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Comparative Exoprotein Profiling of Different *Staphylococcus epidermidis* Strains Reveals Potential Link between Nonclassical Protein Export and Virulence

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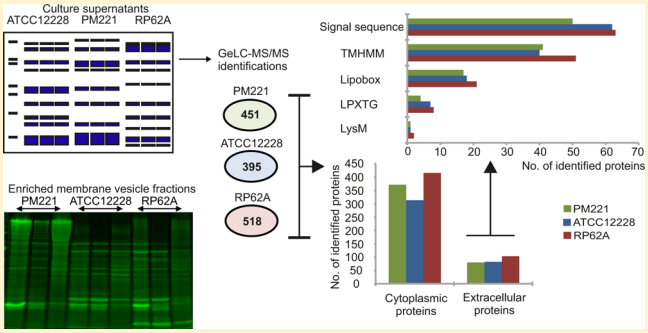
Supporting Information

ABSTRACT: *Staphylococcus epidermidis* (SE) includes commensal and pathogenic strains capable of infecting humans and animals. This study reports global exoproteome profiling of bovine mastitis strain PM221 and two human strains, commensal-type ATCC12228 and sepsis-associated RP62A. We identified 451, 395, and 518 proteins from culture supernatants of PM221, ATCC12228, and RP62A, respectively. Comparison of the identified exoproteomes revealed several strain-specific differences related to secreted antigens and adhesins, higher virulence capability for RP62A, and similarities between the PM221 and RP62A exoproteomes. The majority of the identified proteins (~80%) were predicted to be cytoplasmic, including proteins known to be associated in membrane vesicles (MVs) in *Staphylococcus aureus* and immunogenic/adhesive moonlighting proteins. Enrichment of MV fractions from culture supernatants and analysis of their protein composition indicated that this nonclassical protein secretion pathway was being exploited under the conditions used and that there are strain-specific differences in nonclassical protein export. In addition, several predicted cell-surface proteins were identified in the culture media. In summary, the present study is the first in-depth exoproteome analysis of SE highlighting strain-specific factors able to contribute to virulence and adaptation.



KEYWORDS: *Staphylococcus epidermidis, exoproteome, GeLC−MS/MS, adaptation, virulence, nonclassical secretion, membrane vesicles, moonlighting proteins*

INTRODUCTION

Staphylococcus epidermidis, belonging to coagulase-negative staphylococci (CoNS), is a common colonizer of the human skin that has evolved a benign relationship with the host. Although SE may even balance the healthy microbiota of the human skin and mucous membranes,[1−3] it is an opportunistic pathogen and one of the most frequent causes of nosocomial infections, particularly in immunocompromised patients.[4] These SE nosocomial infections are rarely life-threatening, as SE lacks many virulence determinants, such as the toxins and aggressive exoenzymes often associated with other major pathogens.[1,5,6] However, SE infections are difficult to treat because of the frequent occurrence of multidrug-resistant strains and because of SE's ability to form biofilms that cause resistance both to drugs and the host immune system.[6,7]

In addition to being an opportunistic pathogen, SE together with the more aggressive and coagulase-positive *S. aureus* (SA) is a major causative species of intramammary infections (IMI) in economically important livestock such as cows. Mastitis caused by SE and other CoNS is often indolent and difficult to diagnose, and it is commonly associated with mildly clinical or subclinical persistent IMIs.[8−10] Recently, it has also been suggested that the humans could be the source of IMI in dairy cows because of the similarities in the genotypes of some of the SE strains associated with IMI with strains isolated from human skin.[11−13] The bovine SE also seems to share same virulence determinants and antimicrobial resistance patterns with strains isolated from human infections.[14]

Proteins secreted into the extracellular milieu (i.e., the exoproteome) include factors that help the bacteria to select the host and to adapt to different environments. Exoproteins of human and bovine SA strains have been extensively analyzed in the search for novel strategies to combat staphylococcal infections.[15−24] With respect to SE, proteome-level studies are limited to a few reports focusing on comparisons of the proteins from clinically important human strains.[25,26] The present study was designed to screen for strain-specific proteins that

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could play a role in the adaptation and infection in bovine and human hosts. The analytical technique chosen was GeLC−MS/MS because this has been shown to maximize the number of identifications, leading to near-to-complete proteome coverage,[15,27−31] and because it is also useful for elucidating strain-specific marker proteins in closely related bacteria.[27] Because this present study provides a deeper insight into the expressed exoproteomes of different SE strains, it clearly broadens our understanding of the infectious potential of this opportunistic species.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

SE strains, PM221 isolated from bovine persistent IMI,[32,33] ATCC12228 representing a non-infection-associated and non-biofilm-forming[5] strain, and the virulent ATCC35984 (hereafter, RP62A)[6] strain, were grown on tryptic soy (TS) (Becton Dickinson, NJ, USA) agar at 37 °C o/n. Individual colonies were inoculated in duplicate in TS broth (50 mL; $OD_{600} \approx 0.06-0.07$), with the strains being cultured at 37 °C with shaking at 250 rpm (Certomat, Sartorius, Goettingen, Germany). The production of virulence factors in batch cultures by SA has been reported to be most efficient during the late-exponential phase of growth[34] and therefore supernatants from the selected bovine and human strains were withdrawn at the corresponding growth conditions. Growth curves were first created for the selected SE strains in TS broth to determine the optimal sampling point at which each strain could be assumed to be efficiently secreting virulence factors but showing minimal cell lysis (Supporting Information Figure 1). This showed that PM221, ATCC12228, and RP62A share highly similar patterns of growth until entering the stationary phase that occurs at $OD_{600} \sim 3.0$ for RP62A and ∼4.0 for PM221 and ATCC12228. On the basis of the growth kinetics, samples were withdrawn from exponentially growing cells at an OD_{600} of 1.9 (±0.2) (Supporting Information Figure 1), and the supernatants were harvested by centrifugation (5000*g*, 10 min, 4 °C). In order to inhibit different proteases, the supernatants were supplemented with an appropriate amount of the cOmplete Protease Inhibitor tablets (Roche Applied Science, Basel, Switzerland) prior to additional filtering through 0.2 μm cellulose acetate membranes (Corning Incorporated, NY, USA).

