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Article

Tryptic Shaving of *Staphylococcus aureus* Unveils Immunodominant Epitopes on the Bacterial Cell Surface

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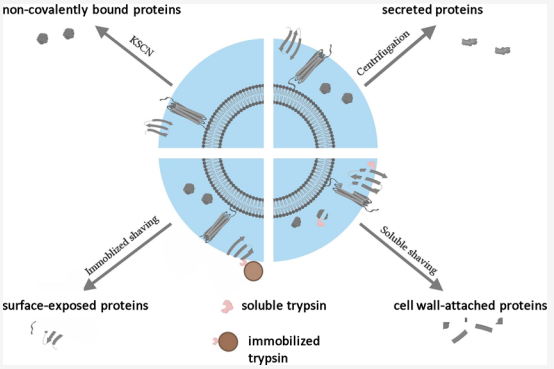
ABSTRACT: The opportunistic pathogen *Staphylococcus aureus* has become a major threat for human health and well-being by developing resistance to antibiotics and by fast evolution into new lineages that rapidly spread within the healthy human population. This calls for development of active or passive immunization strategies to prevent or treat acute phase infections. Since no such anti-staphylococcal immunization approaches are available for clinical implementation, the present studies were aimed at identifying new leads for their development. For this purpose, we profiled the cell-surface-exposed staphylococcal proteome under infection-mimicking conditions by combining two approaches for "bacterial shaving" with immobilized or soluble trypsin and subsequent mass spectrometry analysis of liberated peptides. In parallel, non-covalently cell-wall-bound proteins extracted with potassium thiocyanate and the exoproteome fraction were analyzed by gel-free proteomics. All data are available through ProteomeXchange accession PXD000156. To pinpoint immunodominant bacterial-surface-exposed epitopes, we screened selected cell-wall-attached proteins of *S. aureus* for binding of immunoglobulin G from patients who have been challenged by different types of *S. aureus* due to chronic wound colonization. The combined results of these analyses highlight particular cell-surface-exposed *S. aureus* proteins with highly immunogenic exposed epitopes as potential targets for development of protective anti-staphylococcal immunization strategies.



KEYWORDS: *Staphylococcus aureus, virulence factor, cell wall, surfacome, exoproteome*

INTRODUCTION

The Gram-positive bacterium *Staphylococcus aureus* is an opportunistic pathogen that asymptomatically colonizes approximately 30% of the healthy human population.[1,2] However, upon invasive growth, *S. aureus* can cause a wide variety of diseases ranging from relatively mild skin infections to severe sepsis. A major reason for concern is the high propensity of *S. aureus* to acquire resistance to antibiotics.[3] This is critically underscored by the rapid development of resistance to the antibiotic methicillin.[4−6] While methicillin-resistant *S. aureus* (MRSA) was initially only a threat for hospitalized, elderly, and frail individuals, the last two decades have witnessed the emergence of so-called community-acquired MRSA lineages that rapidly spread within the young and healthy population.[7−9] Importantly, community-acquired MRSA lineages have now also entered nosocomial settings, which gives rise to increased morbidity and mortality rates.[10−15] It is therefore a major societal challenge to develop novel, effective, and long-lasting anti-staphylococcal therapies.[11]

In principle, active or passive immunotherapy approaches can be very effective in protecting individuals at risk against pathogenic microbes.[16] Unfortunately, however, no vaccines or therapeutic antibodies against *S. aureus* in general or MRSA in particular are currently available for clinical implementation.[17−24] This relates to multiple factors, including the ability of *S. aureus* to evade or suppress the human immune system[25−29] as well as the high genome plasticity and adaptability of this widespread pathogen.[6,30−34] Also, the published attempts to develop anti-staphylococcal vaccines

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have been so far focused on a relatively narrow group of known *S. aureus* antigens, including capsular polysaccharides and cell-wall-associated or secreted proteins.[35−37] In this context, it is noteworthy that various proteomics analyses have indicated the presence of at least 121 different proteins in the *S. aureus* cell envelope.[30,38−42] These proteins may include powerful targets for future anti-staphylococcal immunization strategies, especially if they are exposed to the extracellular environment where they are readily recognizable by the human immune system.[43] However, relatively little is known about the *S. aureus* cell-surface-exposed proteome—the "surfacome"—in terms of the particular protein domains that are directly exposed to the extracellular milieu. Even less is known about the presence of possible immunodominant epitopes within such exposed protein domains that could be used for the development of novel immunization approaches.

To pinpoint candidate targets for novel anti-staphylococcal immunization approaches, the present studies were focused on the identification of immunodominant cell-surface-exposed protein domains of *S. aureus*. For this purpose, we first performed an in-depth proteomics analysis of the surfacome based on the incubation of *S. aureus* cells with immobilized trypsin that cannot penetrate the cell wall[30] or with soluble trypsin that can penetrate into the deeper cell wall layers. In parallel, we analyzed proteins released from the cells by spontaneous shedding or by treatment with the chaotropic compound potassium thiocyanate (KSCN), and we analyzed the extracellular proteome (i.e., the "exoproteome") of the investigated cells. Lastly, a screening for immunodominant epitopes in the identified cell-surface-exposed proteins was performed using peptide arrays and serum immunoglobulin G (IgG) from patients with the genetic blistering disease epidermolysis bullosa (EB). As shown in previous research, these patients are exposed to multiple and alternating types of *S. aureus* over long periods of time due to chronic wound colonization.[19,44−46] Altogether, the present "tryptic shaving" of the *S. aureus* cell unveils a set of cell-wall-localized and surface-exposed antigens that may serve as targets for novel active or passive immunization approaches to prevent or treat staphylococcal infections.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Growth Conditions

The strains used in this study are listed in Table 1. *S. aureus* strains were grown overnight in tryptic soy broth (TSB; Oxoid, Basingstoke, U.K.) under vigorous shaking at 37 °C. The cultures were then diluted into a prewarmed RPMI 1640 medium (RPMI; GE Healthcare/PAA, Little Chalfont, United Kingdom) to an OD_{600} of 0.05, and cultivation was continued under the same conditions. Exponentially growing cells were again diluted into a fresh and prewarmed RPMI medium to a final OD_{600} of 0.05, and their cultivation was continued to an OD_{600} of 0.2. *Lactococcus lactis* was grown at 30 °C in M17 broth (Oxoid, Basingstoke, U.K.) supplemented with 0.5% w/v glucose (GM17). For plasmid selection, the GM17 broth was supplemented with chloramphenicol (5 μg/mL).

Table 1. Bacterial Strains Used in This Study[a]

strain	phenotype	ref
S. aureus USA300 (LAC)	community-acquired MRSA isolate	92
S. aureus USA300 (LAC) Δ*spa*Δ*sbi*	*spa sbi* double mutant	93
S. aureus USA300 (LAC) Δ*spa*Δ*sbi*Δ*lytM*	*spa sbi lytM* triple mutant	93
S. aureus Newman	MSSA laboratory strain	94
S. aureus Newman Δ*spa*Δ*sbi*	*spa sbi* double mutant	95
L. lactis PA1001 (pPA180::*lytM*:::his_6)	Cm^R, nisin-inducible expression of *lytM* with C-terminal his_6 directed from the P_{nisA} promotor	50

[a] Cm^R, chloramphenicol resistance gene; P_{nisA} nisin-inducible promoter; his_6, 6 histidine-tag.

