conjugated goat anti-mouse IgG (Sigma) or with peroxidase-conjugated goat anti-mouse isotypes IgG1, IgG2a, IgG2b, and IgG3 (Invitrogen) by incubating the samples for 2 h at room temperature and using the 3,3',5,5'-tetramethylbenzidine system (BD Biosciences) as the substrate according to the manufacturer's instructions. The enzyme reaction was stopped by addition of 2 N H₂SO₄, and this was followed by measurement of the OD₄₅₀ using an enzyme-linked immunosorbent assay plate reader (MRX TC; Dynex Technology, Kaiserslautern, Germany). The serum dilution which resulted in an OD₄₅₀ of ≥ 0.1 after subtraction of the background value was defined as the titer of a serum.

Mouse sepsis model. C57BL/6 mice ($n = 10$) and BALB/c mice ($n = 9$ to 13) were challenged 2 weeks after the last immunization by intravenous (i.v.) injection of ATCC 29213 (3×10^7 CFU) and USA300 (2×10^7 CFU) in 0.3 ml of 1× PBS, respectively. Mice were monitored daily for clinical signs of infection and mortality for 10 days.

In vivo imaging. Mice were infected with the bioluminescent *S. aureus* Xen29 strain according to Xenogen's recommendations. Prior to infection the mice were shaved ventrally to enhance light detection. The animals were challenged i.v. with an inoculum containing 1×10^8 CFU *S. aureus* Xen29 2 weeks after the last booster injection. The infected mice were monitored daily for 3 days postinfection in an anesthetized state (isofluran-oxygen gas mixture) using an in vivo imaging system (IVIS) (Xenogen). The mice were imaged in the ventral position using 5 min of exposure. An untreated control mouse was included in every image to detect the background emission of photons. The infection rate was displayed using a pseudocolor scale representing the number of photons per second emitted from the mice. Very intense bioluminescence signals were displayed as red, and low-intensity signals were displayed as blue. The light intensity in defined regions of interest was quantified using the LivingImage software (Xenogen). Bioluminescence signals were quantified by setting the regions of interest over the ventral side of the animal's body. The extremities, tail, and head were excluded from the analysis.

Bacterial loads in organs. Mice were sacrificed on day 3 after the IVIS imaging to determine the numbers of bacteria in organs. The target tissues (liver, kidney, spleen, heart, and lung) were weighed and homogenized in 2 ml PBS containing 0.05% Triton X-100. Tenfold serial dilutions were spread on Mueller-Hinton agar plates, and the numbers of CFU were determined.

Statistical analyses. Mann-Whitney rank sum tests were performed to analyze the statistical significance of bacterial loads in organs. A Kaplan-Meier survival curve was analyzed by performing a log rank test. Differences for which the *P* value was less than 0.05 were considered statistically significant.

RESULTS

IVIG contains opsonizing antibodies to *S. aureus*. To determine whether commercial IVIG preparations are suitable sources of antibody for identification of immunoreactive antigens, an IVIG preparation (Octagam, Octapharma, or Venim-mun N; Aventis-Behring) was tested in vitro for the presence of opsonizing antibodies to *S. aureus* using a PMN-mediated opsonophagocytosis assay. The efficacy of complement-independent IVIG-mediated phagocytosis was compared to the efficacy of phagocytosis induced by 2.5% HI serum. To minimize IgG-independent phagocytosis, a short incubation time for bacterial uptake (3 min) was chosen. Flow cytometry revealed that IVIG induced strong phagocytic activity of 77.3% of human PMNs, which was slightly higher than the value obtained with HI serum (59.9%), in which specific *S. aureus* IgGs are also likely present. Furthermore, phagocytosis was nearly completely abolished when IVIG was preabsorbed with *S. aureus* (dSaIVIG) (Fig. 1), indicating that opsonizing antibodies are abundant in IVIG.

Detection and identification of immunogenic staphylococcal ACW proteins by 2-DE analysis. ACW proteins from *S. aureus* clinical isolate ATCC 29213 were separated by 2-DE, which was followed by Western blot analysis using IVIG and dSaIVIG to identify antigenic proteins. To obtain high-quality spot resolution and to ensure adequate spot matching and

FIG. 1. In vitro opsonophagocytosis of GFP-expressing *S. aureus* by human PMNs. Human PMNs, bacteria (MOI, 10), and IVIG, dSaIVIG, or HI serum were incubated at 37°C for 3 min, followed by differential centrifugation. Flow cytometric analysis revealed the percentage of GFP-positive human PMNs (x axis). Dead human PMNs were stained by propidium iodide (PI) (y axis).

identification, proteins were separated using two different pH ranges, pH 3 to 10 (Fig. 2A) and pH 4 to 7 (Fig. 2B), in a series of three gels in parallel for each pH range. One gel was used for immunoblotting, and the same blot was probed twice, first with dSaIVIG (Fig. 2, top panel) and then with IVIG (Fig. 2, middle panel). IVIG and dSaIVIG produced reproducibly distinct immunoblot profiles for 2-DE-separated *S. aureus* ACW proteins in at least three individual experiments. The depletion of *S. aureus*-specific, opsonizing IgGs by absorption of IVIG with *S. aureus* resulted in a considerably lower number of spots

compared to immunoblots treated with IVIG (Fig. 2, top and middle panels). We assumed that proteins recognized by complete IVIG but not by dSaIVIG may be vaccine candidates. In contrast, protein spots strongly reacting with both IVIG and dSaIVIG were thought to be recognized by IgGs that lack specificity for *S. aureus* antigens and were therefore excluded from further investigation. The spots which were not detected in all three individual experiments were also excluded from further analysis. For spot matching and identification two other gels were stained either with silver (Fig. 2, bottom panels) or

FIG. 2. Differences in the *S. aureus* antigen recognition profiles obtained using IVIG and dSaIVIG for immunoblotting. ACW proteins isolated from *S. aureus* strain ATCC 29213 were separated according to their isoelectric points on pI 3 to 10 IPG strips (A) and on pI 4 to 7 IPG strips (B). Gels loaded with 100 μ g protein were used for subtractive immunoblotting. Spots not immunoreactive with dSaIVIG (top panels) but immunoreactive with IVIG (middle panels) were spots predicted to be promising vaccine candidates (circles). Spots immunoreactive with both IVIG and dSaIVIG were excluded from further investigation (squares). For MALDI-TOF identification corresponding spots were matched with preparative gels loaded with 500 μ g protein (bottom panels). Circled and numbered spots identified by MALDI-TOF analysis are listed in Table 1.