Enrichment of the Membrane Fraction from Culture Media and Its Protein Composition Analysis

The membrane vesicle fraction from filtered culture supernatants (45 mL) was enriched by ultrafiltration using 100 kDa membrane units (Vivaspin20, GE Healthcare, Little Chalfont, UK). Proteins from filtered culture media, supernatant flow-throughs, and concentrated retentates after ultrafiltration were precipitated by 10% TCA/70% EtOH and centrifugation (8000*g*, 40 min, 4 °C). Proteins were solubilized in 2.5× Laemmli sample buffer.[35] The protein composition was assessed by SDS-PAGE using a 12% polyacrylamide gel with Tris-glycine SDS running buffer (25 mM Tris, 192 mM Glycine, 0,1% SDS, pH 8.3) followed by SYPRO-Orange (Sigma-Aldrich, St. Louis, MO, USA) staining.[36] The fluorescent image was captured with an AlphaImager gel documentation system (ProteinSimple, Santa Clara, CA, USA).

Exoproteome Identification with GeLC−MS/MS

The proteins from filtered culture supernatants were precipitated with 10% TCA/70% EtOH as described above. Prior to GeLC−MS/MS analysis, protein pellets were subjected to additional purification using the 2D CleanUp kit (GE Healthcare). The purified protein samples (∼100 μg of protein, quantified with 2D Quant Kit, GE Healthcare) were dissolved in 2.5× Laemmli buffer. Proteins were separated with SDS-PAGE using Mini-PROTEAN TGX Precast Gel (Any kD, Bio-Rad Laboratories, Hercules,CA, USA) followed by Coomassie Blue staining, and the resulting gel lanes (Supporting Information Figure 2) were subjected to in-gel digestion and nanoLC−MS/MS essentially as described previously.[27] Briefly, each gel lane was sliced into seven fractions; the proteins were reduced, alkylated, and in-gel digested with trypsin (sequencing grade modified trypsin V5111, Promega, Madison,WI, USA). The resulting tryptic peptides were extracted from the gel, with the recovered peptides being present in a final volume of ∼130−150 μL. The samples were dried and dissolved in 0.1% trifluoroacetic acid (TFA). The tryptic peptides were analyzed with nanoLC−MS/MS using an Ultimate 3000 nanoliquid chromatograph (Dionex/Thermo Fisher Scientific, Waltham, MA, USA) and QSTAR Elite hybrid quadrupole time-of-flight mass spectrometer (Applied Biosystems/MDS Sciex, Foster City, CA, USA) with nano-ESI ionization. MS data were acquired automatically with Analyst QS 2.0 Software (Applied Biosystems) using SMART IDA.

Databases of predicted protein sequences encoded by the PM221 (2530 entries), ATCC12228 (2485 entries, AE015929.1−AE015935.1), and RP62A (2526 entries, CP000029.1−CP000028.1) genomes and plasmids were compiled to obtain in-house databases for protein identification. After LC−MS/MS, MS/MS data obtained from three biological replica samples were first searched separately and then combined and searched against protein databases using Mascot (version 2.4.0, Matrix Science, Boston, MA, USA) and Paragon (version 4.0.0.0, Applied Biosystems/MDS Sciex) search algorithms through the ProteinPilot interface (version 4.0, Applied Biosystems/MDS Sciex). The search criteria for Mascot searches were trypsin digestion with one allowed miss-cleavage, carbamidomethyl modification of cysteine as a fixed modification, oxidation of methionine as a variable modification, peptide mass tolerance of 50 ppm, MS/MS fragment tolerance of 0.2 Da, and peptide charges of 1+, 2+, and 3+. Parameters for Paragon searches using the Rapid search mode included the previously mentioned methionine and cysteine modifications. The Compid tool[37] was used to parse significant hits from the compiled Mascot and Paragon output files into tab-delimited data files. Protein identifications that had probability-based Mascot Mowse scores ≥75 and $p < 0.05$ and/or Paragon Unused ProtScores ≥1.3 and $p < 0.05$ were considered to be reliable high-confidence identifications. The mass spectrometry proteomics data have been deposited into the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository[38] under the data set identifier PXD000631. To estimate the false discovery rates (FDRs), all Mascot and Paragon searches were repeated using identical search parameters and validation criteria against PM221, ATCC12228, and RP62A decoy databases containing all of the protein sequences in both forward and reverse orientations. The sequences were reversed using the Compid tool, and the FDR percentages were calculated using the formula $2 \times n_{reverse}/(n_{reverse} + n_{forward})$ given by Elias et al.[39] The calculated FDRs in each of the three strains were as follows: PM221 Mascot 3.4%, Paragon 0%; ATCC12228 Mascot 5.0%, Paragon 0%; and RP62A Mascot 1.7%, Paragon 0%.

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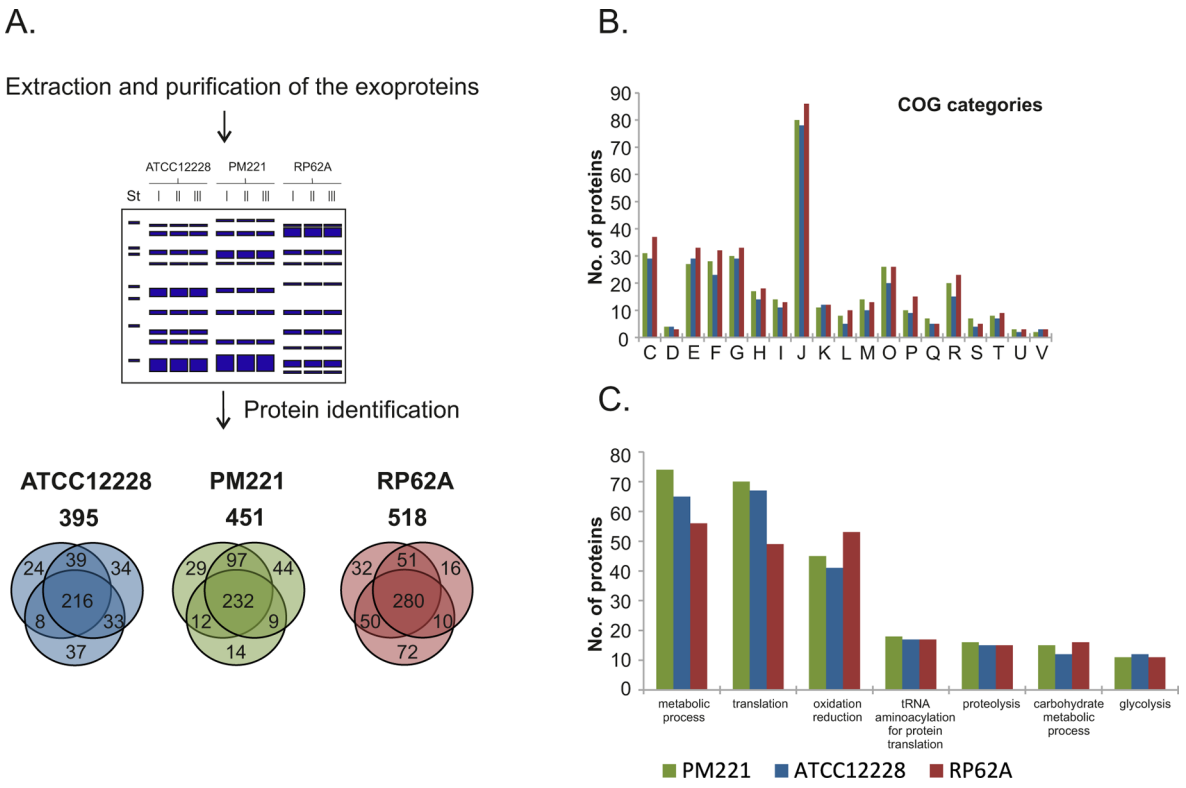