Isolation and Processing of Sub-Proteome Fractions

Exoproteome. Cells were separated from the growth medium by centrifugation (15 min, 6750*g*, 4 °C). The growth medium fraction thus obtained was filtered (pore size 0.22 μm), and the exoproteome present in this fraction was precipitated overnight at 4 °C with 10% trichloroacetic acid (TCA; Sigma-Aldrich, St. Louis, USA). Precipitated proteins were pelleted by centrifugation (20 min, 18,620*g*, 4 °C) and washed with acetone. Protein pellets were dried and resuspended in 50 mM ammonium bicarbonate and digested overnight with trypsin (Promega, Madison, USA) at 37 °C.

Surfacome Shaving with Immobilized Trypsin ("Shaving 1"). Cells were harvested by centrifugation (10 min, 6080*g*, 4 °C) and washed twice with phosphate-buffered saline (PBS) containing 40% sucrose (Acros) and 20 mM sodium azide (Sigma-Aldrich, St. Louis, USA). Immobilized trypsin (Thermo Fisher Scientific, Waltham, MA, USA) was activated with 50 mM ammonium bicarbonate (Sigma-Aldrich, St. Louis, USA), resuspended in 50 μL of PBS with 40% sucrose and 20 mM sodium azide, and added to the washed cells. The shaving reaction was conducted for 45 min at 37 °C.[30,47] Released peptides representing the surfacome were isolated, reduced with 10 mM DTT (30 min), alkylated with iodoacetamide (Sigma-Aldrich, St. Louis, USA), and digested with trypsin overnight at 37 °C.

Surfacome Shaving with Soluble Trypsin ("Shaving 2"). The protocol for cell shaving with soluble trypsin was essentially the same as the protocol for shaving with immobilized trypsin. In this case, immobilized trypsin in 48 μL of PBS with 40% sucrose and 20 mM sodium azide was added to the cells.

Spontaneously Released Proteins ("Control"). The protocol for analysis of proteins spontaneously released from the cells was essentially the same as the protocol for shaving with immobilized trypsin. In this case, 50 μL of PBS with 40% sucrose was added to the cells.

Non-Covalently Cell-Wall-Bound Proteins ("Cell Wall"). Cells were harvested by centrifugation (10 min, 6750*g*, 4 °C), washed twice with PBS with 40% sucrose and 20 mM sodium azide, resuspended in 1 M KSCN, and incubated for 10 min on ice. After centrifugation (10 min, 6750*g*, 4 °C), the resulting supernatant was filtered (pore size 0.22 μm), and proteins in the filtrate were precipitated with TCA. The collected proteins were digested with trypsin overnight at 37 °C.

Mass Spectrometric Analyses

Reduction and alkylation, desalting of the samples, mass spectrometric (MS) analyses, and database searches were performed as previously described.[30] The strain-specific UniProt databases were used for the *S. aureus* strains Newman and USA300, including concatenated reversed databases with

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5250 and 5298 entries, respectively. Validation of MS/MS-based peptide and protein identifications was performed with Scaffold (version Scaffold_2_04_00, Proteome Software Inc., Portland, OR). Peptide identifications were accepted if they exceeded the specific database search engine thresholds. Sequest identifications required at least deltaCn scores greater than 0.10 and XCorr scores greater than 1.9, 2.2, 3.8, and 3.8 for singly, doubly, triply, and quadruply charged peptides, respectively. All experiments were conducted in independent triplicate. Peptides were only accepted as identified if they were detected in at least two out of the three replicates per sample set. With these filter parameters, no false-positive hits were obtained. The identified proteins and peptides of strains USA300 and Newman are listed in Tables S1 and S2, respectively.

Western Blotting

Proteins were separated using NuPAGE gels (Thermo Fisher Scientific, Waltham, MA, USA), and the separated proteins were subsequently transferred to a Protran nitrocellulose membrane (GE Healthcare Life Sciences, Little Chalfont, United Kingdom) by semidry blotting (75 min at 1 mA/cm^2). Immunodetection of the cytoplasmic marker protein thioredoxin A (TrxA) in samples for proteome analyses was achieved with polyclonal rabbit antibodies specific for *S. aureus* TrxA as previously described.[48]

To detect the LytM protein, *S. aureus* cells were separated from the growth medium by centrifugation. Cells were disrupted using 0.1 μm glass beads (Biospec Products, Bartlesville, USA) in a Precellys 24 homogenizer (Bertin Technologies, France), and proteins in the growth medium fraction were precipitated using 10% TCA. Subsequently, the cellular and extracellular proteins were resuspended in LDS sample buffer (Thermo Fisher Scientific, Waltham, MA, USA), separated on NuPAGE gels (Life Technologies, Grand Island, NY. USA), and semidry blotted onto a Protran nitrocellulose membrane. LytM was detected using polyclonal rat antibodies (1:5000) and IRDye 800CW-labeled secondary goat anti-rat antibodies (LI-COR Biosciences, Nebraska, USA). To generate the anti-LytM antibodies, LytM was expressed in *L. lactis* using nisin-induced expression and, subsequently, the protein was purified as previously described.[49,50] Purified LytM was used to immunize rats as described by Timmerman et al and van den Berg et al.[49,50]

Immunofluorescence

S. aureus cells were cultured in RPMI as indicated above, harvested by centrifugation (5 min, 18,620*g*, 4 °C), and washed once in PBS with 20 mM sodium azide. Next, cells were resuspended in 100 μL of PBS with 20 mM sodium azide and IsaA-specific[51] or TrxA-specific antibodies. As a control, cells were incubated with IsaA-specific antibodies that were preincubated with purified IsaA. After 30 min of incubation on ice, the cells were collected by centrifugation (5 min, 18,620*g*, 4 °C) and resuspended in PBS with 20 mM sodium azide containing AlexaFluor 594 labeled goat-anti-rabbit antibodies (Thermo Fisher Scientific, Waltham, MA, USA). After 30 min of incubation on ice, the cells were washed three times in PBS with 20 mM sodium azide, resuspended in MilliQ water, and transferred to poly-L-lysine slides (Thermo Fisher Scientific, Waltham, MA, USA). After drying, a Vectashield mounting medium (Vector Laboratories, Peterborough, U.K.) was applied to the slides to prevent photobleaching. Images were recorded with a Leica DM5500 B fluorescence microscope (Leica Microsystems, Wetzlar, Germany), and image processing was conducted with the ImageJ 1.43 m software.