TABLE 1. Immunogenic anchorless cell wall proteins from *S. aureus* ATCC 29213 identified by MALDI-TOF

GenBank accession no.	Spot	Putative identification	Theoretical pI	Theoretical mol wt (10 ³)	MASCOT total score ^a	Sequence coverage (%) ^b
gi 15927930	2160	Hypothetical protein, similar to esterase	7.2	35.6	92	28
gi 15926453	2338	Enolase	4.6	47.1	206	47
gi 15926814	2357	3-Oxoacyl-(acyl-carrier protein) reductase	5.6	26.2	103	35
gi 15926530	2036/2078	Hypothetical protein, similar to NADH dehydrogenase	5.4	44.4	79	29
gi 15926396	2037/2038	Hypothetical protein, similar to anion-binding protein	9.0	74.4	248	38
gi 2239274	2039/2345	Peptidoglycan hydrolase	6.1	35.2	63	22
gi 15926560	2042	Conserved hypothetical protein	5.6	31.8	121	48
gi 15928148	2043/2050/2096	Immunodominant antigen A	6.1	24.2	80	23
gi 14247692	2053	Aldehyde dehydrogenase	6.6	51.9	56	15
gi 21205110	2056	Truncated beta-hemolysin	8.8	37.5	105	42
gi 15927879	2155	Secretory antigen precursor SsaA homolog	9.0	29.4	112	40
gi 15927798	2158	50S ribosomal protein L13	9.3	16.3	228	66
gi 15926780	2161	Conserved hypothetical protein	5.7	34.6	186	45
gi 15926226	2219/2227	Translational elongation factor TU	4.7	43.1	98	33
gi 15924043	2221	Autolysin	9.7	102.6	77	11
gi 21205078	2222	Hypothetical protein	5.8	65.5	63	13
gi 14247604	2406	Protoporphyrinogen oxidase	6.3	52.2	126	23
gi 15927580	2425	Hypothetical protein, similar to synergohymenotropic toxin precursor	8.6	38.7	78	14
gi 6578924	2070	Glutamyl-tRNA ^{Gln} amidotransferase subunit A	5.0	52.3	143	36
gi 15926504	2077	Aminotransferase NifS homologue	5.3	46.4	346	60
gi 15926226	2084	Translational elongation factor TU	4.4	29.6	153	29
gi 15923845	2089	Hypothetical protein	5.7	29.4	113	38
gi 15928148	2094/2099	Immunodominant antigen A	5.9	24.2	84	23
gi 15927677	2226	ATP synthase beta chain	4.7	51.4	317	55
gi 21204821	2228	Alanine dehydrogenase	5.6	40.1	268	55
gi 15925843	2229/2230	Phosphopentomutase	5.0	43.8	229	60
gi 15926547	2232	NAD-specific glutamate dehydrogenase	5.2	45.9	255	50
gi 15928230	2235	Hypothetical protein, similar to autolysin precursor	6.0	69.2	72	16
gi 14247509	2237	Chorismate mutase homolog	5.8	40.7	112	34
gi 15927670	2239	Hypothetical protein, similar to SceD precursor	5.5	24.1	70	34
gi 15923892	2240	Hypothetical protein	6.5	37.1	89	19
gi 15926841	2241	Uridylate kinase	6.0	26.3	88	26
gi 15926838	2242	Transcription pleiotropic repressor CodY	5.9	28.7	178	49
gi 14247601	2244	Enterotoxin SEM	6.5	27.5	68	28
gi 15926324	2247	Ferrichrome transport ATP-binding protein (FhuC)	5.6	29.7	99	28
gi 15928275	2342	Conserved hypothetical protein	5.3	37.5	188	33
gi 15926178	2346	50S ribosomal protein L25	4.4	23.8	104	34
gi 15926451	2353	Triose phosphate isomerase	4.8	27.4	66	32
gi 13701328	2374	Xaa-Pro dipeptidase	5.2	39.6	241	36

^a Identification probability of the fragment match.^b Sequence coverage based on the total number of amino acids.

with Coomassie brilliant blue (not shown). Spots of interest were cut from the Coomassie brilliant blue-stained gel and used for MALDI-TOF analysis. Since different IVIG preparations have different IgG repertoires, leading to variability in opsonizing activity among pathogens (30), a second IVIG preparation, Venimmun N, was used for Western blot analysis. The results revealed that the protein pattern obtained was nearly the same as the protein pattern obtained with Octagam (data not shown). A total of 39 proteins giving positive signals with both Octagam and Venimmun N were identified using SUPRA (Table 1). Notably, proteins previously described as ACW proteins, like enolase (Eno), were revealed by our screening procedure, supporting the validity of the method. Likewise, except for the abundantly expressed protein A (SpA), no proteins containing the sortase A targeting motif LPXTG were identified.

Except for four candidate proteins, namely, enterotoxin M and three hypothetical proteins (spots 2089, 2222, and 2240),

all identified proteins are highly conserved in *S. aureus*, since their sequences were present in all 14 complete sequences of *S. aureus* strains currently available in the GenBank database. The four proteins mentioned above were excluded from further investigation because ubiquitous occurrence is a major prerequisite for a vaccine candidate. Furthermore, the two most obvious candidates, immunodominant antigen A (IsaA), which was previously reported to be extremely immunogenic (57) and described as an autolytic enzyme involved in cell wall metabolism (54), and SpA (several spots between 45 and 66 kDa), were excluded from further analysis because they are present at high titers in many individuals but do not confer protection against *S. aureus* infection.