Figure 1. Exoproteome identifications form SE strains PM221, ATCC12228, and RP62A. (A) GeLC−MS/MS identification of PM221, ATCC12228, and RP62A exoproteins. Three biological replicates (I−III) were analyzed, and two database search engines, Mascot and Paragon, were used for protein identification. The data from biological replicates were first processed separately followed by a compiled database search. The numbers in circles refer to the identifications of the separately processed biological replicates, whereas the total identification numbers come from the search results of the combined biological replicates. (B) COG (clusters of orthologous groups) classification of all identified proteins. COG categories: C, energy production and conversion; D, cell cycle control, cell division, chromosome partitioning; E, amino acid transport and metabolism; F, nucleotide transport and metabolism; G, carbohydrate transport and metabolism; H, coenzyme transport and metabolism; I, lipid transport and metabolism; J, translation, ribosomal structure and biogenesis; K, transcription; L, replication, recombination and repair; M, cell wall/membrane/envelope biogenesis; O, post-translational modification, protein turnover, chaperones; P, inorganic ion transport and metabolism; Q, secondary metabolites biosynthesis, transport and catabolism; R, general function prediction only; S, function unknown; T, signal transduction mechanisms; U, intracellular trafficking, secretion, and vesicular transport; and V, defense mechanisms. (C) The main GO (Gene Ontology) annotation groups of biological processes of all identified proteins.

Computational Proteome Analyses

Theoretical molecular weights (MW) and isoelectric points (pI) for all PM221, ATCC12228, and RP62A proteins were acquired with ProMoST protein modification screening tool (http://proteomics.mcw.edu/promost.html). The GRAVY values (grand average hydropathy) were calculated using the Protein GRAVY calculation tool (http://www.bioinformatics.org/sms2/protein_gravy.html). The cellular location for each protein was estimated using the PSORTb localization prediction tool at http://www.psort.org/psortb/. The SignalP 4.0 Server (http://www.cbs.dtu.dk/services/SignalP/) was used to detect potential signal sequences in each protein sequence, whereas proteins exploiting nonclassical secretion mechanisms were predicted using the SecretomeP 2.0 Server (http://www.cbs.dtu.dk/services/SecretomeP/). The presence of potential transmembrane domains was identified using the TMHMM Server v. 2.0 at http://www.cbs.dtu.dk/services/TMHMM/. Lipoproteins were identified by scanning for a lipobox with the PRED-LIPO prediction program (http://biophysics.biol.uoa.gr/PRED-LIPO/index.jsp) and a lipoprotein consensus sequence L[AS][AG]C[40] with ScanProsite tool (http://ca.expasy.org/tools/scanprosite/). The presence of several cell wall anchor motifs and domains was obtained with the ScanProsite tool; a leucine-rich repeat region LRR involved in protein−protein and protein−ligand interactions; LysM, a C-terminal lysine that mediates noncovalent attachment to peptidoglycan; and motifs for sortase-mediated protein anchoring: LPXTG, NPQTN, and EVPTG (C-terminal anchoring signal motifs) as well as TLXTC, a sortase signature motif with a catalytic cysteine residue.[40−46] TIGR number and description, Prosite number, InterProScan number and description, biological process from gene ontology (GO), and GO numbers were acquired from InterProScan (http://www.ebi.ac.uk/Tools/pfa/iprscan/) analyses. KEGG annotations for the proteins were obtained from http://www.genome.jp/tools/kaas/, and the resulting KO numbers were converted to COGs (clusters of orthologous groups) at http://www.genome.jp/files/ko2cog.xl and http://www.ncbi.nlm.nih.gov/COG/old/palox.cgi. Two complementary tools were used for the prediction of orthologous groups among the PM221, ATCC12228, and RP62A proteomes. In brief, protein sequences were compared with BLAST,[47] and orthologous groups were predicted with OrthoMCL tool.[48] Alternatively, reciprocal best BLAST hits (RBBH) were extracted and merged with sequences from the same species that are more similar to the RBBH than to any sequences from other species, and fully interconnected RBBH groups were identified using Cliquer.[49] The OrthoMCL tool was also utilized for identifying groups of paralogous genes. Additional

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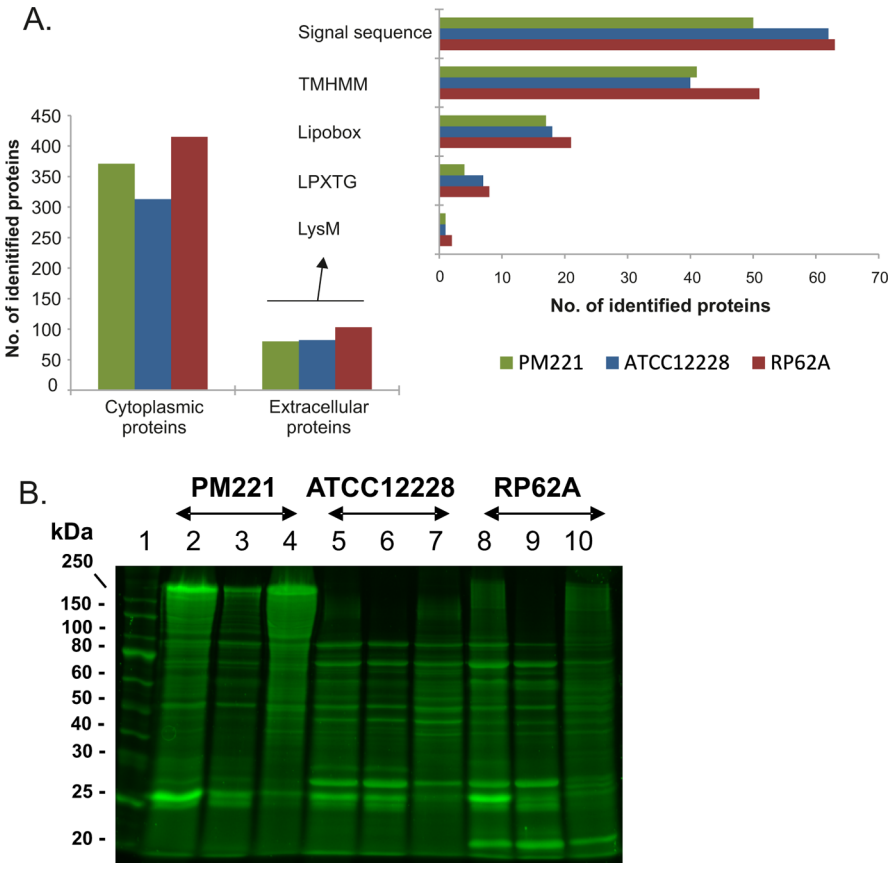