Pepscan Analysis

To map regions of cell-surface-exposed *S. aureus* proteins that are recognized by human IgGs, we synthesized libraries of linear 15-mer peptides with an overlap of 11 amino acids on a solid support (Pepscan, Lelystad, the Netherlands), as previously described.[52] For some proteins, libraries of CLIPS-constrained 15-mers were prepared as previously described.[49] The peptide libraries were probed with heat-inactivated human sera, in a dilution of 1:1000, with a goat-anti-human-HRP conjugate as a secondary antibody, and developed with 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid). A charge-coupled device camera was used to register absorbance at 405 nm. For every single Pepscan data set, the data was normalized to the average signal intensity of the analysis. Furthermore, the signals for every single protein were normalized to the median of the corresponding protein. In addition, the standard deviations of the normalized data sets were calculated for each protein. Peptides with a signal exceeding the median plus twice the standard deviation and a normalized signal intensity higher than 3 were regarded as immunogenic domains.

Human Plasma

Whole blood samples from EB patients were processed immediately after donation by 1:1 dilution in Hanks' Balanced Salt Solution (Gibco). Plasma was obtained after separation from blood cells using Ficoll-Paque PLUS (GE Healthcare, Little Chalfont, United Kingdom) according to the manufacturer's instructions. The collected human plasma was stored at −30 °C prior to use.

Ethics Statements

Blood donations from EB patients were previously collected with the approval of the medical ethics committee of the University Medical Center Groningen (approval no. NL2747104209).[46] All blood donations were obtained after written informed consent from the respective patients, adhering to the Helsinki Guidelines.

Biological and Chemical Safety

S. aureus is a biosafety level 2 (BSL-2) microbiological agent and was accordingly handled following appropriate safety procedures. All experiments involving live *S. aureus* bacteria and chemical manipulations of *S. aureus* protein extracts were performed under appropriate containment conditions, and protective gloves were worn. All chemicals and reagents used in this study were handled according to the local guidelines for safe usage and protection of the environment.

Data Availability

The mass spectrometry data are deposited in the ProteomeXchange repository PRIDE with the data set identifier PXD000156 (http://proteomecentral.proteomexchange.org).[53] All original Western blots are presented in Figure S1.

RESULTS

Complementary Protein and Peptide Identifications in Different Sub-Proteome Fractions

To identify protein domains that are exposed on the cell surface of *S. aureus*, a comprehensive gel-free proteomics analysis was performed on the community-acquired MRSA strain USA300 and the methicillin-sensitive *S. aureus* (MSSA)

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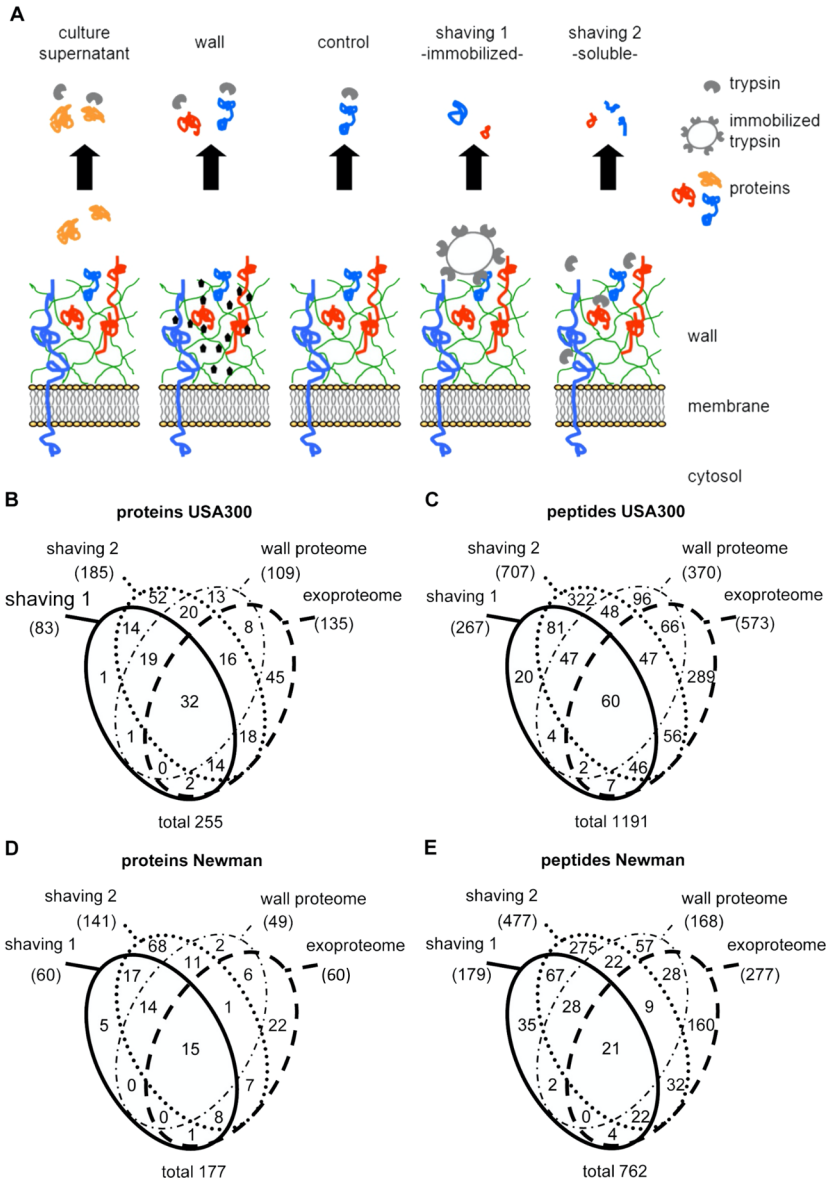

Figure 1. Schematic representation of the analysis of different sub-proteome fractions and proteomics results in numbers. (A) Cells were harvested at identical optical densities, and proteins in the growth medium fraction (culture supernatant) were precipitated and digested with trypsin. Non-covalently cell-wall-attached proteins were extracted with 1 M KSCN and also digested with trypsin (wall). Cells were incubated in PBS with 40% sucrose and 20 mM azide alone (control) or in the same buffer with either immobilized trypsin (shaving 1) or soluble trypsin (shaving 2). The Venn diagrams summarize the results obtained for strains (B,C) USA300 and (D,E) Newman at the (B,D) protein and (C,E) peptide levels. Shaving 1 and shaving 2 mark the results from cell shaving with immobilized or soluble trypsin, respectively, wall proteome marks the results from cell wall extraction with KSCN, and exoproteome marks the results from the analysis of culture supernatants.

laboratory strain Newman. For this purpose, the bacteria were grown in the RPMI medium because a previous study has shown that the global gene expression profile of *S. aureus* grown in this medium resembles that of *S. aureus* grown in human plasma.[54] Hence, the growth conditions in RPMI mimic the conditions encountered by *S. aureus* during bacteremia, including the restricted availability of iron in blood.[54] Upon harvesting of the staphylococcal cells, they were shaved either with trypsin immobilized on agarose beads (Figure 1A, shaving 1) or with soluble trypsin (shaving 2). Liberated peptides were collected and subsequently identified by MS. In parallel, gel-free proteomics was applied to define spontaneously released proteins (control), non-covalently cell-wall-bound proteins extracted with KSCN (cell wall), and proteins in the exoproteome of the analyzed cells. The rationale of this is approach was that the immobilized trypsin would only access protein domains that stick out from the cell surface, whereas the other approaches would help to distinguish the surface-exposed protein domains from proteins or protein domains that are present in the deeper cell envelope layers or in the exoproteome. Overall, 255 unique proteins from the USA300 strain and 177 from the Newman strain were identified in the combined cell-surface-associated and extracellular proteome fractions (Figure 1B,D). Furthermore, 1191 unique peptides were identified in all samples derived from strain USA300, and 762 were identified in the samples derived from strain Newman (Figure 1C,E). Importantly, only two proteins were identified in the control fractions (one protein for each strain), showing that the spontaneous release of proteins from the cells during collection and processing of the