Expression of recombinant proteins and purification of specific antibodies. Three proteins, enolase (Eno), oxoacyl reductase (Oxo), and hypothetical protein hp2160, were randomly chosen to test their abilities to trigger protective immune responses. These proteins were expressed as soluble GST fusion

FIG. 3. In vitro opsonizing activity of enriched human anti-Eno, anti-Oxo, and anti-hp2160 IgGs. Human PMNs, bacteria (MOI, 10), and 2.5% HI serum or 50 μ g/ml IgGs were incubated for 3 min at 37°C for opsonophagocytosis, and this was followed by differential centrifugation. Human PMNs and bacteria without opsonins served as negative controls (dark gray areas). Green fluorescence was measured by flow cytometry immediately after phagocytosis was stopped (t3) (light gray areas) and 30 min later (t33) (open areas). The percentages of GFP-positive human PMNs represent the percentages of cells with internalized ATCC 29213-GFP (M1).

proteins and purified by affinity chromatography. The purity of the recombinant proteins was assessed by SDS-PAGE (see Fig. SA1A in the supplemental material), which showed that rEno and rhp2160 were very pure, whereas contaminants, probably bacterial chaperonins, copurified with rOxo. Copurification of endogenous chaperonins is a known phenomenon that may occur during production of fusion proteins due to stable interactions between fusion proteins and chaperonins (45).

To assess the surface localization of the selected antigens in *S. aureus* and to analyze the opsonic activities of antibodies against rEno, rOxo, and rhp2160, we purified corresponding antibodies from IVIG by affinity chromatography. The efficacy of the enrichment procedure is shown in Fig. SA1B in the supplemental material, and each antibody fraction was able to detect the corresponding antigen, rEno (75.5 kDa), rOxo (54.5 kDa), or rhp2160 (64.0 kDa), showing a minimal reaction with *E. coli* lysate; the only exception was rOxo, where copurification of an endogenous *E. coli* product resulted in copurification of antibodies against this protein from IVIG as well. The specificity for antigen recognition was further demonstrated, for example for hp2160, by immunoblotting using enriched antibodies with ACW protein extract from *S. aureus* ATCC 29213 (see Fig. SA1C in the supplemental material). Except for a signal resulting from cross-reaction with staphylococcal SpA, only a single band of the expected size was detected using the enriched anti-hp2160 antibody.

Moreover, as shown in Fig. SA2 in the supplemental material, flow cytometric analysis revealed specific binding of the enriched human IgGs to the surface of nonpermeabilized SpA-deficient *S. aureus* strain Wood 46. These data support the hypothesis that there is surface localization of the SUPRA-identified proteins. The remarkable weaker fluorescence signal for anti-hp2160-stained *S. aureus* cells may indicate less surface expression of this protein.

Opsonic effect of anti-Eno, anti-Oxo, and anti-hp2160 antibodies. The opsonic activity of affinity-purified antibodies against

rEno, rOxo, and rhp2160 (anti-Eno, anti-Oxo, and anti-hp2160) was analyzed using a human PMN-mediated, complement-independent opsonophagocytosis assay as described above for IVIG. As shown in Fig. 3, antibodies to any of the selected antigens were able to induce strong uptake of bacteria by human PMNs. Within 3 min, 85 to 98% of human PMNs stained positive for *S. aureus*, indicating that there was robust opsonizing activity triggered by affinity-purified antibodies. Comparable phagocytosis rates were obtained for IVIG (92.4%) and HI serum (98.1%). Using dSaIVIG as source of irrelevant antibodies, 5.5% of PMNs were found to be GFP positive, and thus these PMNs were comparable to PMNs in the absence of IgGs.

At a later time point (t33) the percentage of GFP-positive human PMNs was markedly reduced in all samples. To determine whether the reduction in the number of green fluorescent PMNs was caused by elimination of bacteria, a smaller amount of *S. aureus* (MOI, 1) was used for opsonophagocytosis to enable quantification of viable, intracellular bacteria by CFU counting. As shown in Fig. 4, the number of bacteria incubated with PMNs in the presence of IVIG or enriched specific antibodies was markedly reduced within the first 30 min, and there were only marginal differences in killing efficacy among the antibodies. In contrast, bacteria incubated with PMNs alone or nonspecific IgGs (dSaIVIG) survived contact with PMNs. The possibility of a reduction in the number of CFU due to aggregation of *S. aureus* upon antibody binding can be excluded, since no differences in the numbers of CFU were detected when bacteria were incubated with IgGs alone (see Fig SA3 in the supplemental material).

Immunization with rEno, rOxo, and rhp2160 induces significant levels of specific anti-*S. aureus* antibodies. Encouraged by the fact that anti-Eno, anti-Oxo, and anti-hp2160 were able to induce opsonophagocytic killing of *S. aureus* by human PMNs in vitro, we immunized mice with the recombinant proteins to induce protective immune responses against *S. aureus*. One week after the last booster injection, a high titer of total

FIG. 4. In vitro opsonophagocytic killing of *S. aureus* by human neutrophils. *S. aureus* ATCC 29213 was opsonized with either 50 μ g/ml of human anti-Eno (filled triangles), anti-Oxo (filled squares), or anti-hp2160 IgGs (filled diamonds) or 500 μ g/ml of IVIG (filled circles), dSaIVIG (open diamonds), or HI serum (HIser) (dashed line). Human PMNs, bacteria (MOI, 1), and IgGs were incubated at 37°C for 3 min. Cells were lysed immediately after differential centrifugation (t3), as well as 30 min (t33), 60 min (t63), and 90 min (t93) later. PMNs coincubated with bacteria served as negative controls (open circles). The means and standard deviations for triplicate analyses are indicated.