Figure 2. Majority of the identified proteins are intracellular and presumably nonclassically secreted. (A) Classification of the identified proteins according to the predicted cellular location, and signal sequences and anchor motifs of the identified proteins predicted to be extracellular. Signal sequence, N-terminal signal peptide targeting the protein into the secretory pathway; TMHMM, proteins with transmembrane helices; Lipobox, proteins with a N-terminal lipobox mediating covalent binding of a conserved Cys residue to a lipid; LPXTG, proteins with a C-terminal LPXTG cell wall anchoring signal for covalent attachment to peptidoglycan by sortase; and LysM, proteins with a C-terminal lysine mediating noncovalent attachment to peptidoglycan. (B) The membrane vesicle fraction was enriched from culture supernatants using ultrafiltration with 100 kDa cutoff membranes. The protein composition was assessed by SDS-PAGE followed by SYPRO-Orange staining: proteins were precipitated from filtered culture supernatants (lanes 2, 5, and 8), flow-through fraction from ultrafiltration (lanes 3, 6, and 9), and retentate containing enriched membrane vesicles (lanes 4, 7, and 10).

protein sequence searchers were performed against an entire nonredundant protein sequence database using the BLAST with default parameters.

Urease and Lactamase Activity

The activities of β-lactamase (Bla) and urease (Ure) in the culture supernatants were determined with the methods of O'Callaghan and Morris[50] and Christensen,[51] respectively. Bla activity was assessed using 170 μM nitrocefin (Thermo Scientific) as the substrate. Ure activity was assessed using 140 μM urea and 29 μM Phenol red as the substrate and indicator, respectively. Hydrolysis of both substrates was monitored after 20 h of incubation at 37 °C at 560 nm (Ure) and 490 nm (Bla).

RESULTS

Identification Characterization of the Exoproteomes

We compared the exoproteomes from SE strains isolated from bovine (PM221) and human hosts (ATCC12228 and RP62A) in order to identify strain-specific adaptation and virulence proteins. For identification, the precipitated and purified protein samples were analyzed by GeLC−MS/MS as previously described.[27] Three biological replicates were analyzed, and database searches for the biological replicates were initially undertaken separately for each replicate. This revealed that the common identifications between biological replicates accounted for over 50% of the total number of identifications in each strain, and more than 70% of the proteins could be identified from at least two replicates (Figure 1A and Supporting Information Table 1). Next, the data from biological replicates were processed together, and this compilation identified totals of 451, 395, and 518 proteins from PM221, ATCC12228, and RP62A, respectively (Figure 1A and Supporting Information Tables 2−4). The identification of 412 (PM221), 379 (ATCC12228), and 471 (RP62A) proteins was based on at least five distinct peptides, and one-peptide hits were assigned only with four, two, and four proteins from PM221, ATCC12228, and RP62A, respectively (Supporting Information Table 5). The raw proteomic data has been deposited into the ProteomeXchange Consortium (identifier PXD000631).

The overall COG category distribution of all identified proteins (Figure 1B) was similar in all three strains, with the largest COG categories being translation, ribosomal structure and biogenesis (COG, J), energy production and conversion (COG, C), and carbohydrate, amino acid, and nucleotide transport and metabolism (COG, G/E/F). The two infectious strains had more identified proteins associated with nucleotide transport and metabolism, replication, recombination and

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repair (COG, L), cell wall/membrane/envelope biogenesis (COG, M), and post-translational modification, protein turnover, chaperones (COG, O) than the low-virulent ATCC12228 strain. According to the GO annotation (Figure 1C), proportionally more translation-associated proteins were identified from PM221 and ATCC12228 than from RP62A.

The Majority of the Identifications Are Cytoplasmic Proteins, Possibly Entering the Extracellular Milieu via Nonclassical Secretion Pathways

The classifications of the identified proteins into cytoplasmic and extracellular proteins according to the combined results of several prediction tools (PSORTb, SignalP, SecretomeP, TMHMM, PRED-LIPO, and ScanProsite) and manual inspection (Figure 2A) revealed that the highest number of predicted extracellular and cell surface-associated proteins was identified in the RP62A strain, representing a virulent SE. The numbers of the identified proteins with known motifs for secretion were 50, 62, and 63 in PM221, ATCC12228, and RP62A, respectively. Several cell wall-associated proteins were also identified, including a high number of potential lipoproteins and integral membrane proteins (IMPs) carrying one or more transmembrane domains (TMDs). Some of the identified proteins were also predicted to possess a C-terminal LPXTG motif known to mediate the covalent attachment of the protein to the cell wall[42] or to carry a LysM motif that allows for noncovalent attachment of the protein to peptidoglycan.

The majority of the identifications were associated with cytoplasmic proteins (371, 313, and 415 proteins from PM221, ATCC12228, and RP62A, respectively) (Figure 2A). Several of these harbor multitasking functions depending on the subcellular location of the protein and are thus considered to be moonlighting proteins.[52] The existence of membrane vesicle (MV)-mediated protein export could be one reason for the identification of several cytoplasmic proteins in each culture media. To test whether the proteins from SE cells were released within large structures such as MVs, we enriched the proteins from culture media supernatant using centrifugal filter units (100 kDa cutoff) and compared the protein profiles in the original supernatant, the retentate (50× concentrated), and the flow-through fractions by SDS-PAGE and SYPRO-Orange staining (Figure 2B). This revealed that the protein composition in the retentate contained proteins with a broad range of molecular masses, and not only large, >100 kDa proteins, which should be enriched in the retentate. This suggests that a large proportion of the smaller proteins in the retentate was secreted within large vesicular structures. No overall enrichment of low-molecular-weight (<100 kDa) proteins in the flow-through fraction compared to retentate was observed (Figure 2B), suggesting that the majority of the cytoplasmic proteins in the supernatants had been exported via high-molecular-weight structures like MVs.