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different sub-proteome fractions was negligible in the present experimental setup (Tables S1 and S2). Therefore, the control fraction is not further specified in what follows. It is also noteworthy that shaving with immobilized trypsin identified fewer proteins/peptides than shaving with soluble trypsin, suggesting that the soluble trypsin does indeed reach targets in the deeper layers of the cell wall (Figure 1). The complementarity of the four sub-proteome fractionation approaches is underscored by the relatively small numbers of proteins/peptides that were identified in all samples from each strain (Figure 1). In fact, this underscores the need to combine multiple approaches for a comprehensive description of the cell-wall-, cell-surface-, and extracellular proteomes of *S. aureus*.

Comparison of KSCN-Extracted Wall Proteins with Proteins Identified by Tryptic Shaving

The analysis of the proteins that were extracted from the bacterial wall using KSCN resulted in 370 and 168 different peptides for strains USA300 and Newman, respectively, representing 109 and 49 different proteins (Figure 1B−E). Interestingly, the numbers of proteins that are specific for the wall extracts are rather low (19 and 16%), while the numbers of peptides that are specific for this fraction are much higher (41 and 50%). These findings indicate that the majority of the cell wall proteins are located at the cell surface but that certain domains of these proteins most likely protrude into the cell envelope.

Comparison of the Exoproteome with the Wall-Attached Proteins

In the exoproteome of strain USA300, we identified 573 peptides from 135 different proteins, whereas in the exoproteome of strain Newman, we identified only 277 peptides from 60 unique proteins (Figure 1B−E). The comparison of these proteins with the proteins identified in the three approaches addressing the wall proteome (cell wall, shaving 1 and 2) revealed that 30−40% of the proteins are shared between the fractions (50−57% on the peptide level). This comparison reveals that the shaving 2 approach with soluble trypsin has the highest complexity, thereby implying either unspecificity of the approach or an extraordinarily high complexity of the wall proteome.

Characteristics of Proteins in the Different Sub-Proteomes

The *S. aureus* cell wall contains, besides the peptidoglycan meshwork, also negatively charged teichoic acids. We therefore wondered whether the possible interaction of positively charged protein domains with negatively charged cell wall components might cause an over-representation of negatively charged protein domains on the cell surface. To address this question, we analyzed the p*I* distribution of the identified peptides and proteins in the different subcellular fractions. This analysis revealed bimodal distributions of the p*I* values of the identified proteins of *S. aureus* strains Newman and USA300, having peaks in the basic as well as the acidic p*I* ranges (Figure S2A,B). Interestingly, the p*I* distribution of identified proteins from strain USA300 tended toward the acidic range, while the p*I* distribution of proteins from strain Newman tended toward the basic range. This observation likely reflects the higher number of cytoplasmic protein identifications in all strain USA300-derived samples as the majority of cytoplasmic proteins exhibit acidic p*I* values. Furthermore, the proteins identified with the shaving 2 approach based on soluble trypsin revealed a lower representation of basic proteins, which can be explained by the higher proportion of cytoplasmic proteins. Nevertheless, the distribution of the p*I* values at the peptide level resembled a unimodal distribution with a peak in the acidic range (Figure S2C,D). Both in samples from the Newman and USA300 strains, we observed predominantly peptides with a p*I* below 5 and a much lower representation of peptides above p*I* 8 for the shaving-based approaches. While ∼70% of peptides from the shaving-derived samples have a p*I* below 5, this is only the case for about 40 and 50% for the cell-wall-extracted and exoproteome samples, respectively. This indicates that the tryptic shaving approaches preferentially result in the identification of negatively charged peptides. This could either be due to a reassociation of positively charged peptides with the negatively charged cell wall during the shaving reaction or to an over-representation of negatively charged amino acids on the cell surface.

Strain-Specific Protein Identifications in Sub-Proteome Fractions

A comparison of the total protein identifications revealed that the different analyzed sub-proteome fractions from strain USA300 were more complex than those from strain Newman (Figure 2, Figure S3). Furthermore, a search for potential

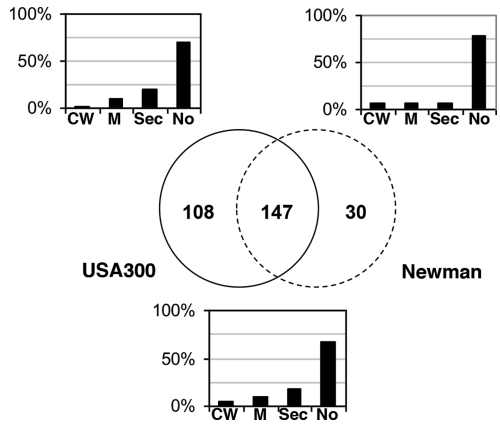

Figure 2. Predicted subcellular localization of the proteins identified for strains USA300 and Newman. The overlapping and unique proteins identified in the four sub-proteome fractions from strains USA300 and Newman were analyzed with respect to their predicted subcellular localization as previously described.[30] CW, covalently wall-bound proteins; M, transmembrane and lipoproteins; Sec, secreted proteins; No, proteins with no predicted motif for subcellular localization.

signal peptides, transmembrane domains, and cell-wall-binding domains revealed that most proteins that were exclusively identified in samples from strains USA300 or Newman lack such signals, which suggests a predominant cytoplasmic localization (Figure 2, upper panels). Since about 4 times more unique proteins were identified in samples from strain USA300 than in samples from strain Newman, it seems that strain USA300 has a higher propensity for "extracellular cytoplasmic protein" (ECP) localization or cell lysis. This view is supported by the observation that the bifunctional staphylococcal autolysin Atl—a typical cell-wall-bound protein—was identified by MS in the exoproteome of strain USA300 but not in the exproteome of strain Newman. To investigate the possible lysis of strain USA300, Western blotting experiments were performed in which the localization of the cytoplasmic marker protein TrxA was assessed. As

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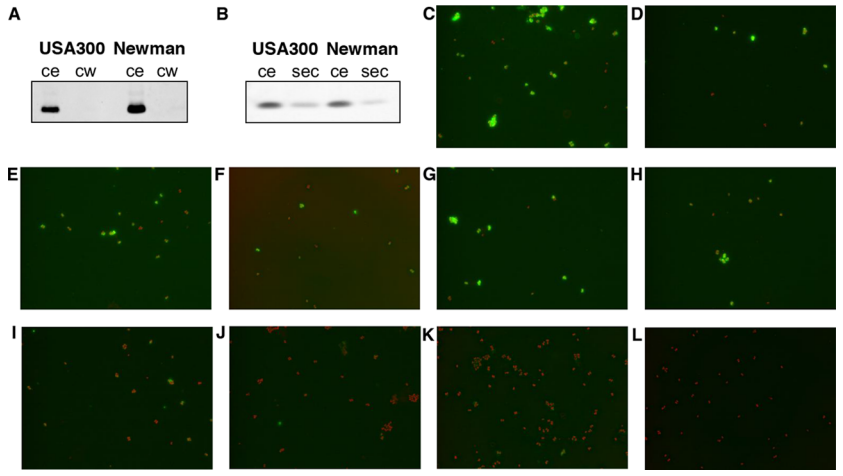

