

Comparative Exoproteomics and Host Inflammatory Response in *Staphylococcus aureus* Skin and Soft Tissue Infections, Bacteremia, and Subclinical Colonization

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The exoproteome of *Staphylococcus aureus* contains enzymes and virulence factors that are important for host adaptation. We investigated the exoprotein profiles and cytokine/chemokine responses obtained in three different *S. aureus*-host interaction scenarios by using two-dimensional gel electrophoresis (2-DGE) and two-dimensional immunoblotting (2D-IB) combined with tandem mass spectrometry (MS/MS) and cytometric bead array techniques. The scenarios included *S. aureus* bacteremia, skin and soft tissue infections (SSTIs), and healthy carriage. By the 2-DGE approach, 12 exoproteins (the chaperone protein DnaK, a phosphoglycerate kinase [P_{gk}], the chaperone GroEL, a multisensor hybrid histidine kinase, a 3-methyl-2-oxobutanoate hydroxymethyltransferase [PanB], cysteine synthase A, an *N*-acetyltransferase, four isoforms of elongation factor Tu [EF-Tu], and one signature protein spot that could not be reliably identified by MS/MS) were found to be consistently present in more than 50% of the bacteremia isolates, while none of the SSTI or healthy-carrier isolates showed any of these proteins. By the 2D-IB approach, we also identified five antigens (methionine aminopeptidase [MetAPs], exotoxin 15 [Set15], a peptidoglycan hydrolase [LytM], an alkyl hydroperoxide reductase [AhpC], and a haptoglobin-binding heme uptake protein [HarA]) specific for SSTI cases. Cytokine and chemokine production varied during the course of different infection types and carriage. Monokine induced by gamma interferon (MIG) was more highly stimulated in bacteremia patients than in SSTI patients and healthy carriers, especially during the acute phase of infection. MIG could therefore be further explored as a potential biomarker of bacteremia. In conclusion, 12 exoproteins from bacteremia isolates, MIG production, and five antigenic proteins identified during SSTIs should be further investigated for potential use as diagnostic markers.

Staphylococcus aureus is capable of causing a wide range of infections, including skin and soft tissue infections (SSTIs), bacteremia, osteomyelitis, and more. However, certain sequence types (STs) of *S. aureus* may better colonize and infect patients. For instance, necrotizing pneumonia or sepsis is commonly associated with ST1 of clonal cluster 1 (CC1) (1). In addition, the burden of multidrug-resistant (MDR) *S. aureus* renders infection control challenging in hospital settings. Furthermore, non-MDR strains may also cause severe staphylococcal infections (2–4).

In *S. aureus*, exoproteins play a major role in virulence, particularly during invasion and host tissue damage. *S. aureus* produces exogenous phenol-soluble modulins that exhibit strong cytolytic activity against human neutrophils, erythrocytes, and monocytes (5). The exoprotein LukGH was recently reported to exhibit synergistic effects with Panton-Valentine leukocidin on human neutrophil lysis (6). Similarly, the exoprotein SasX facilitates intercellular aggregation and promotes biofilm formation (7). A continuous search for new *S. aureus* virulence factors is ongoing, and comparative exoproteomics of strains isolated from different infection types may help in the identification of additional virulence factors.

Several studies have reported heterogeneous virulence gene expression in strains from different infection types and different clones (8, 9). These studies also reported exoproteome heterogeneity likely due to genetic regulation, posttranslational modification, or targeted protein degradation or stabilization. Such heterogeneity complicates the identification of potential biomarkers or vaccine candidates for *S. aureus*.

It is well known that some exoproteins are antigenic. This an-

tigenicity has been shown in both *S. aureus*-infected patients and healthy carriers (10, 11). The exoproteins Hla, IsdB, IsaA, and ClfA elicit significantly higher levels of antibodies in patients than in healthy carriers. Such immunogens play key roles in host protection by enhancing opsonic activity and inducing cytokine and chemokine production to promote immune cell recruitment (12, 13). Unfortunately, the efficacy of these protective actions has not been transformed into a licensed vaccine, underscoring the need for a better understanding of host responses to the different types of infection caused by *S. aureus*. A comprehensive analysis of exoproteome variations and immunoproteomics may allow correlations between different infection types and host immune profiling to be discovered. In turn, this knowledge may help to identify new disease-specific protein markers.

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Here, we described the first pilot study of differentially expressed *S. aureus* exoproteins from different strains and detected during different infections. Until now, most staphylococcal immunoproteomic studies have focused mainly on proteins in the pI range of 6 to 11, as this range is known to cover the majority of well-known virulence factors (11, 14, 15). We investigated exoproteins at lower pI values of 4 to 7 in order to get a clearer picture of all of the proteins involved. In order to investigate the *S. aureus*-induced patterns of cytokines or chemokines, we analyzed their concentrations during SSTIs, bacteremia, and healthy colonization events by using the cytometric bead array (CBA) and the BD LSRFortessa flow cytometer. Ultimately, our study aimed to determine the protein signatures corresponding to various infection types and to identify biomarkers for the clinical detection of *S. aureus* infections.

MATERIALS AND METHODS

***S. aureus* strains.** This study was conducted with the approval of the Faculty of Medicine and Health Sciences of the Universiti Putra Malaysia, the Clinical Research Centre of Hospital Serdang, and the Ministry of Health Malaysia Medical Research Ethics Committee. It was conducted at the Universiti Putra Malaysia, and the samples were obtained from patients at Hospital Serdang. Six isolates each were collected from patients with bacteremia and SSTIs and from healthy carriers. SSTIs included superficial skin infections (such as impetigo, folliculitis/furunculosis, and mastitis) that can progress to more complicated skin infections (such as cellulitis, surgical wound infections, subcutaneous abscesses, and necrosis). All isolates were confirmed as being *S. aureus* by standard methods, which included Gram staining (Gram-positive cocci in clusters), mannitol fermentation, and coagulase and DNase production. All *S. aureus* isolates were stored in Luria-Bertani broth containing 20% (vol/vol) sterile glycerol at -70°C .

***S. aureus* strain characterization.** All 18 isolates were subjected to *mecA* PCR for the detection of methicillin resistance. Staphylococcal cassette chromosome *mec* (SCC*mec*) typing was performed for the *mecA*-positive isolates according to a protocol optimized by Ghaznavi-Rad et al. (16).