Identification of Strain-Specific Factors Related to Virulence and Adaptation

On the basis of the combined results from RBBH and OrthoMCL, the exoproteomes from the three SE strains were categorized into common (i.e., protein orthologues/paralogues) or unique (i.e., without an evolutionary counterpart in the other strains) fractions. This analysis revealed over 330 proteins that were commonly identified in each three strains (Figure 3). The numbers of unique and strain-shared proteins (protein orthologues) that were identified from only one of the strains were 48, 22, and 98 in PM221, ATCC12228, and RP62A, respectively (Figure 3 and Tables 1−3).

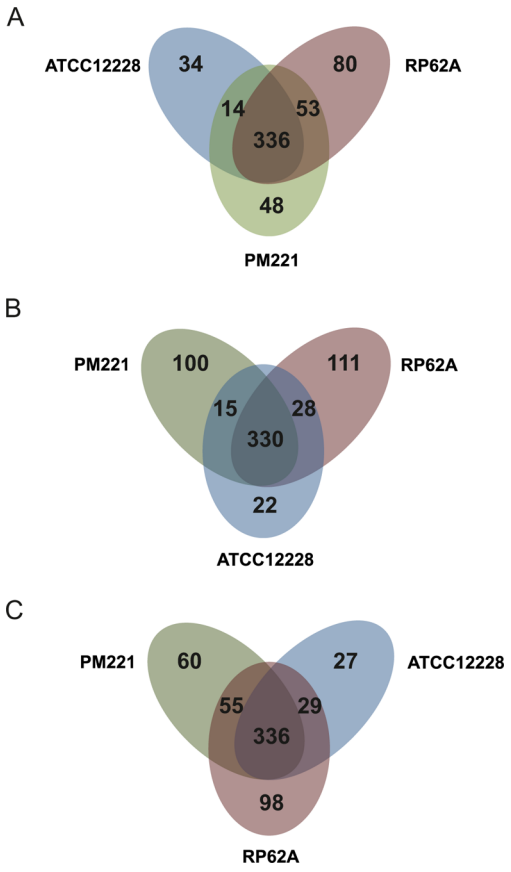

Figure 3. Common and strain-specific identifications from PM221, ATCC12228, and RP62A. Venn diagrams displaying the identified proteins obtained after search against all predicted proteins in PM221 (A), ATCC12228 (B), and RP62A (C). Differences in the total numbers are a consequence of the minor differences in orthologue analysis caused by the order of comparison (PM221 vs ATCC12228/ATCC12228 vs PM221, PM221 vs RP62A/RP62A vs PM221, etc.).

A comparison of the specifically identified proteins revealed several interesting differences, for example, among proteins contributing virulence, adaptation, macromolecule metabolism, and defense mechanism (Tables 1−3). In PM221, the unique identifications related to virulence and adaptation proteins included glutamyl aminopeptidase with likely moonlighting functions,[53] TRAP-binding OpuCA,[54] and proteins contributing to methicillin resistance (LytH, HmrA, and FmhA) (Table 1). ATCC12228-specific identifications with potential roles in virulence and adaptation included two surface-associated proteins harboring LPXTG motifs (Table 2). Under the conditions used here, RP62A was the most efficient strain in exporting/releasing adhesive proteins (SesE and SesG), immunomodulatory antigens and peptides (IsaB and PSMα), and peptidolytic, proteolytic, lipolytic, and hydrolytic (urease) enzymes as well as hypothetical proteins (Table 3). Most of the hypothetical proteins were predicted to have phage-related functions.

Urease is a multicomplex pathogenicity-related enzyme,[55] and its α-, β- and γ-subunits were identified from RP62A. These findings suggested that the culture medium of RP62A shows higher urease activity as compared to culture media from PM221 or ATCC1228. In order to verify this, the presence of urease in each of the three culture media was tested by

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Table 1. Strain-Specific Proteins Identified from PM221, Including Protein Orthologues and Unique Proteins with No Genetic Counterpart in RP62A or ATCC12228[a]

ID	protein	protein location
Exoenzymes, Hydrolytic Enzymes, Toxins, Virulence, Adhesion, Antigenic Proteins		
SEB01442	Glutamyl aminopeptidase; Deblocking aminopeptidase	cyto
SEB02037	*M42 glutamyl aminopeptidase, cellulase*	*cyto*
SEB02352	*Merozoite surface protein 3b*	*cyto*
Regulatory Proteins		
SEB01650	AgrD	cyto
SEB01515	*HTH domain protein SA1665, binds to mecA promoter region*	*cyto*
SEB01513	*putative transcriptional regulator*	*cyto*
Cell Wall/Membrane/Envelope Biogenesis, Defense Mechanisms		
SEB00263	6-phospho-3-hexuloisomerase	cyto
SEB01335	LytH protein involved in methicillin resistance/*N*-acetylmuramoyl-L-alanine amidase domain	sec
SEB02025	Osmotically activated L-carnitine/choline ABC transporter, ATP-binding protein OpuCA	cyto
SEB00603	*D-alanine-poly(phosphoribitol) ligase subunit 2*	*cyto*
SEB01742	*HmrA protein involved in methicillin resistance/amidohydrolase of M40 family*	*cyto*
SEB01995	*FmhA protein of FemAB family*	*sec*
Signaling, Intracellular Trafficking		
SEB00459	HPr kinase/phosphorylase	cyto
Translation, Ribosomal Structure, Transcription, Replication and Repair		
SEB01378	LSU ribosomal protein L35p	cyto
SEB00665	*Tryptophanyl-tRNA synthetase, proteobacterial type*	*cyto*
SEB01363	*DNA-3-methyladenine glycosylase*	*cyto*
SEB01382	*Helicase loader DnaI*	*cyto*
SEB02287	*Putative deoxyribonuclease YcfH*	*cyto*
Energy Production and Cell Cycle		
SEB00843	FIG001960: FtsZ-interacting protein related to cell division	cyto
SEB00846	Cell division initiation protein DivIVA	cyto
SEB01713	ATP synthase gamma chain	cyto
Macromolecule, Ion, and Metabolite Transport and Metabolism		
SEB00049	Potassium-transporting ATPase C chain	sec
SEB00160	Glycerol dehydrogenase	cyto
SEB00270	Phosphomethylpyrimidine kinase	cyto
SEB00430	Ribonucleotide reductase of class Ib (aerobic), alpha subunit	cyto
SEB00720	2-succinyl-5-enolpyruvyl-6-hydroxy-3-cyclohexene-1-carboxylic-acid synthase	cyto
SEB02048	Poly(glycerol-phosphate) alpha-glucosyltransferase	cyto
SEB02515	Ornithine decarboxylase	cyto
SEB00514	*Glycine cleavage system H protein*	*cyto*
SEB00855	*Aspartate carbamoyltransferase*	*cyto*
SEB01021	*Low-specificity L-threonine aldolase*	*cyto*
SEB01141	*Peptide methionine sulfoxide reductase MsrB*	*cyto*
SEB01755	*oxidoreductase ylbE*	*cyto*
SEB01841	*Glucose 1-dehydrogenase*	*cyto*
SEB01932	*Isopentenyl-diphosphate delta-isomerase, FMN-dependent*	*cyto*
SEB02164	*Acyl-coenzyme A synthetases/AMP-(fatty) acid ligases*	*cyto*
Hypothetical Proteins and Proteins with General Prediction Only		
SEB00280	hypothetical protein	cyto
SEB00439	hypothetical protein	sec
SEB00824	hypothetical protein	sec
SEB00831	BH2577 unknown conserved protein in B. subtilis	cyto
SEB02036	hypothetical protein	sec
SEB02414	hypothetical protein	cyto
SEB02416	hypothetical protein	sec
SEB02525	hypothetical protein	cyto
SEB00021	*hypothetical protein SA_21*	*sec*
SEB00541	*hypothetical protein*	*cyto*
SEB01723	*Hypothetical protein ywlG*	*cyto*
SEB01403	*FIG146085: Phosphoesterase, DHH family protein*	*cyto*