IgG to all three antigens (OD_{450} at 1:10⁶ serum dilution, >0.1) was detected in serum of immunized mice compared to the titer in preimmune sera (see Fig. SA4A in the supplemental material). Moreover, as determined for hp2160 (see Fig. SA4B in the supplemental material), high levels of subclass IgG1, IgG2a, IgG2b, and IgG3 IgGs were detected, resulting in an OD_{450} of >0.3 at a serum dilution of 1:10⁵.

Immunization reduces the spread of bacteria in mouse tissues. Immunized mice were challenged i.v. with bioluminescent *S. aureus* strain Xen 29 using an inoculum containing 1×10^8 CFU to determine whether the immune response elicited by rEno, rOxo, and rhp2160 was able to protect mice against *S. aureus*. Mice were monitored by an IVIS to follow the spread of bacteria from day 1 to day 3 postinfection. In vivo bioluminescence images of the ventral side of uninfected control, mock-immunized, and recombinant protein-immunized mice are shown in Fig. 5A. In contrast to the progressive increase in bioluminescence observed in mice immunized with BSA, no significant increase of bioluminescence was detected in mice treated with rEno, rOxo, or rhp2160. The results of a quantitative analysis of the bioluminescence signals are shown in Fig. 5B. Whereas low bioluminescence values were observed for vaccinated mice over the course of infection, there was remarkable spread of bacteria, resulting in an up-to-10-fold increase

FIG. 5. In vivo imaging of *S. aureus* Xen29 infection in C57BL/6 mice immunized with rEno, rOxo, rhp2160, or BSA as a control. *S. aureus* Xen29 (1×10^8 CFU) was injected i.v. Immunized mice ($n = 7$) were shaved to avoid loss of photon emission and imaged ventrally. (A) The infection was monitored daily by using 5 min of exposure over a 3-day course using the IVIS. (B) Quantitative analysis of bioluminescence. The total number of photons per second in a region of interest was calculated using the IVIS computer software. The means and standard deviations for groups of seven mice are indicated. (C) Bacterial loads in organs of immunized mice 3 days after *S. aureus* challenge. The bars and error bars indicate the means and standard deviations, respectively, for groups of seven mice. An asterisk indicates that a treated group is statistically significantly different than the BSA control ($P < 0.05$).

FIG. 6. Survival of mice immunized with rhp2160. Mice immunized with rhp2160 (solid line) and a BSA control (dashed line) were challenged i.v. with *S. aureus* strains. Survival was monitored for 10 days. (A) C57BL/6 mice ($n = 10$) challenged with 3×10^7 CFU of the *S. aureus* ATCC 29213 strain. (B) BALB/c mice ($n = 9$) challenged with 3×10^7 CFU of the *S. aureus* ATCC 29213 strain. (C) BALB/c mice ($n = 12$ to 13) challenged with 2×10^7 CFU of the CA-MRSA strain USA300.

in bioluminescence signals, in the group of mock-immunized mice.

Furthermore, quantification of the CFU present in individual organs of immunized mice at day 3 postinfection demonstrated that vaccination with rEno, rOxo, or rhp2160 yielded lower *S. aureus* densities (10- to 100-fold lower) in the heart, lung, liver, and spleen than the densities observed for BSA controls. Statistically significant reductions in the level of *S. aureus* were observed in the liver (for Oxo and hp2160) and in lungs (hp2160) ($P < 0.05$). In contrast, just a modest difference in the bacterial load was detected in kidneys.

Mice immunized with rhp2160 are partially protected against lethal *S. aureus* infection. The protective activity of Eno, Oxo, and hp2160 was further evaluated by monitoring the survival of immunized mice after challenge with a sublethal dose of *S. aureus* ATCC 29213 previously shown to be virulent to mice (26) or with a lethal dose of clinically relevant CA-MRSA strain USA300. The course of infection was monitored for 10 days. All mice developed clinical signs of infection. Immunization of mice with *S. aureus* proteins Eno and Oxo did not confer any significant protection against *S. aureus* sepsis compared to BSA-immunized controls (see Fig. SA5 in the supplemental material). In contrast, the survival rates of mice immunized with rhp2160 were statistically significantly higher than the survival rates of mock-immunized animals for all mouse or *S. aureus* strain combinations (Fig. 6). As shown in Fig. 6A and B, after challenge with *S. aureus* ATCC 29213 the survival rates for immunized mice were about 40% higher than the survival rates for control animals. When mice were challenged with the highly virulent strain *S. aureus* USA300 (Fig. 6C) the protection was less pronounced, but still significant ($P < 0.006$).

DISCUSSION

In this paper we propose SUPRA as a method for identification of immunogenic cell wall-associated proteins, an initial decisive step for vaccine development. By combining a proteomic approach with subtractive Western blot analysis using IVIG, we were able to identify 39 *S. aureus* ACW proteins which may serve as novel vaccine candidates. Finally, we char-

acterized some of these proteins as targets of opsonic antibodies in vitro and as vaccine candidates in a murine model of sepsis.

The source of the antibody used for identification of immunogenic proteins is an important issue in vaccine development. Antibodies to several components of the staphylococcal cell wall are frequently present in a population (12). However, they do not confer protection against reinfection, probably due to impaired activation of adaptive immunity by superantigens (16). Since IVIG preparations are successfully used for treatment of patients with antibody deficiencies in order to reduce their high levels of susceptibility to bacterial infections (6, 27), it is obvious that protective antibodies specific for various human pathogens are present in IVIG. Therefore, we hypothesize that IVIG preparations, which consist of purified IgGs pooled from hundreds of healthy individuals, might represent an antibody repertoire for identification of potential vaccine candidates. Further support for the potential use of IVIG for identification of protective antibodies comes from recent studies in which humoral immune responses to *S. aureus* in different populations were analyzed. Marked differences in antibody repertoires between patients and healthy individuals, with different levels in individual sera, were observed (12). Furthermore, it has been shown that antibodies against protective vaccine determinants (IsdA or IsdH) were underrepresented in nasal carriers but were present at high levels in sera of healthy individuals (10). Another methodological advantage of utilizing IVIG is avoidance of the antibody limitation that comes with individual sera, thereby solving the reproducibility problem connected to proteomic analyses. SUPRA employing IVIG purchased from different manufacturers revealed that pools from more than 1,000 healthy individuals appear to be random enough with regard to the repertoire of antibodies against *S. aureus* ACW proteins.