Multilocus sequence typing (MLST) and staphylococcal protein A (*spa*) typing were performed as previously described (17, 18). Pulsed-field gel electrophoresis (PFGE) was performed to investigate the genetic relationships among the 18 isolates (19). The *Sma*I restriction fragment patterns were analyzed with BioNumerics software (Applied Maths, Ghent, Belgium), and the percent similarities were determined by the unweighted-pair group method using average linkages based on Dice coefficients.

Each isolate was screened by PCR for 15 virulence factors involved in attachment, tissue invasion, evasion of host defense, and possible toxin production. The panel of genes included *cna* (20), *fmbA* (21), *icaA*, *icaD* (22), *pvl* (23), arginine catabolic mobile element-associated *arcA* (24), *seg*, *seh*, *sei* (25), *sea*, *seb*, *sec*, *eta*, *etb*, and *tssT* (26).

Sera. Patients admitted to the hospital were randomly chosen for this study. For the bacteremia study, the patients were monitored daily on the basis of their symptoms, which included persistent high fever, chills, low blood pressure, and a high total white blood cell count. Only patients who had no symptoms of bacteremia during their first day in the hospital were selected. Blood was drawn from the patients once they were suspected to have bloodstream infections. Generally, serum samples were collected from two groups (those with SSTIs and those with bacteremia) at day 1 and at day 14, after the infection was considered cured. Serum was collected once from healthy carriers upon their identification as carriers. Samples were collected only from those participants who had provided signed informed consent.

The criteria for inclusion in this study were an age of >18 years, consent to be included in the study, and willingness to participate in regular

clinical follow-ups. Immunocompromised subjects and patients with renal insufficiency were not included in this study. Additionally, patients who died during the study or were diagnosed with bacteremia, diabetes mellitus, eczema, or polymicrobial infection at the time of admission were excluded.

Exoprotein extraction. An overnight broth culture of the *S. aureus* strains collected during the clinical study was pipetted into 500 ml of tryptic soy broth supplemented with 0.001 M 2,2'-dipyridyl. The optical density at 600 nm of the culture was adjusted to 0.03 to 0.04, and the culture was grown at 37°C with constant agitation at 150 rpm. Once the culture reached the postexponential phase, the exoproteins from 500 ml of culture were precipitated by the addition of ice-cold ethanol-trichloroacetic acid. The precipitated exoproteins were dried at room temperature and solubilized in rehydration buffer containing 8 M urea, 2 M thiourea, 2.0% (wt/vol) 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), 0.2% (vol/vol) Bio-Lyte 3/10 ampholytes, and 50 mM dithiothreitol (DTT) to a final volume of 150 μl . The exoprotein solution was then centrifuged at $21,000 \times g$ at room temperature for 10 min to remove insoluble proteins. The concentration of the exoprotein was determined with the RC-DC (reducing agent- and detergent-compatible) Protein Assay (Bio-Rad).

2-DGE. Analytical two-dimensional (2D) gel electrophoresis (2-DGE) was performed as described previously (27). A total of 6 μg of exoproteins solubilized in 125 μl of rehydration buffer (8 M urea, 2 M thiourea, 2.0% [wt/vol] CHAPS, 0.2% [vol/vol] Bio-Lyte 3/10 ampholytes, 50 mM DTT) was loaded onto 7-cm immobilized pH gradient (IPG) strips (linear, pI 4 to 7; Bio-Rad) and passively rehydrated for 14 h. Subsequent isoelectric focusing, followed by second-dimension electrophoresis and silver staining, was carried out as previously described (27). Briefly, the absorbed protein inside the IPG strips was isoelectrically focused for a total of 14,000 Vh in a PROTEAN isoelectric focusing cell (Bio-Rad) and processed to equilibration steps with equilibration buffer I (6 M urea, 2% [wt/vol] SDS, 20% [vol/vol] glycerol, 2% [wt/vol] DTT, 50 mM Tris-HCl, pH 8.8) and equilibration buffer II (buffer I with 2.5% [wt/vol] iodoacetamide instead of 2% DTT). For second-dimension separation, equilibrated IPG strips were embedded in a 1-mm-thick SDS-PAGE gel (12.5% T) with overlay agarose (Bio-Rad) and subjected to electrophoresis in a Mini Dodeca-Cell (Bio-Rad) for 45 min at a constant 200 V in Laemmli SDS-PAGE running buffer. The gels were subjected to silver staining with the ProteoSilver Silver Stain kit (Sigma-Aldrich), scanned with a calibrated densitometer GS-800 (Bio-Rad), and analyzed with the PDQuest Advanced 8.0.1 2D Gel Analysis software. Technical triplicates were performed with each sample.

2D immunoblotting (2D-IB). A total of 12 μg of pooled exoproteins for each strain in the different groups was separated by 12.5% 2-DGE as described above. The separated exoproteins and methanol-pretreated polyvinylidene difluoride (PVDF) membranes were incubated in Towbin buffer (25 mM Tris, 190 mM glycine, 20% [vol/vol] methanol [pH 8.3]) for 10 min. Trans-Blot membranes were also rinsed with Towbin buffer. The exoproteins on the 2D electrophoresis gels were then blotted onto PVDF membranes for 30 min at a constant 25 V with a Trans-Blot SD semidry blotting device according to the manufacturer's instructions (Bio-Rad). A prestained protein ladder was used to confirm transfer efficiency. The PVDF membranes with the blotted exoproteins were then blocked overnight in a solution of 5% nonfat dry milk in TBST (Tris-buffered saline-Tween containing 20 mM Tris-HCl, 137 mM NaCl, and 0.1% [vol/vol] Tween 20 [pH 7.6]) at 4°C . This blocking step was followed by five washes with TBST buffer prior to overnight incubation of the PVDF membranes with various human sera at a 1:10,000 dilution in 5% nonfat dry milk-TBST buffer at 4°C in the dark. Five washes with TBST buffer were performed, and the bound human IgG was detected by incubation with peroxidase-conjugated goat anti-human IgG (H+L) diluted 1:50,000 in 5% nonfat dry milk-TBST buffer for 1 h at room temperature. Five washes with TBST buffer were then performed. The membranes were then incubated with SuperSignal West Pico substrate for 5 min. Visual-

ization was performed with an AlphaMager with a 5-min recording time. Three independent experiments were performed for each patient, and acute- and convalescent-phase serum samples were always analyzed in the same experiment. The acquired images were imported into PDQuest for quantitative comparison.

Pooled human serum samples from each clinical group were used to determine the total immune proteome. The 2D-IB image was matched with silver-stained 2-DGE images of the corresponding pooled exoproteins. We aimed to ensure that the immunoreactive protein spots of interest were accurately excised and subjected to tandem mass spectrometry (MS/MS) analysis.