[a]Proteins with normal font were identified from at least two biological replicates, and those with italic font were identified from one biological replicate. Relevant information related to protein conservation, cellular location, the presence of different cell-membrane or cell-wall anchoring motifs, and identification criteria are described in Table S1.

supplementing equal amounts of the filtered culture medium with urea as the substrate and phenol-red as the indicator with overnight incubation at 37 °C. This in vitro analysis (Figure 4A) confirmed the presence of higher urease activity in the culture supernatant samples of RP62A as compared to the other two samples, although some activity was also observed in the ATCC12228 supernatant.

The comparison of the identifications shared by two strains (Supporting Information Table 6) indicates that the exoproteomes of the PM221 and RP62A strains are more similar to each other than to that of ATCC12228. Among the PM221- and RP62A-shared identifications, some proteins with predicted motifs for secretion are involved, for example, in immunomodulatory functions (67 kDa myosin cross-reactive antigen), inorganic ion transport and metabolism, and cell cycle and cellular responses to stress (e.g., ALP, FtsH, and MreC). The highest number of identified proteins predicted to be extracellular was shared by the human strains, and of these, delta hemolysin and proteins with TMDs (fibronectin binding proteins Ebh and hemagglutinin), LPXTG (SesA, SesH), and lipobox (Ferrichrome ABC transporter, two potential lipoproteins) are potential virulence-associated factors.

Higher identification scores and sequence coverage can be used to estimate changes in protein relative abundances between two closely related organisms.[27,56] In the present study, we identified β-lactamase from all three strains, but with markedly higher identification scores for PM221 and RP62A as compared to that for ATCC12228 (Supporting Information Tables 2−4). Our identification data suggest that β-lactamase activity (Bla), conferring resistance to the β-lactam group of antibiotics, is higher in the culture supernatants of PM221 and RP62A. This hypothesis was tested by the addition of nitrocefin, a chromogenic cephalosporin substrate routinely used to detect the presence of this enzyme, to the filtered culture supernatants, with the reactions progressing overnight at 37 °C. Only the PM221 and RP62A samples contained detectable nitrocefin-hydrolyzing activity (Figure 4B), which confirms that compared to ATCC12228, the secretion of Bla is more efficient in PM221 and RP62A under the conditions used.

Nonclassical Protein Export May Be One of the Virulence Mechanisms Exploited by SE

Most of the protein identifications specific to the virulent SE strains (PM221 and RP62A) were associated with predicted cytoplasmic proteins (Tables 2 and 3), several of which have

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Table 2. Strain-Specific Proteins Identified from ATCC12228, Including Protein Orthologues and Unique Proteins with No Genetic Counterpart in PM221 or RP62A[a]

ID	protein	protein location
Exoenzymes, Hydrolases, Toxins, Virulence, Adhesion, Antigenic Proteins		
SE_2395	SdrF/BbP (Ser-Asp rich fibrinogen-binding, bone sialoprotein-binding protein)	sec
SE_p517	LPXTG-motif cell wall anchor	sec
SE_0172	*Peptidase, cysteine*	*sec*
Cell Wall/Membrane/Envelope Biogenesis, Defense Mechanisms		
SE_1674	alanine racemase	cyto
SE_0170	*galactosamine-containing minor teichoic acid biosynthesis protein*	*cyto*
Translation, Ribosomal Structure, Transcription, Replication and Repair		
SE_1355	translation initiation factor IF-3	cyto
SE_0536	*peptide chain release factor RF-2*	*cyto*
SE_1293	*transcription elongation factor*	*cyto*
Transformation		
SE_0925	*DNA processing Smf protein*	*cyto*
Energy Production and Cell Cycle		
SE_1198	*branched-chain alpha-keto acid dehydrogenase E1*	*cyto*
Macromolecule, Ion, and Metabolite Transport and Metabolism		
SE_0763	phosphoribosylaminoimidazole carboxylase ATPase subunit	cyto
SE_2086	*ribokinase*	*cyto*
SE_2268	*7,8-dihydroneopterin aldolase*	*cyto*
SE_1639	*fructokinase*	*cyto*
Hypothetical Proteins		
SE_0447	conserved hypothetical protein	sec
SE_1536	conserved hypothetical protein	cyto
SE_1753	conserved hypothetical protein	sec
SE_p201	hypothetical protein SE_p201 plasmid	sec
SE_p202	hypothetical protein SE_p202 plasmid	sec
SE_p601	hypothetical protein SE_p601 plasmid	sec
SE_0114	*conserved hypothetical protein*	*sec*
SE_0569	*conserved hypothetical protein*	*sec*

[a]Proteins with normal font were identified from at least two biological replicates, and those with italic font were identified from one biological replicate. Relevant information related to protein conservation, cellular location, the presence of different cell-membrane or cell-wall anchoring motifs, and identification criteria are described in Table S2.