Figure 3. Lysis controls and verification of cell surface localization of IsaA. (A) Cells were harvested from cultures with an OD_{600} of 0.2 and washed, and non-covalently cell-wall-bound proteins were extracted with KSCN. The extracted wall proteins (cw) as well as crude cell extracts (ce) were analyzed by Western blotting with specific antibodies against the cytosolic marker protein TrxA. (B) Cultures were harvested at an OD_{600} of 0.2. Cells were separated from the growth medium by centrifugation, and the presence of TrxA in crude cell extracts (ce) or growth medium fractions (sec) was assessed by Western blotting with specific antibodies. (C) Cells of *S. aureus* Newman Δ*spa*Δ*sbi* were harvested at an OD_{600} of 0.2, incubated with an IsaA-specific antibody plus a secondary antibody labeled with AlexaFluor 594, and inspected by fluorescence microscopy. As a control for specific antibody binding, the IsaA-specific antibody was preincubated with increasing amounts of purified IsaA prior to fluorescence microscopy: (D) 10 pg IsaA, (E) 100 pg IsaA, (F) 1 ng IsaA, (G) 10 ng IsaA, (H) 100 ng IsaA, (I) 1 μg IsaA, (J) 10 μg IsaA. Antibodies directed against (K) the secreted thermonuclease Nuc or (L) the cytosolic marker protein TrxA were applied as negative controls for immunofluorescence.

shown in Figure 3A, no TrxA was extracted from the cells with KSCN under conditions that were similar to those applied for cell surface shaving with trypsin. This implies that little if any lysis occurred during the isolation of the different cell-associated sub-proteome fractions. On the other hand, about 2-fold more TrxA was detectable in growth medium fractions of strain USA300 than in the equivalent fractions of strain Newman (Figure 3B). This suggests that strain USA300 has a higher propensity for ECP than strain Newman and that this phenomenon had occurred already during culturing. This is an important observation because it implies that the cytoplasmic proteins identified on the surface of staphylococcal cells used for our present sub-proteome analyses had reached the cell surface during culturing. This would be consistent with studies that have implicated membrane weakening by phenol-soluble modulins[55,56] and autolysis mediated by Atl[57] or prophage activity[58] during culturing as the main processes that direct ECPs to the cell surface and growth medium of *S. aureus*.

Interestingly, we observed a differential localization of the fibronectin-binding protein FnbpA in the strains Newman and USA300, where most of the FnbpA was secreted by strain Newman, whereas most of the FnbpA in the USA300 strain was cell-surface-localized but only accessible to soluble trypsin (Figure S5, Tables S1 and S2). These observations are consistent with a previous study showing that strain Newman produces a truncated form of FnbpA that is mostly secreted into the extracellular milieu because it has lost the C-terminal LPxTG motif for covalent cell wall anchoring.[59] Nonetheless, at least some FnbpA was shown to be retained in the cell wall of strain Newman from which it could be liberated by protoplasting.[60]

Proteins Common to *S. aureus* Strains USA300 and Newman

Despite clear differences, there are also many overlaps in the proteins identified in the respective sub-proteome fractions from *S. aureus* strains Newman and USA300 (Figure 2, Table S3). This is in line with the fact that most of the proteins identified in the present studies are encoded by the genomes of both investigated *S. aureus* strains. Interestingly, seven proteins were identified in all four sub-proteome samples from the USA300 and Newman strains (Tables S1−S3). These are the IgG-binding protein Spa, the secretory antigen SsaA, the transglycosylase IsaA, the fructose-bisphosphate aldolase class 1, the DNA-binding protein HU, and the ribosomal proteins L29 and L30. To verify cell surface exposure, an immunofluorescence control experiment was performed for IsaA, against which the highly specific monoclonal antibody 1D9 was previously developed.[24,58] As shown in Figure 3C, the IsaA-specific antibody bound effectively to the cells of a Δ*spa*Δ*sbi* mutant of strain Newman, which lacks the staphylococcal immunoglobulin-binding proteins Spa and Sbi. Titration of the antibodies with increasing amounts of purified recombinant IsaA confirmed the specificity of the IsaA antibody binding to the cells (Figure 3D−J). Furthermore, antibodies against the secreted thermonuclease, which was not detected on the cell surface of strains Newman or USA300, did not bind to the cells (Figure 3K), and the same was true for antibodies against the cytoplasmic marker protein TrxA (Figure 3L), which was also not identified as being bound to the cell surface (Figure 3A). Based on these findings, we conclude that the surface shaving approach does indeed yield specific information on the surface exposure of particular *S. aureus* proteins.

In a previous study, the commonly produced *S. aureus* peptidoglycan hydrolase LytM was proposed to be cell-surface-located based on immune electron microcopy experiments.[61] Nevertheless, others were previously unable to demonstrate binding of the purified mature LytM protein to crude cell wall preparations of *S. aureus*,[62] and in our present proteomics analyses, LytM was only detectable in the growth medium fractions of both the USA300 and Newman strains (Tables S1−S3). Furthermore, the identified LytM peptides all mapped to the C-terminal domain of the protein (Figure 4A). Since this could be due to protection of the N-terminal domain of

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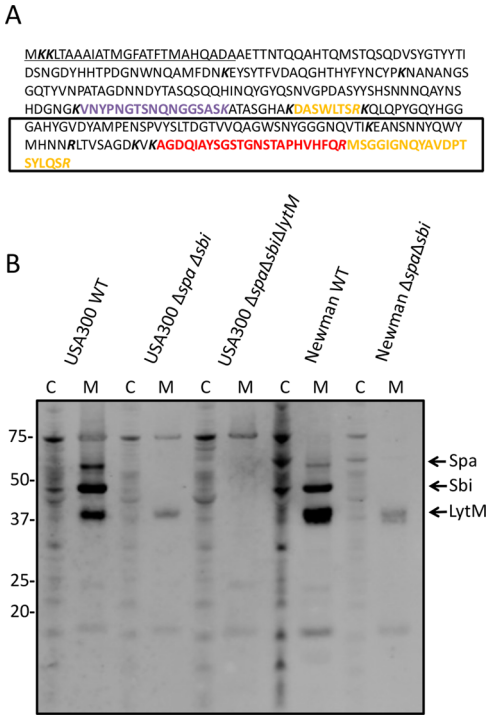

Figure 4. Peptide identification and localization of LytM. (A) The amino acid sequence of LytM and the peptides identified by MS in the growth medium fractions of *S. aureus* strains USA300 and Newman are shown. Peptides identified for both strains are marked in yellow, a peptide uniquely identified for strain Newman is marked in purple, and a peptide uniquely identified for strain USA300 is marked in red. The signal peptide is underlined, and the catalytic domain is boxed. (B) Detection of LytM in cell (C) and growth medium (M) fractions of different strains by Western blotting. Equal amounts of the different fractions of early exponential cultures of USA300 (LAC) wild-type (wt), USA300 (LAC) Δ*spa*Δ*sbi*, USA300 (LAC) Δ*spa*Δ*sbi*Δ*lytM*, Newman wt, and Newman Δ*spa*Δ*sbi* were loaded as described in the Experimental Procedures. Note that LytM (32 kDa) has a slightly aberrant mobility in LDS-PAGE.