Protein identification. For identification of proteins by liquid chromatography (LC)-electrospray ionization (ESI) MS/MS, the silver-stained protein spots were manually excised from 2D electrophoresis gels. The proteins were trypsin digested, and the peptides were extracted according to standard techniques (28). ESI MS analysis of the peptides was performed with an Ultimate 3000 Nano high-performance liquid chromatography system (Dionex) coupled to a 4000 Q TRAP mass spectrometer (Applied Biosystems, Foster City, CA). Briefly, peptides were eluted with an increasing gradient of acetonitrile with the mobile phase containing 0.1% formic acid at 300 nl/min. The mass spectrometer was set to perform information-dependent acquisition to acquire fragmentation data on the three most intense ions with the following criteria: a mass range of 400 to 2,800, a charge 1 to 3, an intensity of $>5^4$ counts, and exclusion of former target ions for 300 s. The spectra were analyzed to identify proteins of interest with the Mascot sequence matching software (Matrix Science, London, United Kingdom) and the Ludwig NR database. An individual ion score of >60 was considered significant to identify a protein spot with a *P* value of ≤ 0.05 .

Cytokine and chemokine assays. To investigate cytokine and chemokine production patterns, patient serum was analyzed with the BD CBA human Th1/Th2/Th17 cytokine kit and the BD CBA Human Chemokine kit as recommended by the manufacturer. The cytokine and chemokine molecules detected by this kit include gamma interferon (IFN- γ), interleukin-10 (IL-10), IL-2, IL-4, IL-6, IL-17A, IL-8, tumor necrosis factor (TNF), IFN- γ -inducible protein 10 (IP-10), monocyte chemoattractant protein 1 (MCP-1), monokine induced by IFN- γ (MIG), and regulated upon activation of normal T cell expressed and secreted protein (RANTES). Before the CBA was started, the cytometer setup beads provided by the manufacturer were used for the photomultiplier tube voltage and compensation settings in the BD LSRFortessa flow cytometer. The singlet bead population was obtained by adjustment of the forward and side light scatter voltages. The individual beads were then separated by using the optimal allophycocyanin and fluorescein isothiocyanate voltages, approximately 70,000 and 75 V, respectively. After the setting, a mixture of the recombinant standards (standards included all of the recombinant cytokines or chemokines tested) was reconstituted and serially diluted in assay diluent to obtain a standard curve (either 20 to 5,000 pg/ml of the cytokine standards or 10 to 2,500 pg/ml of the chemokine standards). For the cytokine assay, 50 μ l of the diluted recombinant cytokine standards or 50 μ l of individual human sera (diluted 1:8 or undiluted) was incubated with 50 μ l of serum-enhanced pretreated cytokine-mixed capture beads and human Th1/Th2/Th17 phycoerythrin (PE) detection reagent (containing PE-conjugated detection antibodies) for 3 h at room temperature and protected from light. Following incubation, washing, and acquisition of fluorescence data (through FACSDiva software), the results were analyzed with the FCAP Array software. In order to facilitate the analysis of samples with the FCAP Array software, recombinant cytokine standards were acquired from the lowest to the highest concentration, followed by the most concentrated test sample. The level of each cytokine was measured on the basis of PE and 300 events and was recorded per analyte by using the singlet population as the storage and stopping gates. The standard curve was plotted by using a four-parameter logistic model (FCAP Array software). During analysis, the mean fluorescence intensity (MFI) of each population of cytokine capture beads was

recorded. The intensity measurement with PE is proportional to the cytokine concentration. The cytokine concentrations were then quantified by comparison of the MFIs of samples to a standard curve. Most of the steps in the chemokine assay were similar to those used in the cytokine assay; however, serum enhancement for capture beads was omitted and signal detection was dependent on the human chemokine PE detection reagent instead of the human Th1/Th2/Th17 PE detection reagent.

Statistics. Statistical analyses were conducted with SPSS Statistics (version 17.0). For each protein spot, antigenic protein signal, or cytokine concentration, the mean value of technical replicates was calculated from three independent experiments. The spot intensities or cytokine concentrations of the different study groups were then assessed with Student's *t* test. The Mann-Whitney U test or Wilcoxon signed-rank test for paired samples was performed if the data were not normally distributed. Fisher's exact test was also used for categorical data. A *P* value (two tailed) of ≤ 0.05 was considered statistically significant.

RESULTS

Eighteen subjects of mixed ethnicity (Chinese, Indian, and Malay) participated in this study. Twelve participants developed *S. aureus* infections, while six were only nasally colonized without any clinical symptoms (see Table S1 in the supplemental material).

Molecular characterization of *S. aureus* isolates. According to the DNA sequence-based typing and PFGE results, the isolates were diverse. Eighteen isolates comprised 10 STs, eight clonal lineages (Table 1), and 11 pulsotypes (A to K). Of these 18 isolates, 3 were resistant to methicillin and harbored the *mecA* gene; 2 of these 3 were hospital associated (2W and 5W; ST239-t037-SCC*mec* III hospital-associated methicillin-resistant *S. aureus* [MRSA]) and genetically indistinguishable, while the other isolate was community-associated MRSA (4B; ST80-t4152-SCC*mec* IVc).

The combinations of virulence genes harbored by the isolates tested are listed in Table 1 (see also Table S2 in the supplemental material). Most of the isolates, even those with similar genetic backgrounds, harbored clearly different combinations of virulence genes (Table 1). Only a few isolates that shared the same genotype (methicillin-susceptible *S. aureus* ST1-t127, isolates 5B and 4W) showed the same virulence gene pattern (*sea seh can fnbA icaA icaD pvl*).

Acidic and neutral exoproteome of *S. aureus*. All 18 of the isolates studied showed a vast heterogeneity in their *in vitro* exoproteome (2-DGE). This exoprotein expression heterogeneity was more commonly observed among isolates from different infection types, even when the isolates belonged to the same clonal complex, such as CC1 (Fig. 1). In this study, we observed that at least half of the bacteremia isolates contained a large number of extracellular proteins with complex spot patterns and high-intensity signals when expressed *in vitro*, in contrast to SSTIs and carrier isolates (Fig. 1). Exoprotein spots specific to each infection group (either SSTI or bacteremia isolates) were very difficult to define. However, we still identified 12 exoprotein spots with moderate intensity that were expressed only by the bacteremia isolates. Eleven of these unique exoprotein spots were successfully identified by LC MS/MS (Fig. 2); these exoproteins included the chaperone protein DnaK, a phosphoglycerate kinase (SAO11_2521, also referred as P_{gk}), the molecular chaperone GroEL, a multisensor hybrid histidine kinase (Anae109_2543), a 3-methyl-2-oxobutanoate hydroxymethyltransferase (PanB), cysteine synthase A (SATG_02657), an *N*-acetyltransferase (SA21209_0471), and elongation factor Tu (EF-Tu).