We observed that antibodies recognizing IsaA and 45- to 66-kDa proteins (isoforms of IgG binding SpA) turned out to be highly abundant in IVIG. This is consistent with previous observations of their in vivo immunogenicity (10, 29, 57). However, highly abundant antibodies that are ubiquitous in sera are not likely to provide protection against *S. aureus* infection, since

the majority of individuals are reinfected. Therefore, we concentrated our efforts on targets for less abundant, yet reproducibly identified antibody species.

Our screen for potential vaccine targets focused on proteins that represent a novel class of surface proteins in gram-positive bacteria, proteins lacking conventional secretory and surface anchor signals. Due to the lack of a conserved signature, these proteins cannot be identified by genome sequence screening and are underrepresented in proteomic analyses of covalently bound proteins upon extraction by lysostaphin treatment. For instance, it is important to note that apart from IsaA and EF-Tu, which were identified previously by serological proteome analysis (6, 57), the proteins identified by our screen are distinct from 15 *S. aureus* proteins identified previously in a proteomic screen by Vytvytska et al. Furthermore, proteins like staphylococcal enolase, alanine dehydrogenase, or EF-Tu, which are characterized as predominantly cytosolic proteins, were shown to be located on the surface of *S. aureus* and other bacterial pathogens as well (2, 8, 46). A certain number of the proteins identified are enzymes that play a crucial role in the biogenesis of the bacterial cell wall, supporting their surface localization. The precise mechanism of the extracellular translocation of these proteins is unknown; however, secretion followed by noncovalent, receptor-mediated reassociation with the cell wall was suggested for pneumococcal α -enolase (3). Therefore, it is not surprising that some of the proteins identified in our screen were also found in secreted or cytosolic protein fractions (4).

So far, the impact of ACW proteins on staphylococcal pathogenicity has not been studied. However, a number of proteins identified by SUPRA have been reported to be staphylococcal virulence factors. Autolysin was shown to bind extracellular matrix components like fibrinogen, fibronectin, and vitronectin (22), and enolase enables adherence by binding to laminin and plasminogen (8, 35). Thus, an important role of enolase as a staphylococcal virulence factor seems to be indisputable. However, the fact that enolase is one of the key enzymes in glycolysis complicates the creation of an isogenic deletion mutant, thus hampering direct estimation of its virulence potential in a mouse model. For mutant *S. aureus* strains lacking FhuC, an ATP-binding cassette transporter important for iron acquisition, it was demonstrated that deletion resulted in decreased virulence in a murine kidney abscess model (53). Recently, autolytic activity and involvement in cell wall metabolism were described for IsaA. However, IsaA mutants were just slightly attenuated for pathogenicity in a mouse septic arthritis model (54). The role of other candidate proteins in the virulence of *S. aureus* is still less obvious and requires detailed investigation.

The genes encoding many *S. aureus* virulence factors, like adhesins and toxins, are not in the core genome of *S. aureus*; thus, their presence may vary from isolate to isolate (42). Additionally, the expression of virulence factors like FnBP or capsular polysaccharides (CPs) is highly regulated during the bacterial growth cycle by the *agr* system (39). In this context it is unlikely that bacterial pathogenicity factors involved in cell wall biogenesis or other essential metabolic pathways are restricted to certain *S. aureus* isolates.

Among the proteins that have been identified, we evaluated enolase (Eno), oxoacyl reductase (Oxo), and hypothetical pro-

tein hp2160 as vaccine candidates. Similar to Eno, the latter two proteins are involved in metabolism in *S. aureus*. Oxo is an enzyme involved in the fatty acid biosynthesis pathway, and hp2160 is predicted to be an esterase. Whether Oxo and hp2160 are also able to act as bacterial adhesins, which has already been proven for Eno, remains to be investigated. In our experiments using enriched specific antibodies for Oxo and hp2160 we obtained support for their surface localization, but the precise mechanism of their translocation from the cytoplasm remains to be defined.

Affinity-purified antibodies against each of these three proteins were shown to be able to opsonize bacteria, thus leading to a potent opsonophagocytosis and killing effect in vitro. A strong humoral immune response was elicited in mice immunized with the individual proteins. The high levels of specific antibodies correlated with a reduction in bacterial infection in vaccinated animals upon challenge with bioluminescent *S. aureus* strain Xen29. However, vaccination with rEno, as well as vaccination with rOxo, does not protect mice from lethal challenge with clinically relevant *S. aureus* strains in a murine sepsis model. In contrast, mice immunized with rhp2160 were partially protected against lethal *S. aureus* challenge, and there was a statistically significant increase in survival compared to control animals. The reason for the observed discrepancy in the apparent antistaphylococcal activities of Eno and Oxo demonstrated by IVIS analysis and lethal challenge of mice has not been determined yet. One possibility is that even though the bacterial load was reduced during the first 3 days of infection, as demonstrated by IVIS analysis, in Eno- and Oxo-immunized mice, some remaining *S. aureus* cells may have established infectious foci, eventually leading to death of the animals at later time points. Finally, *S. aureus* Eno possesses significant sequence homology to murine and human enolases (70% homology), and Oxo shares 63% homology with murine or human estradiol 17 beta-dehydrogenases. Therefore, we speculate that an autoimmune reaction in immunized animals triggered by *S. aureus* infection might result in the death of animals. Consistently, cross-reactive autoantibodies to streptococcal and human enolases have been suggested to be potential initiators of poststreptococcal rheumatic heart disease as cross-reactive antigens (15). Therefore, we propose that Eno and Oxo are not appropriate protein candidates for vaccination against *S. aureus* infection. Hence, of the three candidates tested so far, hypothetical protein hp2160 is the most promising vaccine candidate, having induced statistically significant protection against *S. aureus* in a murine sepsis model with all different mouse or *S. aureus* strain combinations used. Even though full protection of the immunized mice was not achieved, the level of protection was considerable. Because of the multiplicity of *S. aureus* virulence factors, it is rather unlikely that immunization with a single protein would confer absolute protection. Previously published data impressively demonstrate this notion (21, 32, 33, 37, 50).