LytM by the cell wall or to its degradation by *S. aureus* proteases, we tested the localization of LytM by Western blotting using a LytM-specific polyclonal rat antibody. As shown in Figure 4B, full-size mature LytM (~32 kDa) was detected solely in the growth medium fractions of the USA300 wild-type strain, a USA300 Δ*spa*Δ*sbi* mutant, the Newman wild-type strain, and a Newman Δ*spa*Δ*sbi* mutant. In contrast, the LytM protein was not only absent from the growth medium fractions of a USA300 Δ*spa*Δ*sbi*Δ*lytM* triple mutant but also from the cell fractions of all investigated USA300 and Newman strains (Figure 4B). These observations show that, at least in our present experimental setup, LytM was secreted into the growth medium, consistent with the results obtained by cell surface shaving and exoproteome analyses. In turn, this implies that the lack of identified N-terminal peptides of LytM is either due to the inability of trypsin to cleave the N-terminal domain of LytM or a lack of detectability of peptides liberated from the N-terminal domain.

Epitope Mapping in Surface Proteins of *S. aureus*

Altogether, our proteomics analyses led to the identification of 285 unique proteins (Figure 2). Thereof, we selected 54 proteins for further analysis by Pepscan epitope mapping. The selected proteins include predicted cytosolic, membrane, lipid-modified, cell-wall-associated, and extracellular proteins (Table S4, sheets A and B). For all of these proteins, linear 15-mer peptide arrays with 11-mer overlaps were prepared. Additionally, arrays with CLIPS-constrained 15-mers were prepared for the IsaA, LytM, and Nuc proteins. Plasma donated by seven different EB patients, who have high IgG responses against staphylococcal proteins,[46] was then used for the detection of immunogenic domains. In total, we analyzed the interaction of 5911 peptides with IgGs in the plasma of EB patients (Table S4, sheet A). This revealed 358 human IgG-binding peptides from 47 different *S. aureus* proteins (Table S4, sheets B and C). Merging of overlapping sequences finally resulted in the delineation of 201 immunodominant domains (Table S4, sheet B). The most conserved peptides recognized by IgGs from human serum belong to a zinc-binding lipoprotein (A6QJP6), the extracellular-matrix-binding protein (Emp), and the iron-regulated surface determinant (IsdB) (Table S4, sheet A; Figure S4). The relative localization of immunodominant protein regions and peptides identified in the different investigated sub-proteomes is schematically presented in Figure 5, Figure S5, and Table S5. Notably, the peptides from certain other cell-surface-exposed proteins did not bind IgGs from EB patient plasma (i.e., FtsL, RS7, the DNA-binding protein HU, a putative thioredoxin, two CsbD-like proteins, and an uncharacterized protein), and these proteins can thus be regarded as negative controls for our epitope mapping analysis. Of note, this lack of binding of IgGs from EB patients to FtsL was also observed in our previous study where we assessed antibody responses to non-covalently cell-wall-bound *S. aureus* proteins by enzyme-linked immunosorbent assays.[19]

The highest number of IgG-binding peptides was identified for IsdB, and a close analysis of their location within this protein revealed a highly immunodominant N-terminal domain between amino acids 47 and 129. This domain was also identified by shaving with immobilized trypsin as being cell-surface-exposed (Figure 5A, shaving 1). A second immunogenic region was detected in the C-terminal part of IsdB between amino acids 395 and 565, which is also identified by surface shaving. Notably, surface shaving with immobilized trypsin (shaving 1) identified only peptides from the N- and C-terminal regions in IsdB, whereas shaving with soluble trypsin (shaving 2) also identified peptides from the central region of IsdB. The observation that the central region of IsdB is only a substrate for soluble trypsin suggests that it is protected by the cell wall. This view is supported by the observation that peptides from the central region were also identified in extracellular IsdB, which is no longer protected by the cell wall due to its release into the growth medium.

A remarkable finding was that especially the surface-exposed N-terminal pro-region of the autolysin Atl was very well recognized by the IgGs from EB patients (Figure 5B). It should be noted that this region is removed from Atl during the processing of the exported pro-Atl into the active amidase and glucosaminidase domains.[63] Furthermore, we detected strong immunogenic signals in apparently surface-exposed domains of other proteins, such as the extracellular-matrix-binding protein (Emp), coagulase (Coa), fibronectin-binding protein A (FnbpA), and clumping factor B (ClfB) (Figure S5). However, we also observed that for some proteins, such as the chemotaxis inhibitory protein (CHIPS), different protein regions were identified by cell surface shaving and epitope mapping. In the case of CHIPS, we observed an IgG-binding domain in the N-terminus, next to the signal peptide, whereas

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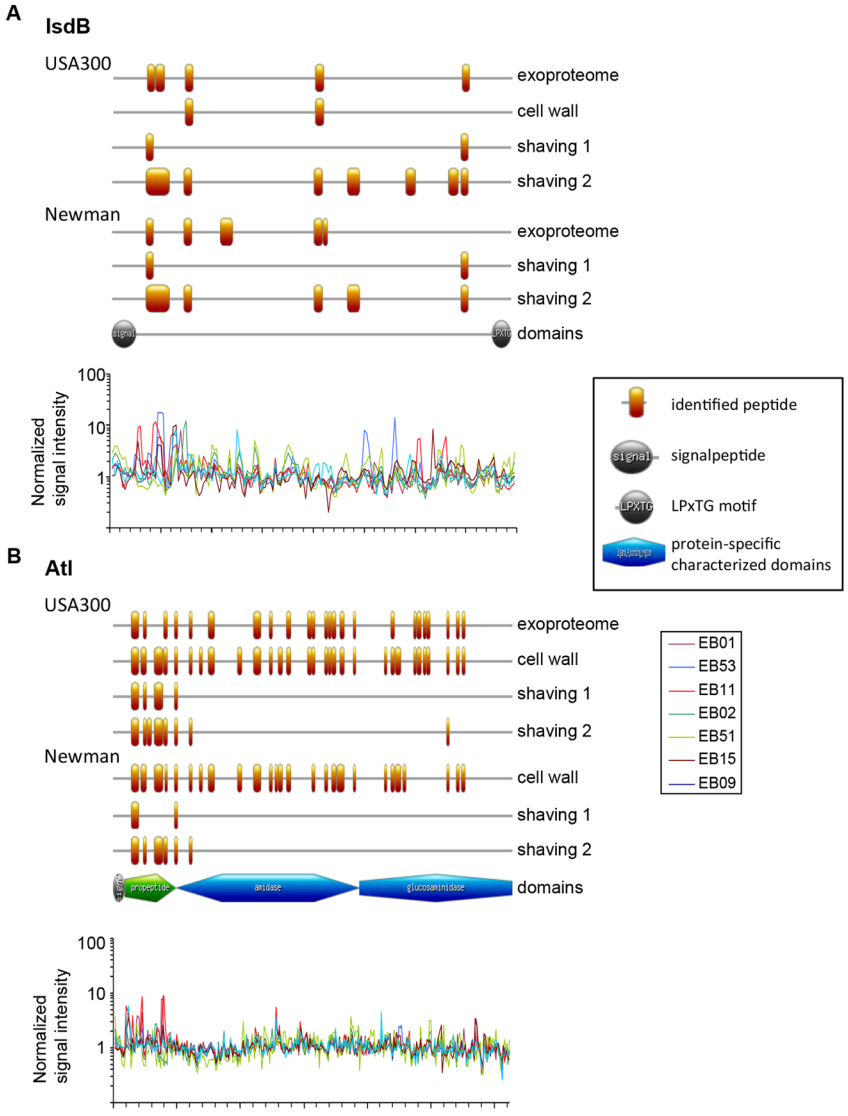