Humoral response to *S. aureus* exoproteins. The antibodies

TABLE 1 Characterization of *S. aureus* isolates

Isolate ^a	MLST ^c result	CC ^d	<i>spa</i> type ^e	Allelic profile	<i>spa</i> repeat succession	Kreiswirth ID ^f	Virulence gene profile
1B	ST1899	101	t7760	3-1-14-15-11-212-3	04-13-12-17-20-17-12-17-17	ZEGMDMGMM	<i>fnbA, icaA, icaD</i>
2B	NA ^b	NA	t127	NA	07-23-21-16-34-33-13	UJFKBPE	<i>fnbA, icaA, icaD</i>
3B	ST1179	97	t359	144-1-1-1-1-5-3	07-23-12-21-17-34-34-33-34	UJGFMBBPB	<i>fnbA, icaA, icaD</i>
4B	ST80	80	t4152	1-3-1-14-11-51-10	07-23-12-02-12-34-34-33-34	UJGAGBBPB	<i>fnbA, icaA, icaD, pvl, mecA</i>
5B	ST1	1	t127	1-1-1-1-1-1-1	07-23-21-16-34-33-13	UJFKBPE	<i>sea, seh, cna, fnbA, icaA, icaD, pvl</i>
6B	ST1	1	t127	1-1-1-1-1-1-1	07-23-21-16-34-33-13	UJFKBPE	<i>sea, seb, seg, seh, sei, cna, fnbA, icaA, icaD</i>
1W	ST30	30	t021	2-2-2-2-6-3-2	15-12-16-02-16-02-25-17-24	WGKAKAOMQ	<i>sei, cna, fnbA, icaA, icaD, pvl</i>
2W	ST239	239	t037	2-3-1-1-4-4-3	15-12-16-02-25-17-24	WGKAOMQ	<i>sea, cna, fnbA, icaA, icaD, mecA</i>
3W	ST1290	Singletons	t131	1-4-1-1-11-1-3	07-23-12-34-33-34	UJGBPB	<i>sea, fnbA, icaA, icaD, pvl</i>
4W	ST1	1	t127	1-1-1-1-1-1-1	07-23-21-16-34-33-13	UJFKBPE	<i>sea, seh, cna, fnbA, icaA, icaD, pvl</i>
5W	ST239	239	t037	2-3-1-1-4-4-3	15-12-16-02-25-17-24	WGKAOMQ	<i>sea, fnbA, icaA, icaD, mecA</i>
6W	ST1	1	t127	1-1-1-1-1-1-1	07-23-21-16-34-33-13	UJFKBPE	<i>sea, sec, seh, cna, fnbA, icaA, icaD</i>
1H	ST1963	101	t714	69-1-14-1-11-19-3	04-13-21-12-17	ZEFGM	<i>seb, sec, seh, fnbA, icaA, icaD</i>
2H	ST8	8	t7758	3-3-1-1-4-4-3	11-19-21-17-21-34-23-25	YHFMBJJO	<i>sea, seh, fnbA, icaA, icaD, tssT</i>
3H	ST1964	121	t159	3-5-6-2-7-14-5	14-44-13-12-17-17-23-18-17	I2Z2EGMMJH2 M	<i>seb, seg, sei, cna, fnbA, icaA, icaD</i>
4H	ST8	8	t7759	3-3-1-1-4-4-3	11-10-34-33-25	YC2BPO	<i>seb, seh, fnbA, icaA, icaD</i>
5H	NA ^b	NA	t7463	NA	259-25-17-17-16-16-16-17-16-16-16	OMMKKKMKKKK	<i>sea, seg, seh, sei, fnbA, icaA, icaD</i>
6H	ST30	30	t318	2-2-2-2-6-3-2	15-12-16-16-02-16-02-25-17-24	WGKKAKAOMQ	<i>sea, seg, seh, sei, cna, fnbA, icaA, icaD</i>

^a Cultured *S. aureus* isolates from 1B-6B patients were characterized as bacteremia strains, *S. aureus* isolates from 1W-6W patients were grouped as SSTI strains, and *S. aureus* isolates harvested from healthy 1H-6H participants were recognized as colonizer strains.

^b NA, not available. Seven sequences of internal fragments of housekeeping genes in the 2B *S. aureus* isolate were totally different from the alleles in the database; the *aroE* gene of the 5H isolate was not detected in this study.

^c MLST is a nucleotide sequence-based characterization of isolates that uses the sequences of internal fragments of seven housekeeping genes that are translated into a specific ST for each isolate.

^d CCs are defined as clusters of closely related STs with up to a single difference in the allelic profile.

^e *spa* type, *Staphylococcus* protein A gene typing is defined by polymorphic direct repeat regions in the *spa* gene. The repeats are assigned a numerical code, and the order of specific repeats is translated into a specific *spa* type for each isolate by the Ridom system (17).

^f Kreiswirth ID (18) is another nomenclature-based method of *spa* typing that assigns letters (A to Z, A2, B2, and more) to each repeat sequence rather than a numerical code. Subsequently, the combination of all of the letters is translated into a *spa* type motif.

from more than half of the participants in each group frequently reacted with nine exoprotein spots (see Fig. S1 and Tables S3 and S4 in the supplemental material). These exoproteins included staphylococcal secretory antigen (SsaA), flavin mononucleotide-dependent NADH-azoreductase (AzoR), and several isoforms of the immunodominant antigen A (IsaA). We found highly significant ($P \leq 0.05$) signals for the majority of the IsaA isoforms in patients, in contrast to healthy carriers (Fig. 3; see Fig. S1 in the supplemental material). Although very few antigenic protein spots were observed in bacteremia patients, in contrast to SSTI patients and healthy carriers, bacteremia strains generated comparatively more signals during the convalescent phase (see Fig. S1 in the supplemental material). While the newly developed spots were not very intense, they correlated with various proteins, such as IsaA isoforms 3, 4, and 6 and DnaK isoform 1, as well as two unidentified proteins (SSP0002 and SSP6001). Superimposition of 2-DGE and 2D-IB images successfully identified the immuno-

proteome signatures of distinct infection types (see Fig. S2 in the supplemental material). For example, serum IgG against methionine aminopeptidase (MetAps), peptidoglycan hydrolase (LytM), haptoglobin-binding heme uptake protein (HarA), alkyl hydroperoxide reductase (AhpC), and exotoxin 15 (Set15) were specifically observed in SSTI patients. Healthy-carrier and SSTI isolates commonly showed antigenic proteins for EF-Tu isoform 5 and IsaA isoform 7. Two antigenic protein spots (DnaK and SSP0002) were found to be selectively present in the bacteremia group. None of the bacteremia isolates expressed Isd system proteins *in vivo* (Fig. 4), but these proteome signatures were found to be produced *in vitro* (from cultures) by both carrier and infection isolates, showing that the coding potential was there.