A recently reported breakthrough in the development of adhesin-based vaccines demonstrated that a combination of multiple proteins confers significant protection against *S. aureus* sepsis (55) compared to immunization with a single antigen. A combination of several antigenic determinants is required not only for optimal strength of protection but also for avoidance of escape mutants (i.e., strains lacking a particular

antigen), as shown by Tuchscher et al. (56). This study demonstrated that passive immunization using antibodies to a particular antigen (e.g., CPs) might result in very quick emergence of nonencapsulated mutants. In contrast, the appearance of such escape mutants was inhibited when a combination of antibodies to both CPs and ClfA was used for passive immunization. In contrast, immunization with a bivalent vaccine consisting of Cna and FnBP did not result in better survival rates than immunization using Cna or FnBP alone (60). Therefore, we assume that a combination of targets belonging to the same functional group may not provide the desired synergistic effect. Based on the protective effect observed upon immunization with rhp2160, evaluation of further proteins identified by SUPRA may lead to identification of additional antigens as promising targets for formulation of a multivalent vaccine.

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REFERENCES

- Antelmann, H., H. Yamamoto, J. Sekiguchi, and M. Hecker. 2002. Stabilization of cell wall proteins in *Bacillus subtilis*: a proteomic approach. *Proteomics* 2:591–602.
- Balasubramanian, S., T. R. Kannan, and J. B. Baseman. 2008. The surface-exposed carboxyl region of *Mycoplasma pneumoniae* elongation factor Tu interacts with fibronectin. *Infect. Immun.* 76:3116–3123.
- Bergmann, S., M. Rohde, G. S. Chhatwal, and S. Hammerschmidt. 2001. Alpha-enolase of *Streptococcus pneumoniae* is a plasmin(ogen)-binding protein displayed on the bacterial cell surface. *Mol. Microbiol.* 40:1273–1287.
- Bernardo, K., N. Pakulat, S. Fleer, A. Schnaith, O. Utermöhlen, O. Krut, S. Müller, and M. Krönke. 2004. Subinhibitory concentrations of linezolid reduce *Staphylococcus aureus* virulence factor expression. *Antimicrob. Agents Chemother.* 48:546–555.
- Boucher, H. W., and G. R. Corey. 2008. Epidemiology of methicillin-resistant *Staphylococcus aureus*. *Clin. Infect. Dis.* 46(Suppl. 5):S344–S349.
- Brady, R. A., J. G. Leid, A. K. Camper, J. W. Costerton, and M. E. Shirtliff. 2006. Identification of *Staphylococcus aureus* proteins recognized by the antibody-mediated immune response to a biofilm infection. *Infect. Immun.* 74:3415–3426.
- Bredius, R. G., C. E. de Vries, A. Troelstra, L. van Alphen, R. S. Weening, J. G. van de Winkel, and T. A. Out. 1993. Phagocytosis of *Staphylococcus aureus* and *Haemophilus influenzae* type B opsonized with polyclonal human IgG1 and IgG2 antibodies. Functional hFc gamma RIIa polymorphism to IgG2. *J. Immunol.* 151:1463–1472.
- Carneiro, C. R., E. Postol, R. Nomizo, L. F. Reis, and R. R. Brentani. 2004. Identification of enolase as a laminin-binding protein on the surface of *Staphylococcus aureus*. *Microbes Infect.* 6:604–608.
- Chhatwal, G. S. 2002. Anchorless adhesins and invasins of Gram-positive bacteria: a new class of virulence factors. *Trends Microbiol.* 10:205–208.
- Clarke, S. R., K. J. Brummell, M. J. Horsburgh, P. W. McDowell, S. A. Mohamad, M. R. Stapleton, J. Acevedo, R. C. Read, N. P. Day, S. J. Peacock, J. J. Mond, J. F. Kokai-Kun, and S. J. Foster. 2006. Identification of in vivo-expressed antigens of *Staphylococcus aureus* and their use in vaccinations for protection against nasal carriage. *J. Infect. Dis.* 193:1098–1108.
- Diep, B. A., S. R. Gill, R. F. Chang, T. H. Phan, J. H. Chen, M. G. Davidson, J. Lin, J. Lin, H. A. Carleton, E. F. Mongodin, G. F. Sensabaugh, and F. Perdreau-Remington. 2006. Complete genome sequence of USA300, an epidemic clone of community-acquired methicillin-resistant *Staphylococcus aureus*. *Lancet* 367:731–739.
- Dryla, A., S. Prustomersky, D. Gelbmann, M. Hanner, E. Bettinger, B. Kocsis, T. Kustos, T. Henics, A. Meinke, and E. Nagy. 2005. Comparison of antibody repertoires against *Staphylococcus aureus* in healthy individuals and in acutely infected patients. *Clin. Diagn. Lab. Immunol.* 12:387–398.
- Etz, H., D. B. Minh, T. Henics, A. Dryla, B. Winkler, C. Triska, A. P. Boyd, J. Sollner, W. Schmidt, U. von Ahsen, M. Buschle, S. R. Gill, J. Kolonay, H. Khalak, C. M. Fraser, A. von Gabain, E. Nagy, and A. Meinke. 2002. Identification of in vivo expressed vaccine candidate antigens from *Staphylococcus aureus*. *Proc. Natl. Acad. Sci. USA* 99:6573–6578.
- Fattom, A. I., G. Horwith, S. Fuller, M. Propst, and R. Naso. 2004. Development of StaphVAX, a polysaccharide conjugate vaccine against *S. aureus* infection: from the lab bench to phase III clinical trials. *Vaccine* 22:880–887.
- Fontan, P. A., V. Pancholi, M. M. Nociari, and V. A. Fischetti. 2000. Antibodies to streptococcal surface enolase react with human alpha-enolase: implications in poststreptococcal sequelae. *J. Infect. Dis.* 182:1712–1721.