Figure 5. Comparison of proteome and epitope mapping results. Peptides of the (A) IsdB and (B) Atl proteins as identified by proteomics analyses of four different sub-proteomes of *S. aureus* strains USA300 and Newman are highlighted in the linearly depicted protein sequence. In addition, known protein domains are indicated. The graphs display the signals from the epitope mapping normalized to the median signal of the respective protein and serum for the seven different EB patients.

proteomics identified most peptides in the C-terminal half of this protein (Figure S5). Interestingly, immunogenic domains positioned next to the signal peptides were observed also for other proteins, such as Atl, Emp, the FPRL1 inhibitory protein (FLIPr), the lipoprotein YkyA, a peptide-binding protein (Q2FKI7), and the MHC class II analog protein (Omp7; Figure S5). Lastly, the epitope mapping revealed also 67 IgG-binding domains in typical cytoplasmic proteins that were found to be exposed on the *S. aureus* cell surface. These include the fructose-bisphosphate aldolase Alf1, the enolase Eno, the triosephosphate isomerase TpiS, the elongation factors G and Ts, the ribosomal proteins S5, S13, and L25, the phosphoglycerate kinase Pgk, and the glyceraldehyde-3-phosphate dehydrogenase GAPDH (Figure S5).

DISCUSSION

The worldwide spread of highly antibiotic-resistant lineages of *S. aureus*, resulting in increased morbidity and increased health-care costs, calls for the development of novel anti-staphylococcal therapies. Such therapies could very well include active or passive immunization.[20,36] Unfortunately, attempts to develop anti-staphylococcal immunotherapy have so far remained unsuccessful, which may relate to the selected antigens. We therefore set out to define cell-surface-exposed immunodominant epitopes in two important model strains of *S. aureus*, namely, the laboratory MSSA strain Newman and the community-acquired MRSA strain USA300.

Various elegant studies on the staphylococcal exoproteome, wall proteome, and surfacome were published in recent years.[30,34,40−42] However, the usefulness of these studies for the rational design of novel anti-staphylococcal immunotherapy was limited by the fact that they were based on different *S. aureus* isolates grown in different media under different conditions and that very different technical approaches were applied.[38,64] Therefore, we designed an integrated workflow in which the cell wall proteomes, surfacomes, and exoproteomes of two different strains (Newman and USA300) grown under identical conditions were analyzed in parallel following exactly the same protocol. An important outcome was that, despite

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substantial differences, there was indeed a significant overlap in the identified proteins of both investigated strains. A major pool of differentially identified proteins was formed by typical cytoplasmic proteins that were detected in and on the wall of both strains. Such ECPs have been shown to leave the cytoplasm to become localized to the cell wall or the extracellular milieu by a variety of mechanisms, including autolysis,[57] membrane weakening by cytolytic peptides,[55,58] and prophage activity.[65−67] Importantly, it is becoming increasingly clear that some of them have so-called "moonlighting functions" in staphylococcal virulence and epidemiology.[55,68,69] In line with this notion, our present studies show that the plasma of EB patients suffering from chronic staphylococcal wound colonization does indeed contain IgGs that specifically bind to a range of cytoplasmic proteins and that there is a significant overlap between the IgG-binding domains and domains identified in our proteomic analysis (e.g., TpiS, RS5, Eno, GAPDH). Notably, antibodies directed against Eno have been demonstrated to cause opsonophagocytic killing of *S. aureus*, and this protein is a known protective antigen on the cell surface of *Streptococcus suis*.[39,70] Furthermore, vaccination of rats with recombinant Eno caused protection against dental caries.[71]

The direct comparison of the shaving approaches 1 and 2 using immobilized and soluble trypsin, respectively, demonstrates how different the outcome of these two closely related approaches is. The shaving reaction with the soluble trypsin resulted in the liberation of 2.6 times more peptides representing 2.2/2.35 times more proteins than the assay with the immobilized trypsin. However, the majority of the peptide and protein identifications from the assays with the immobilized trypsin overlap with the results from the experiments using the soluble trypsin. The majority of the protein identifications that were unique for the soluble trypsin shaving approach are cytosolic proteins, like ribosomal and metabolic enzymes and also the pre-protein translocase unit SecA1. This suggests that the soluble trypsin penetrates the whole cell wall and digests also membrane proteins, thereby enabling leakage of cytosolic proteins into the environment. However, with the soluble trypsin approach, we were also able to identify known cell-envelope-associated proteins like penicillin-binding protein 3 (PbpC), the iron-regulated surface determinant protein IsdB, subunits of the ATP synthetase complex (α, β, b), and the iron-compound-binding protein Q2FEK8/A6QJC5. In the corresponding negative controls, only two peptides were reproducibly identified per strain (Tables S1 and S2). From these control experiments, we conclude that the shaving assays are essentially free of false-positive identifications due to cell lysis or spontaneous liberation of proteins from the bacterial cell envelope.