Serum cytokine levels in patients and healthy carriers. Cytokine and chemokine expression patterns were investigated to see if there were any biomarkers specific to staphylococcal infections in addition to the antigenic protein signatures corresponding to the

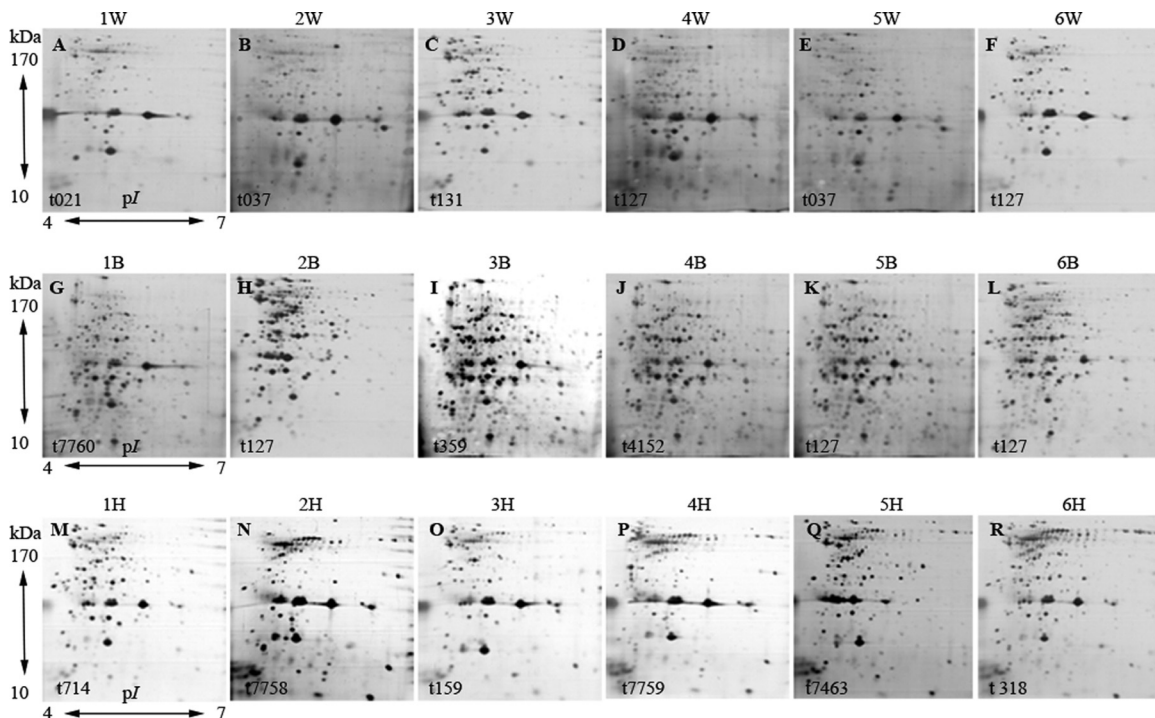


FIG 1 Characterization of *in vitro* expression of *S. aureus* exoproteins by 2-DGE. Isolates from healthy carriers expressed a pattern of acidic exoproteins (1H to 6H) completely different from the patterns of exoproteins secreted by SSTI isolates (1W to 6W) and bacteremia isolates (1B to 6B). Shown is one of three independent replicates analyzed.

defined infection types. As shown in Fig. 5, IL-6 expression levels were significantly higher in most of the *S. aureus*-infected patients than in healthy carriers ($P < 0.035$). Most of the patients showed reduced IL-6 levels during the convalescent phase of infection. However, bacteremia patients still displayed higher serum IL-6 levels (59.53 ± 34.48 pg/ml) than SSTI patients in the convalescent phase (21.45 ± 7.02 pg/ml, $P = 0.044$). We found that the majority of the *S. aureus*-infected patients exhibited higher concentrations of IL-17A during the acute phase of infection (Fig. 5A). In the convalescent phase, the IL-17A production in bacteremia patients was reduced 4.9-fold and was significantly different from that observed in SSTI patients ($P = 0.043$).

Serum chemokine levels in patients and healthy carriers. Significantly higher IL-8 levels were observed in the patient groups than in healthy carriers ($P = 0.016$). In SSTI patients, a rise in the chemokine MCP-1 concentration (254.7 ± 91.21 pg/ml) was detected during the convalescent phase that was significantly different from the values of healthy carriers (143.59 ± 55.66 pg/ml). Conversely, elevation of MIG and IP-10 levels during the convalescent phase was observed in the bacteremia patients, and these increased levels were significantly different from the levels observed in both the SSTI group and the healthy carriers (Fig. 5B). The MIG levels in the bacteremia patients were significantly higher than those observed in SSTI patients in both the acute and convalescent phases ($P = 0.036$ and 0.028 , respectively).

DISCUSSION

To date, several proteomic studies have been reported and this improved our understanding of staphylococcal cell physiology, virulence, and associated host responses (9, 13, 29, 30). However, most of these studies were carried out with a single strain or infec-

tion type with limited follow-up using data on patient serum antibodies. In addition, most of these studies used IPG strips with pHs ranging from 3 to 10 to cover a wide range of proteins or pH 6 to 11, as most of the immunoreactive proteins are resolved in this range (9, 15). 2-DGE in a pH range of 3 to 10 displayed a crowded appearance and low resolution of protein spots, especially for pI values between 4 and 7 (31). Thus, in this study, we compared different infection types and healthy carriers with 7-cm IPG strips at pHs of 4 to 7 by using a modified 2-DGE silver staining method (27) to get better resolution and to identify the acidic and neutral proteins for which information is still limited. For example, in this study, we identified HarA, MetAPs, Set15, AhpC, DnaK, EF-Tu, LytM, PanB, AzoR, and SsaA, which have not been identified in earlier studies that focused on the pH range of 6 to 11. In previous studies, the changes in the human serum cytokine or chemokine profiles over the disease course or between different infection types have also been relatively underexplored. It is important to target the cytokine or chemokine patterns, as these might generate novel diagnostic information to implement early and appropriate treatment. Therefore, the present study addressed SSTIs, bloodstream infections, and subclinical colonization, albeit in a small sample. Hence, our observations are exploratory and generate hypotheses that need to be validated in a larger patient cohort.