previously been identified as moonlighting proteins and/or in MVs isolated from SA.[57,58] To examine the potential link between protein export and the clinical features ascribed to PM221 and RP62A, we next compared the identified exoproteomes with the previously conducted total proteome profiling study of PM221 and ATCC12228.[59] This comparison revealed that more than 90% of the proteins identified from the PM221 and ATCC12228 exoproteomes were also identified from their total proteomes. Interestingly, 97 proteins that were identified in the exoproteome of PM221 were identified only in the total proteome of ATCC12228 but not in its exoproteome (Figure 5 and Supporting Information Table 7A). RP62A was not analyzed for its total proteome composition; however, the comparison of the identified exoproteins from this strain with the total proteome of ATCC12228 indicated 107 proteins that were present in the exoproteome of RP62A and in the total proteome of ATCC12228 but not in the ATCC12228 exoproteome (Figure 5 and Supporting Information Table 7B). In summary, this indicates that these proteins are expressed but not exported by ATCC12228 under the conditions used here. These groups of 97 and 107 proteins identified from PM221 and RP62A exoproteomes, respectively, included a high number of shared identifications, suggesting that the virulent strains release a similar set of intracellular proteins (Supporting Information Table 7B) potentially contributing to virulence and/or adaptation.

DISCUSSION

SE is a common inhabitant of the human skin and mucous membranes, which, under certain circumstances, can behave like an opportunistic pathogen for humans and animals such as dairy cattle. Humans are considered to be carriers of the bovine IMI-causing SE strains, which is why we screened for strain-specific differences between selected bovine (IMI-causing PM221) and human (sepsis-associated strain RP62A and the commensal-type and mildly infectious strain ATCC12228) SE strains. From the point of view of infection, the compositions of the bacterial exoproteome are extremely interesting because this compartment contains proteins directly interacting with the host and that are responsive to many environmental stress factors. Therefore, the present study focused on the identification and comparison of the proteins exported out of the cell from the three SE strains in an attempt to find strain-specific proteins making potential contributions to adaptation and virulence. As a result, 451, 395, and 518 proteins from the culture media of logarithmically growing PM221, ATCC12228, and RP62A cells could be identified, respectively. As far as we are aware, this represents the first comprehensive exoprotein catalog of this species.

The majority of the identified proteins (~80%) were predicted to be cytoplasmic. Also, in previous studies with SA, the majority of the exoproteins (60%) have been associated with cytoplasmic proteins.[16] However, proportionally more cytoplasmic proteins were identified in the present study, which can be due to the differences in the growth media (rich medium vs minimal growth medium) and the sampling time point used for exoprotein isolations (in the present study, the SE cells were cultured to higher cell densities). In addition, the culture media in our study were supplemented with protease inhibitors immediately before separating the supernatants from cell cultures, which, by improving protein stability, is likely to improve the identification of certain proteins with high turnover rates (e.g., transcriptional regulators).

Many of the identified cytoplasmic proteins in the present study have been considered to be anchorless moonlighting proteins at the cell surface in SA and in several other bacteria.[60] This group includes many conserved proteins involved in central metabolic pathways, cellular responses to stress, and/or virulence.[15,16,60] Several of these proteins have also been reported to be immunogenic and/or to bind to different host components and other bacteria, which, in the case of pathogenic bacteria, has clear implications for virulence.[52] Some studies have demonstrated that moonlighting proteins are released from bacterial cell surfaces under neutral or alkaline conditions.[61−63] Thus, it could be that several of these surface-exposed moonlighting proteins were attached only loosely to the SE surfaces and were released into the culture medium during centrifugation or that they can be released via some still unknown mechanism as described for some bacteria.[64−66]

Large numbers of ribosomal proteins were identified from each of the three SE supernatants. Cell lysis has been proposed to be one of the mechanisms by which these proteins end up at the cell-surface.[67] It has also been hypothesized that some of

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Table 3. Strain-Specific Proteins Identified from RP62A, Including Protein Orthologues and Unique Proteins with No Genetic Counterpart in PM221 or ATCC12228[a]

ID	protein	protein location
Exoenzymes, Hydrolytic Enzymes, Toxins, Virulence, Adhesion, Antigenic Proteins		
SERP0083	phenol soluble modulin alpha	cyto
SERP1271	proline dipeptidase	cyto
SERP1640	M23/M37 peptidase domain protein [*Staphylococcus epidermidis* RP62A phage SP-beta]	sec
SERP1649	lipase/acylhydrolase domain protein [*Staphylococcus epidermidis* RP62A phage SP-beta]	sec
SERP1870	urease, beta subunit	cyto
SERP1871	urease, alpha subunit	cyto
SERP0719	*SesE, cell wall surface anchor family protein*	*sec*
SERP0996	*carboxyl-terminal protease*	*sec*
SERP1482	*SesG, cell wall surface anchor family protein*	*sec*
SERP1544	*lipoprotein, putative*	*sec*
SERP1650	*prophage, amidase, putative [Staphylococcus epidermidis RP62A phage SP-beta]*	*cyto*
SERP1874	*urease accessory protein UreG*	*cyto*
SERP2295	*intercellular adhesion protein B*	*sec*
SERP2372	*peptide ABC transporter, peptide-binding protein*	*sec*
Regulatory Proteins		
SERP1398	*transcriptional regulator, Fur family*	*cyto*
SERP1681	*transcriptional regulator, PemK family*	*cyto*
SERP1876	*Staphylococcal accessory regulator R*	*cyto*
Cell Wall/Membrane/Envelope Biogenesis, Defense Mechanisms		
SERP1221	Tn554, streptomycin 3″-adenylyltransferase	cyto
SERP1344	Tn554, streptomycin 3″-adenylyltransferase	cyto
SERP2509	streptomycin 3″-adenylyltransferase	sec
SERP2521	penicillin-binding protein 2′	sec
SERP1706	*UDP-N-acetylglucosamine 1-carboxyvinyltransferase 1*	*cyto*
Signaling, Intracellular Trafficking		
SERP0786	*serine/threonine protein kinase, putative*	*sec*
Translation, Ribosomal Structure, Transcription, Replication and Repair		
SEA0010	aminoglycoside 3′-phosphotransferase	cyto
SERP1195	D-tyrosyl-tRNA(Tyr) deacylase	cyto
SERP1511	truncated ribonucleoside-diphosphate reductase 2 alpha subunit	cyto
SERP1513	truncated ribonucleoside-diphosphate reductase 2, alpha subunit	cyto
SERP2459	CRISPR-associated protein, TM1792 family	cyto
SERP0837	*ribosome-binding factor A*	*cyto*
SERP0918	*exonuclease SbcC*	*cyto*
SERP1204	*S-adenosylmethionine:tRNA ribosyltransferase-isomerase*	*cyto*
SERP1206	*Holliday junction DNA helicase RuvA*	*cyto*
SERP1590	*single-stranded-DNA-specific exonuclease RecJ, putative*	*cyto*
SERP2553	*chromosomal replication initiator protein DnaA*	*cyto*