Notably, several surface-exposed proteins that were subjected to epitope mapping revealed clusters of epitopes that were specifically localized to certain domains within these proteins. This is best exemplified by the wall-anchored IsdB protein, the autolysin Atl, the adhesin Emp, and the transglycosylase IsaA. The significance of these results is underlined by efforts to develop an IsdB-based vaccine, which showed that this protein is highly immunogenic,[72,73] and studies in which the binding sites of human monoclonal antibodies were located to IsdB domains between residues 50 and 285[74] and between residues 323 and 441.[75] In the present studies, we identified the IsdB domain between residues 47 and 129 as a hot spot for recognition by IgGs from EB patients. Moreover, epitopes recognized by two distinct monoclonal antibodies against IsdB[75] were also precisely identified in our present analysis (Table S4, marked in red), and another epitope recognized by one of the investigated monoclonal antibodies was located precisely between two presently mapped IsdB epitopes separated by only five residues (Table S4, marked in blue). These overlaps are striking, especially because of the very different approaches that have been applied. Of note, in the context of efforts to develop vaccines against *S. aureus*, it should be mentioned that a recent trial to apply IsdB for vaccination to protect patients against postoperative staphylococcal infection failed because recipients of the vaccine showed higher mortality than the placebo group upon *S. aureus* infection.[76] Possibly, this relates to an absence of interleukin-2 at the time of vaccination of the respective patients, which may have led to ineffective or misdirected cell-mediated immune responses to the IsdB vaccine.[76] Less effort has as yet been put into the development of vaccines containing Emp or Atl, but passive immunization with antibodies against Emp did show a reduction of staphylococcal loads in an animal model.[77] Furthermore, Atl was previously identified as a strong antigen through gel-based immunoproteomic approaches on growth medium fractions of *S. aureus*.[78,79] However, inclusion of pro-Atl in an octavalent antigen mixture did not provide protection against *S. aureus* in a murine infection model.[50] Interestingly, passive immunization with a monoclonal antibody against IsaA was shown to give protection against *S. aureus* in a central venous catheter-related infection model and a sepsis survival model.[80,81] Likewise, prophylactic treatment of mice with another IsaA-specific human monoclonal antibody (1D9) improved the survival of mice in a bacteremia model.[24] We have recently identified the binding domain of 1D9 to be located in the N-terminal part of the mature IsaA protein.[19] Remarkably, in the present study, only the C-terminal region of IsaA was identified and, in fact, this region was detected in all fractions analyzed (Figure S5). This finding can be explained by the fact that the N-terminal region of IsaA has a relatively low number of arginine and lysine residues resulting in peptides that are difficult to identify by MS. However, the lack of identification of N-terminal IsaA peptides may also indicate a three-dimensional structure of the N-terminal domain that is resilient to cleavage by trypsin. In fact, a similar situation was encountered for LytM, where the N-terminal region also remained undetected in our proteome analyses, which was shown not to be related to a possible protection by the bacterial cell wall. Importantly, the present investigation of LytM exemplifies the value of a combined MS and epitope profiling approach for the identification of possible vaccine targets. Based on the epitope mapping alone, one could argue that the LytM protein harbors several immunogenic epitopes that could be useful targets for vaccination. However, our MS analyses showed that LytM is exclusively secreted into the growth medium, and this was subsequently verified by Western blotting. This makes LytM a less attractive vaccine target because cell-surface-exposed targets are generally preferable for this purpose.[43] On the other hand, the combined MS and epitope mapping analyses pinpoint IsaA as a potentially useful target for vaccination approaches, which is in line with previous studies showing that certain monoclonal antibodies against this protein are protective against staphylococcal infections, at least in murine infection models.[24,80,81]

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Interestingly, the IgGs from different EB patients did not always bind to the same *S. aureus* proteins or protein domains. This may have different reasons. First, the Pepscan approach was based mainly on linear peptides, and therefore, certain conformational epitopes of particular proteins recognized by IgGs of the EB patients might be overlooked. For example, the aforementioned human monoclonal antibody 1D9 recognizes a conformational epitope in IsaA and does not bind to linear IsaA-derived peptides.[82] A second reason for the observed variability in IgG responses could be that the different patients did not carry the same *S. aureus* types.[46] Thus, it is conceivable that different surface-exposed antigens were either produced at low levels or not at all. This is a realistic possibility since an analysis of 58 different *S. aureus* genome sequences showed large variations in the composition and presence of genes for 25 surface-bound and/or immune-evasive proteins.[33] Eighteen of these proteins were identified by our proteomic analyses of strains USA300 and Newman (i.e., ClfA, ClfB, Coa, Eap, Efb, EbpS, Emp, EsxA, FLIPr, FnbA, IsdA, IsdB, SasG, Sbi, SCIN, SdrD, Spa, VWbp), and 12 were analyzed by our Pepscan approach showing that they were recognized by antibodies from at least one EB patient (i.e., ClfB, Coa, EbpS, Efb, Emp, EsxA, FLIPr, FnbA, IsdB, SasG, Sbi, SdrD). It is relevant to note that some of the latter proteins, such as Coa, IsdA, IsdB, and Spa, have been implicated as potentially effective targets for immunotherapy.[83−91]

CONCLUSIONS

Altogether, our present analyses highlight several immunodominant cell-surface-exposed proteins of *S. aureus* and specific subdomains of these proteins as potential targets for novel active or passive immunization approaches. These include the covalently cell-wall-bound proteins ClfB and IsdB, a YkyA-like cell-wall-binding lipoprotein, the membrane proteins EbpS and LtaS, the non-covalently cell-wall-bound and secreted proteins Atl, Emp, IsaA, and Sbi, and the cytoplasmic proteins Afl1, GAPDH, and Eno. Future studies will show whether any of these proteins can indeed serve as effective targets for anti-staphylococcal immunotherapy.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jproteome.0c00043.

Figure S1. Original Western blots. Figure S2. Comparison of the p*I* values of identified proteins and peptides in the different sub-proteome fractions. Figure S3. Predicted subcellular localization of the proteins identified by the different approaches for strains USA300 and Newman. Figure S4. Comparative overview of the results from the Pepscan analysis. Figure S5. Comparison of the results from the proteomic studies and epitope mapping (PDF)

Table S1. Results from the mass spectrometric analyses for the samples derived from *S. aureus* strain USA300 (XLS)

Table S2. Results from the mass spectrometric analyses for the samples derived from *S. aureus* strain Newman (XLS)

Table S3. Comparison of the peptide identifications for *S. aureus* strains USA300 and Newman XLS)

Table S4. Results from the Pepscan analysis (XLS)

Table S5. Comparison of epitope mapping with the peptide identifications from the shaving and exoproteome analyses (XLSX)

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Author Contributions

[#]A.D., M.W., M.M.v.d.K.-P., and E.R. contributed equally to this work.

Notes

The authors declare the following competing financial interest(s): J.J.B. and J.W.B. were employed by Pepscan Therapeutics BV. H.P.J.B. and H.G. were employed by IQ Therapeutics. The other authors declare no conflicts of [▽]interest.

[▽]Deceased (M.J.).

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■ ABBREVIATIONS

ACN, acetonitrile; BSL-2, biosafety level 2; CC, clonal complex; CFU, colony forming unit; DTT, dithiothreitol; ECP, extracellular cytoplasmic protein; IAA, iodoacetamide; KSCN, potassium thiocyanate; LDS, lithium dodecyl sulfate; MLST, multi-locus sequence typing; MLVA, multiple-locus variable number tandem repeat analysis; MRSA, methicillin-resistant *S. aureus*; MSSA, methicillin-sensitive *S. aureus*; MS, mass spectrometry; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; OD_{600}, optical density at 600 nm; PBS, phosphate-buffered saline; p*I*, isoelectric point; RPMI, Roswell Park Memorial Institute 1640 medium; ST, sequence type; TCA, trichloroacetic acid; TFA, trifluoroacetic acid; TSB, tryptic soy broth

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