First, we performed a molecular characterization of isolates. In the limited number of isolates studied, we were not able to define any specific correlation between clones and disease types. Although two studies have reported that no single staphylococcal genotype is specifically associated with a defined infection type (32, 33), two other studies have shown CC5 and

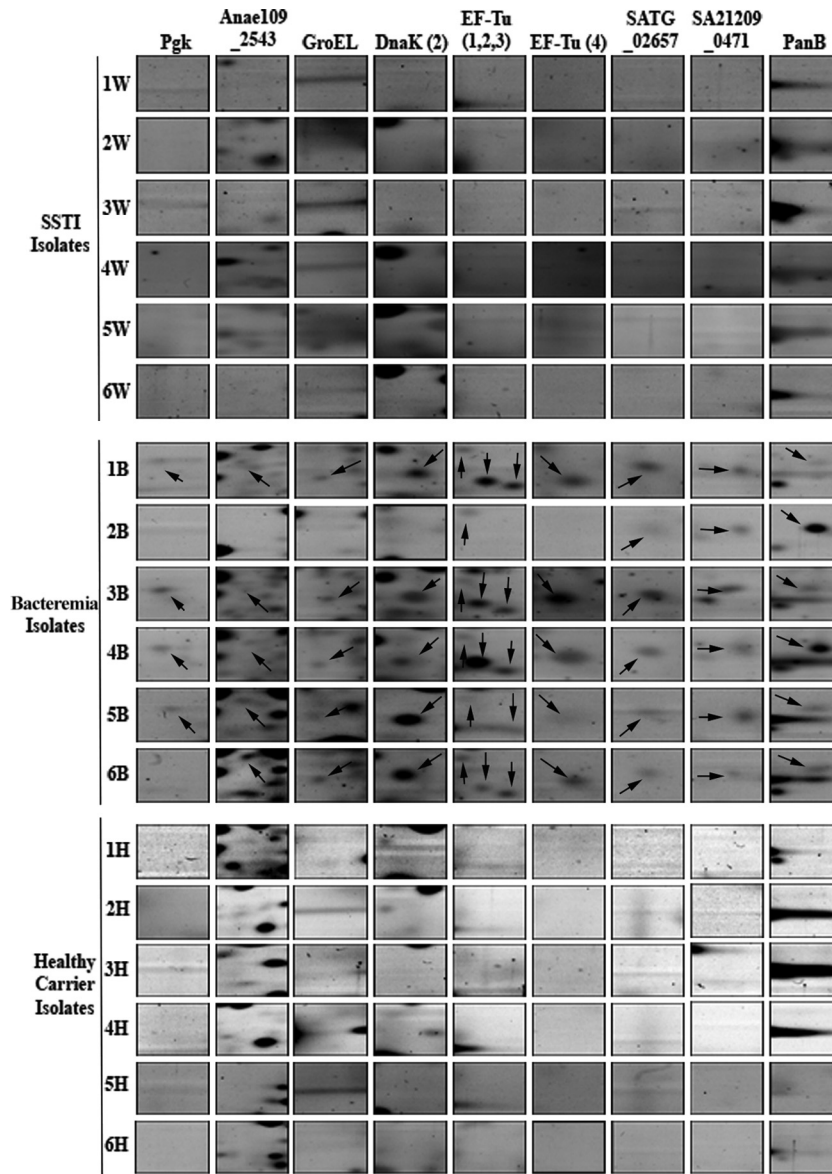


FIG 2 The proteome signatures of bacteremia isolates detected by 2-DGE.

CC30 to be associated with infectious endocarditis and osteomyelitis (34, 35).

Second, the heterogeneous exoprotein expression patterns of *S. aureus* strains made it difficult to identify exoproteins specific to strains or particular infection types. Here we successfully identified only 12 protein spots that were specifically found in bacteremia isolates. Surprisingly, most of these proteins (EF-Tu, DnaK, PanB, Pgg, GroEL, SATG_02657, SA21209_0471, and Ana109_2543) were found to be located in the cytoplasm and play roles in protein synthesis and degradation, metabolism, stress response, amino acid biosynthesis, transcription, and replication. Possibly, this is caused by cellular autolysis in patient blood or these proteins may be released through nonclassical secretion pathways (36–38). In addition, these proteins may be involved in other functions. For example, EF-Tu is also known to be a plasminogen or fibronectin binding protein (39, 40) and might be involved in

reducing the phagocytosis activity of immune cells (41). Plasminogen is a glycoprotein that circulates in the blood. In this study, EF-Tu, which binds host plasminogen on its surface, was found in strains that cause bacteremia. It was speculated that this activity could help *S. aureus* to disseminate in the blood and cause invasive infections. On the other hand, DnaK and GroEL are very important in ensuring the survival of *S. aureus* under various stress conditions such as heat, oxidative, and antibiotic stress (42, 43). For the individual bacteremia patient, the heat shock protein of *S. aureus* (DnaK), which mediates the solubilization of denatured protein, might have been induced in response to the elevated temperature during fever. More recently, DnaK has been shown to be involved in biofilm formation on medical devices and this contributes to intravascular-catheter-related bacteremia in patients (44). Unfortunately, there is not much information about the relevance of PanB in *S. aureus*. PanB has been proven to be an essen-