- Foster, T. J. 2005. Immune evasion by staphylococci. *Nat. Rev. Microbiol.* 3:948–958.
- Foster, T. J., and M. Hook. 1998. Surface protein adhesins of *Staphylococcus aureus*. *Trends Microbiol.* 6:484–488.
- Francis, K. P., D. Joh, C. Bellinger-Kawahara, M. J. Hawkinson, T. F. Purchio, and P. R. Contag. 2000. Monitoring bioluminescent *Staphylococcus aureus* infections in living mice using a novel *luxABCDEF* construct. *Infect. Immun.* 68:3594–3600.
- Gatlin, C. L., R. Pieper, S. T. Huang, E. Mongodin, E. Gebregeorgis, P. P. Parmar, D. J. Clark, H. Alami, L. Papazisi, R. D. Fleischmann, S. R. Gill, and S. N. Peterson. 2006. Proteomic profiling of cell envelope-associated proteins from *Staphylococcus aureus*. *Proteomics* 6:1530–1549.
- Götz, F. 2004. Staphylococci in colonization and disease: prospective targets for drugs and vaccines. *Curr. Opin. Microbiol.* 7:477–487.
- Greenberg, D. P., A. S. Bayer, A. L. Cheung, and J. I. Ward. 1989. Protective efficacy of protein A-specific antibody against bacteremic infection due to *Staphylococcus aureus* in an infant rat model. *Infect. Immun.* 57:1113–1118.
- Heilmann, C., J. Hartleib, M. S. Hussain, and G. Peters. 2005. The multifunctional *Staphylococcus aureus* autolysin Aaa mediates adherence to immobilized fibrinogen and fibronectin. *Infect. Immun.* 73:4793–4802.
- Huizinga, T. W., F. van Kemenade, L. Koenderman, K. M. Dolman, A. E. von dem Borne, P. A. Tetteroo, and D. Roos. 1989. The 40-kDa Fc gamma receptor (FcRII) on human neutrophils is essential for the IgG-induced respiratory burst and IgG-induced phagocytosis. *J. Immunol.* 142:2365–2369.
- Kadurugamuwa, J. L., L. Sin, E. Albert, J. Yu, K. Francis, M. DeBoer, M. Rubin, C. Bellinger-Kawahara, T. R. Parr Jr., and P. R. Contag. 2003. Direct continuous method for monitoring biofilm infection in a mouse model. *Infect. Immun.* 71:882–890.
- Klevens, R. M., M. A. Morrison, J. Nadle, K. Petit, K. Gershman, S. Ray, L. H. Harrison, R. Lynfield, G. Dumyati, J. M. Townes, A. S. Craig, E. R. Zell, G. E. Fosheim, L. K. McDougal, R. B. Carey, and S. K. Fridkin. 2007. Invasive methicillin-resistant *Staphylococcus aureus* infections in the United States. *JAMA* 298:1763–1771.
- Krut, O., O. Utermöhlen, X. Schlossherr, and M. Krönke. 2003. Strain-specific association of cytotoxic activity and virulence of clinical *Staphylococcus aureus* isolates. *Infect. Immun.* 71:2716–2723.
- Lamari, F., N. K. Karamanos, E. Papadopolou-Alataki, F. Kanakoudi-Tsakalidou, G. Dimitracopoulos, and E. D. Anastassiou. 2000. Monitoring of two intravenous immunoglobulins. Preparations for immunoglobulin G subclasses and specific antibodies to bacterial surface antigens and relation with their levels in treated immunodeficient patients. *J. Pharm. Biomed. Anal.* 22:1029–1036.
- Lee, J. C. 1996. The prospects for developing a vaccine against *Staphylococcus aureus*. *Trends Microbiol.* 4:162–166.
- Lorenz, U., K. Ohlsen, H. Karch, M. Hecker, A. Thiede, and J. Hacker. 2000. Human antibody response during sepsis against targets expressed by methicillin resistant *Staphylococcus aureus*. *FEMS Immunol. Med. Microbiol.* 29:145–153.
- Lowy, F. D. 1998. *Staphylococcus aureus* infections. *N. Engl. J. Med.* 339:520–532.
- Magnani, E., L. Bartling, and S. Hake. 2006. From gateway to multisite gateway in one recombination event. *BMC Mol. Biol.* 7:46.
- Mamo, W., P. Jonsson, J. I. Flock, M. Lindberg, H. P. Muller, T. Wadstrom, and L. Nelson. 1994. Vaccination against *Staphylococcus aureus* mastitis: immunological response of mice vaccinated with fibronectin-binding protein (FnBP-A) to challenge with *S. aureus*. *Vaccine* 12:988–992.
- Menzies, B. E., and D. S. Kernodle. 1996. Passive immunization with anti-serum to a nontoxic alpha-toxin mutant from *Staphylococcus aureus* is protective in a murine model. *Infect. Immun.* 64:1839–1841.
- Middleton, J. R. 2008. *Staphylococcus aureus* antigens and challenges in vaccine development. *Expert Rev. Vaccines* 7:805–815.
- Molkanen, T., J. Tynnelä, J. Helin, N. Kalkkinen, and P. Kuusela. 2002. Enhanced activation of bound plasminogen on *Staphylococcus aureus* by staphylokinase. *FEBS Lett.* 517:72–78.
- Nandakumar, R., M. P. Nandakumar, M. R. Marten, and J. M. Ross. 2005. Proteome analysis of membrane and cell wall associated proteins from *Staphylococcus aureus*. *J. Proteome Res.* 4:250–257.
- Nilsson, I. M., J. M. Patti, T. Bremell, M. Hook, and A. Tarkowski. 1998. Vaccination with a recombinant fragment of collagen adhesin provides protection against *Staphylococcus aureus*-mediated septic death. *J. Clin. Invest.* 101:2640–2649.
- Nimmerjahn, F., and J. V. Ravetch. 2008. Fc gamma receptors as regulators of immune responses. *Nat. Rev. Immunol.* 8:34–47.
- Novick, R. P. 2003. Autoinduction and signal transduction in the regulation of staphylococcal virulence. *Mol. Microbiol.* 48:1429–1449.
- Otto, M. 2008. Targeted immunotherapy for staphylococcal infections: focus on anti-MSCRAMM antibodies. *BioDrugs* 22:27–36.
- Pappin, D. J., P. Hojrup, and A. J. Bleasby. 1993. Rapid identification of proteins by peptide-mass fingerprinting. *Curr. Biol.* 3:327–332.