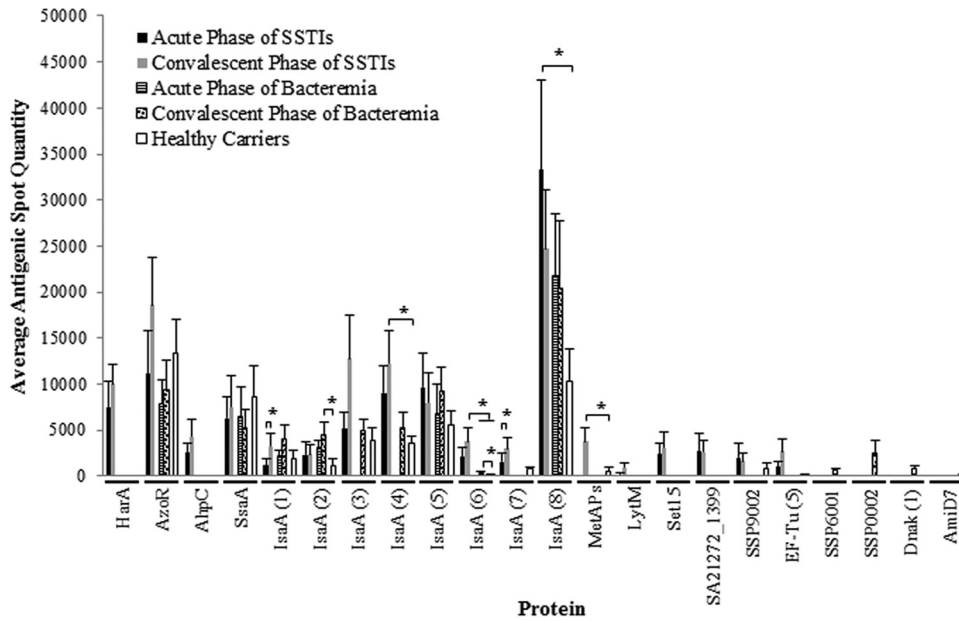


FIG 3 Variability in serum IgG responses to *S. aureus* exoproteins. New antigenic proteins with weak signals are observed between the acute and convalescent phases of infection. *, $P \leq 0.05$.

tial virulence factor in *Mycobacterium tuberculosis* pathogenesis (45). It is interesting that these data indicate that the secreted cytoplasmic proteins may also be involved in staphylococcal virulence.

Unfortunately, none of the 12 protein spots unique to the bacteremia isolates appeared to be antigenic. One of the explanations for this could be denaturation of epitopes during 2D-IB because of the reducing agent DTT. Moreover, there is no native 2D-IB approach with high resolution that could be performed. However, some proteins (such as IsaA isoforms 1 to 8) that were commonly present in both infection types and healthy carriers showed antigenicity (2D-IB) but with various intensities. This result is in agreement with data from other studies (11, 46), where serum from patients showed antistaphylococcal antibody titers higher than those of serum from healthy carriers. Therefore, it is likely that healthy carriers do not depend solely on the humoral immune responses for protection against (auto)infection.

In the present study, anti-IsaA antibody was detected in almost all individual human sera as this protein is expressed by the majority of *S. aureus* strains (11, 47, 48). Some of the antibodies against IsaA isoforms appeared only in the convalescent phase after bacteremia. This clearly shows that all isoforms of a protein should be taken into account during the design of vaccines or

therapeutic agents. IsaA is one of the major antigens of *S. aureus* that are located either in the cell wall or secreted extracellularly. IsaA has a putative hydrophilic lytic domain and may have specific peptidoglycan hydrolysis activity when close to the cell wall, which indirectly assists in cell wall turnover, staphylococcal growth, and hence microbial survival (49). Therefore, IsaA is produced by most *S. aureus* strains during colonization or infection. Consequently, high titers of antibody to IsaA protein in patients have been reported in several earlier studies (50, 51). In addition, SsaA- and AzoR-specific IgGs were also found to be common in our study subjects. The high levels of anti-SsaA antibody observed in the 2D-IB assay were in accordance with the results of a previous study (11). The role of anti-SsaA antibody in pathogenesis is not clearly understood; however, SsaA has been postulated to be involved in biofilm-associated infections (11, 52). AzoR was recently proven to play a role in providing resistance to thiol-specific stress (53).

Here, we report for the first time the antigenicity of MetAPs and Set15 in immunoblot assays of SSTI isolates. In addition, antigenicity for LytM, AhpC, and HarA (known as IsdH or SasI) was also specifically found in SSTI isolates. The presence of antigenicity indicates that certain exoproteins are expressed *in vivo* and may be prerequisites for infection. For example, MetAPs play a main

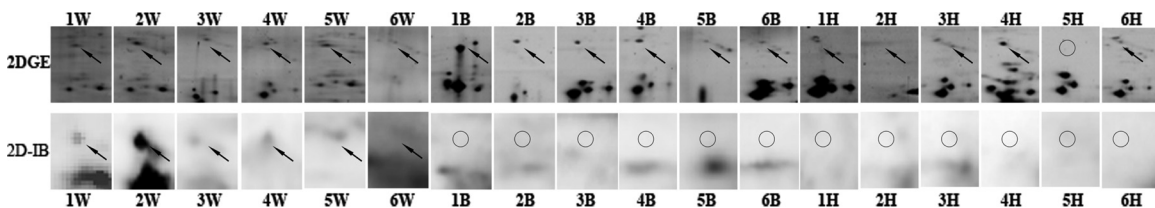


FIG 4 Presence of a HarA (IsdH) protein spot and binding of antibodies to the HarA protein in 2-DGE and 2D-IB images. Most of the *S. aureus* isolates expressed the HarA protein during *in vitro* culture, as shown on the 2-DGE gel images. In the 2D-IB images, the HarA protein was not produced *in vivo* by any of the bacteremia or healthy-carrier isolates. An arrow indicates the presence of HarA, while an open circle indicates the absence of HarA.

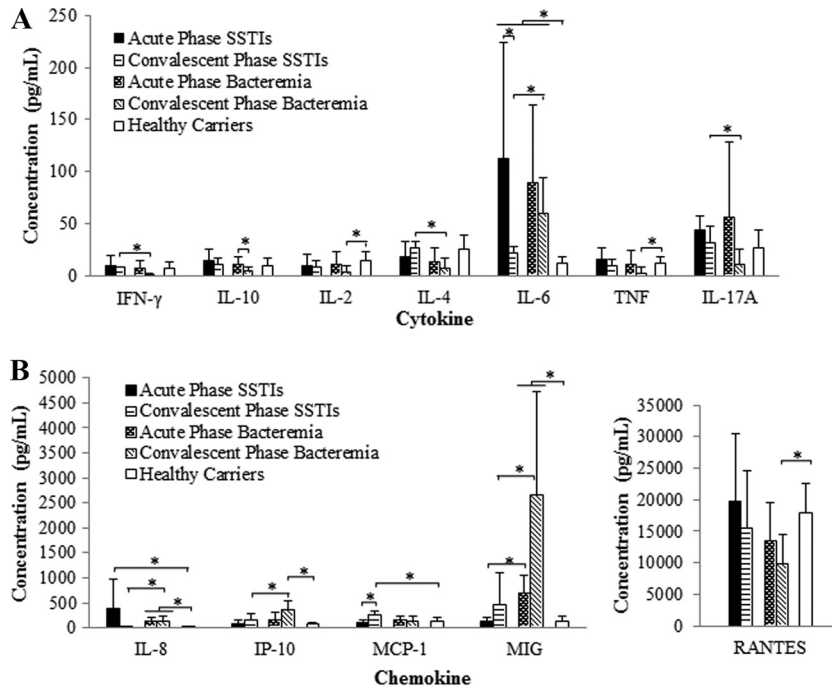


FIG 5 Profiling of cytokines and chemokines. (A) Concentrations of the cytokines IFN- γ , IL-10, IL-2, IL-4, IL-6, TNF, and IL-17A in various groups. (B) Concentrations of the chemokines IL-8/CXCL8, IP-10/CXCL10, MCP-1/CCL2, MIG/CXCL9, and RANTES/CCL5 in various groups. *, P value (two tailed) of ≤ 0.05 , as determined by a Mann-Whitney or Wilcoxon signed-rank test.

role in protein maturation by catalyzing N-terminal methionine excision from newly synthesized proteins. Inhibitors of MetAPs have been shown to significantly limit the growth of MRSA (54). Additionally, MetAPs were also reported to be involved in the recycling pathway of methionine and production of polyamines which are important for SSTIs (55, 56). Although very few data on Set15 are available, it has been shown to be immunoreactive and important in the survival of *S. aureus*. *set15* mutants isolated from murine kidney abscesses were found to be low in number compared with the wild type (57, 58). It was recently demonstrated that Set proteins act as inhibitors of polymorphonuclear leukocyte adherence. Consequently, this might prevent the recruitment of circulating neutrophils to the site of skin infection. Infected skin produces hydrogen peroxide (H_2O_2), which is an important factor in wound healing (59). Therefore, *S. aureus* might produce AhpC to protect itself against H_2O_2 (60). In the present study, antibody against LytM was detected in patients with SSTIs, but only during the convalescent phase. A previous study has shown that higher levels of IgGs against LytM may prevent an *S. aureus* infection from becoming bacteremia (61), and none of our SSTI patients progressed to bacteremia. Interestingly, HarA was found to be produced only *in vivo* by SSTI isolates. HarA is a protein in the Isd system (including IsdABCDEFGI) that is upregulated under iron-restricted conditions. HarA has also been shown to protect *S. aureus* from opsonophagocytosis, and it inhibits neutrophil rolling on the endothelial surface of blood vessels close to the site of infection (62). Immunization with IsdH induced protection against *S. aureus* mastitis (63). However, Isd was not shown to be antigenic in all of the patients and controls in our study. This is in agreement with the previous findings of Kolata et al. demonstrating that Isd proteins such as IsdH, IsdA, and IsdB could not be

identified in bacteremia patients (15). Taken together, these results may help to explain the failure of V710 (vaccine containing IsdB) in clinical trials (64). Vaccine preparations using Isd proteins should be reevaluated to determine their coverage against an array of staphylococcal infections.

Ultimately, the kinetics of cytokine and chemokine production indicated that a variety of human immune responses may contribute to the outcome of *S. aureus*-human interactions. The serum IL-6 levels were higher in patients than in healthy carriers. IL-6 plays a major role in inflammation and acute-phase reactions, affecting both B and T cells (65, 66). The IL-17A level was significantly higher in SSTI patients than in bacteremia patients, even after 2 weeks of infection. An increase in the IL-17A titer during cutaneous *S. aureus* infections has been previously described (67). Abnormal functioning of IL-17A may increase host susceptibility to *S. aureus* skin infection (68, 69). IL-17A stimulates the production of proinflammatory cytokines, chemokines, and adhesion molecules in keratinocytes to promote neutrophil maturation and to recruit additional neutrophils to infection sites (70). Overall, the production of cytokines may trigger the release of chemokines to recruit additional immune cells to the infection site.

We found that bacteremia patients possessed higher average MIG and IP-10 titers than SSTI patients and healthy carriers during the convalescent phase. This result is in agreement with previous reports showing that both of these specific chemokines are highly induced in *S. aureus* bacteremia, where they act as specific chemoattractants for Th1 cells (71–73). Luster et al. showed that elevated expression of IP-10 in the epidermis causes delayed wound healing (74).

Significantly elevated MIG titers in bacteremia patients during the acute phase suggest that MIG could be a candidate diagnostic

marker for early detection of bacteremia. However, further investigation is warranted to confirm its significance, especially during early stages of bacteremia. Similarly, the convalescent-phase MCP-1 level in SSTI patients was significantly higher than that in healthy carriers. Our study represents the first demonstration of MCP-1 expression in SSTI patients. MCP-1 is known to be crucial for polarizing Th cells toward Th2 differentiation (73).

Our study confirmed that the *in vitro* and *in vivo* expression of *S. aureus* exoproteins is highly variable, irrespective of strain relatedness, and documents new common protein signatures that warrant further study. Most of the immunoproteome signatures were found to be produced *in vitro* by most of the isolates. The differential expression of Isd proteins in different infection types should be taken into consideration when designing future therapeutics. The diversity in the cytokine and chemokine response pattern will aid the understanding of staphylococcal disease types over the course of infection. Production of the chemokine MIG was found to be significantly high during bacteremia onset, which shows its potential for use as a marker for early diagnosis. However, whether MIG is specific for *S. aureus* or whether it could be used as a more generic marker of bacterial sepsis must be evaluated by using larger sample panels. In conclusion, we confirm here that the interactions between *S. aureus* and its human host are very complex. The fact that we did not find reliable prospective infection markers suggests that widening the scope of the present study will, in the end, result in the definition of highly individual infection and colonization scenarios that will be difficult to predict on the basis of both bacterial and host marker molecules. Although our current understanding of the importance of proteome signatures for bacteremia and SSTIs is incomplete, the present study has revealed the pattern of protein expression during the course of an infection. In light of the observations listed above, these protein signatures need to be validated in large populations comprising different infection types. Each protein signature should then be characterized by using *in vivo* models to determine its potential for use as a vaccine or a biomarker. Overall, this strategy has important clinical implications since targeting of proteome signatures shows promise as a preventive and therapeutic strategy to achieve complete bacterial clearance.

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