42. Peacock, S. J., C. E. Moore, A. Justice, M. Kantzanou, L. Story, K. Mackie, G. O'Neill, and N. P. Day. 2002. Virulent combinations of adhesin and toxin genes in natural populations of *Staphylococcus aureus*. *Infect. Immun.* **70**: 4987–4996.
43. Projan, S. J., M. Nesin, and P. M. Dunman. 2006. Staphylococcal vaccines and immunotherapy: to dream the impossible dream? *Curr. Opin. Pharmacol.* **6**:473–479.
44. Rivas, J. M., P. Speziale, J. M. Patti, and M. Hook. 2004. MSCRAMM-targeted vaccines and immunotherapy for staphylococcal infection. *Curr. Opin. Drug Discov. Dev.* **7**:223–227.
45. Rohman, M., and K. J. Harrison-Lavoie. 2000. Separation of copurifying GroEL from glutathione-S-transferase fusion proteins. *Protein Expr. Purif.* **20**:45–47.
46. Rosenkrands, I., R. A. Slayden, J. Crawford, C. Aagaard, C. E. Barry III, and P. Andersen. 2002. Hypoxic response of *Mycobacterium tuberculosis* studied by metabolic labeling and proteome analysis of cellular and extracellular proteins. *J. Bacteriol.* **184**:3485–3491.
47. Sakoulas, G., and R. C. Moellering, Jr. 2008. Increasing antibiotic resistance among methicillin-resistant *Staphylococcus aureus* strains. *Clin. Infect. Dis.* **46**(Suppl. 5):S360–S367.
48. Schaffer, A. C., and J. C. Lee. 2008. Vaccination and passive immunisation against *Staphylococcus aureus*. *Int. J. Antimicrob. Agents* **32**(Suppl. 1):S71–S78.
49. Schagger, H., and G. von Jagow. 1987. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal. Biochem.* **166**:368–379.
50. Schennings, T., A. Heimdahl, K. Coster, and J. I. Flock. 1993. Immunization with fibronectin binding protein from *Staphylococcus aureus* protects against experimental endocarditis in rats. *Microb. Pathog.* **15**:227–236.
51. Schnaith, A., H. Kashkar, S. A. Leggio, K. Addicks, M. Krönke, and O. Krut. 2007. *Staphylococcus aureus* subverts autophagy for induction of caspase-independent host cell death. *J. Biol. Chem.* **282**:2695–2706.
52. Shevchenko, A., M. Wilm, O. Vorm, and M. Mann. 1996. Mass spectrometric sequencing of proteins from silver-stained polyacrylamide gels. *Anal. Chem.* **68**:850–858.
53. Speziali, C. D., S. E. Dale, J. A. Henderson, E. D. Vines, and D. E. Heinrichs. 2006. Requirement of *Staphylococcus aureus* ATP-binding cassette-ATPase FhuC for iron-restricted growth and evidence that it functions with more than one iron transporter. *J. Bacteriol.* **188**:2048–2055.
54. Stapleton, M. R., M. J. Horsburgh, E. J. Hayhurst, L. Wright, I. M. Jonsson, A. Tarkowski, J. F. Kokai-Kun, J. J. Mond, and S. J. Foster. 2007. Characterization of IsaA and SceD, two putative lytic transglycosylases of *Staphylococcus aureus*. *J. Bacteriol.* **189**:7316–7325.
55. Stranger-Jones, Y. K., T. Bae, and O. Schneewind. 2006. Vaccine assembly from surface proteins of *Staphylococcus aureus*. *Proc. Natl. Acad. Sci. USA* **103**:16942–16947.
56. Tuchscher, L. P., F. R. Buzzola, L. P. Alvarez, J. C. Lee, and D. O. Sordelli. 2008. Antibodies to capsular polysaccharide and clumping factor A prevent mastitis and the emergence of unencapsulated and small-colony variants of *Staphylococcus aureus* in mice. *Infect. Immun.* **76**:5738–5744.
57. Vytvytska, O., E. Nagy, M. Bluggel, H. E. Meyer, R. Kurzbauer, L. A. Huber, and C. S. Klade. 2002. Identification of vaccine candidate antigens of *Staphylococcus aureus* by serological proteome analysis. *Proteomics* **2**:580–590.
58. Weichhart, T., M. Horky, J. Sollner, S. Gangl, T. Henics, E. Nagy, A. Meinke, A. von Gabain, C. M. Fraser, S. R. Gill, M. Hafner, and U. von Ahsen. 2003. Functional selection of vaccine candidate peptides from *Staphylococcus aureus* whole-genome expression libraries in vitro. *Infect. Immun.* **71**:4633–4641.
59. Yang, G., Y. Gao, J. Dong, Y. Xue, M. Fan, B. Shen, C. Liu, and N. Shao. 2006. A novel peptide isolated from phage library to substitute a complex system for a vaccine against staphylococci infection. *Vaccine* **24**:1117–1123.
60. Zhou, H., Z. Y. Xiong, H. P. Li, Y. L. Zheng, and Y. Q. Jiang. 2006. An immunogenicity study of a newly fusion protein Cna-FnBP vaccinated against *Staphylococcus aureus* infections in a mice model. *Vaccine* **24**:4830–4837.

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