Table 3. continued

ID	protein	protein location
Energy Production and Cell Cycle		
SERP0731	succinate dehydrogenase, flavoprotein subunit	sec
SERP0856	pyruvate ferredoxin oxidoreductase, alpha subunit	cyto
SERP1079	2-oxoisovalerate dehydrogenase, E3 component, lipoamide dehydrogenase	cyto
SERP0646	*quinol oxidase, subunit II*	*sec*
SERP1713	*ATP synthase F0, B subunit*	*sec*
SERP2327	*acetoin dehydrogenase, E3 component, dihydrolipoamide dehydrogenase*	*cyto*
Macromolecule, Ion, and Metabolite Transport and Metabolism		
SERP0246	ABC transporter, substrate-binding protein, putative	sec
SERP0545	ornithine aminotransferase	cyto
SERP0653	phosphoribosylformylglycinamidine synthase I	cyto
SERP0769	carbamoyl-phosphate synthase, large subunit	cyto
SERP2186	sulfate adenylyltransferase	cyto
SERP0511	*lipoate synthase*	*cyto*
SERP0652	*phosphoribosylformylglycinamidine synthase, PurS protein*	*cyto*
SERP0792	*DAK2 domain protein*	*cyto*
SERP1264	*acetyl-CoA carboxylase, carboxyl transferase, beta subunit*	*cyto*
SERP1325	*riboflavin synthase, beta subunit*	*cyto*
SERP1495	*sucrose-6-phosphate hydrolase*	*cyto*
SERP2190	*sulfite reductase (NADPH) hemoprotein beta-component*	*cyto*
SERP2383	*ABC transporter, substrate-binding protein*	*sec*
Hypothetical Proteins and Proteins with General Prediction Only		
SERP1137	conserved hypothetical protein TIGR00043	cyto
SERP1351	conserved hypothetical protein	sec
SERP1505	hypothetical protein SERP1505	cyto
SERP1506	conserved hypothetical protein	cyto
SERP1514	hypothetical protein SERP1514	cyto
SERP1517	hypothetical protein SERP1517	cyto
SERP1526	hypothetical protein SERP1526	cyto
SERP1540	conserved hypothetical protein [*Staphylococcus epidermidis* RP62A phage SP-beta]	sec
SERP1594	conserved hypothetical protein	cyto
SERP1595	conserved hypothetical protein	cyto
SERP1596	hypothetical protein SERP1596	cyto
SERP1597	hypothetical protein SERP1597	cyto
SERP1623	conserved hypothetical protein [*Staphylococcus epidermidis* RP62A phage SP-beta]	cyto
SERP1624	conserved hypothetical protein [*Staphylococcus epidermidis* RP62A phage SP-beta]	cyto
SERP1626	hypothetical protein SERP1626 [*Staphylococcus epidermidis* RP62A phage SP-beta]	cyto
SERP1627	hypothetical protein SERP1627 [*Staphylococcus epidermidis* RP62A phage SP-beta]	cyto
SERP1628	hypothetical protein SERP1628 [*Staphylococcus epidermidis* RP62A phage SP-beta]	cyto
SERP1629	hypothetical protein SERP1629 [*Staphylococcus epidermidis* RP62A phage SP-beta]	cyto

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Table 3. continued

ID	protein	protein location
Hypothetical Proteins and Proteins with General Prediction Only		
SERP1630	hypothetical protein SERP1630 [*Staphylococcus epidermidis* RP62A phage SP-beta]	cyto
SERP1634	conserved hypothetical protein [*Staphylococcus epidermidis* RP62A phage SP-beta]	cyto
SERP1636	conserved hypothetical protein [*Staphylococcus epidermidis* RP62A phage SP-beta]	cyto
SERP1641	conserved hypothetical protein [*Staphylococcus epidermidis* RP62A phage SP-beta]	cyto
SERP1642	conserved hypothetical protein [*Staphylococcus epidermidis* RP62A phage SP-beta]	sec
SERP1645	conserved hypothetical protein [*Staphylococcus epidermidis* RP62A phage SP-beta]	cyto
SERP2212	conserved domain protein	sec
SERP2456	hypothetical protein SERP2456	cyto
SERP0074	*hypothetical protein SERP0074*	*sec*
SERP0159	*conserved hypothetical protein*	*cyto*
SERP0214	*conserved hypothetical protein*	*cyto*
SERP0316	*conserved hypothetical protein*	*cyto*
SERP0366	*conserved hypothetical protein*	*sec*
SERP0514	*conserved hypothetical protein*	*cyto*
SERP0696	*GTP-binding protein TypA*	*cyto*
SERP0774	*conserved hypothetical protein*	*sec*
SERP1509	*hypothetical protein SERP1509*	*cyto*
SERP1530	*hypothetical protein SERP1530*	*cyto*
SERP1531	*hypothetical protein SERP1531*	*cyto*
SERP1533	*hypothetical protein SERP1533*	*sec*
SERP1542	*hypothetical protein SERP1542 [Staphylococcus epidermidis RP62A phage SP-beta]*	*cyto*
SERP1543	*hypothetical protein SERP1543*	*cyto*
SERP1614	*hypothetical protein SERP1614*	*cyto*
SERP1620	*hypothetical protein SERP1620 [Staphylococcus epidermidis RP62A phage SP-beta]*	*cyto*
SERP1631	*hypothetical protein SERP1631 [Staphylococcus epidermidis RP62A phage SP-beta]*	*cyto*
SERP1637	*conserved domain protein [Staphylococcus epidermidis RP62A phage SP-beta]*	*cyto*
SERP1643	*hypothetical protein SERP1643 [Staphylococcus epidermidis RP62A phage SP-beta]*	*cyto*

[a]Proteins with normal font were identified from at least two biological replicates, and those with italic font were identified from one biological replicate. Relevant information related to protein conservation, cellular location, the presence of different cell-membrane or cell-wall anchoring motifs, and identification criteria are described in Table S3.

the ribosomal proteins are released from dead or stressed cells, which then remain attached and decorate the cell walls of intact bacteria.[53] Ribosomal proteins appear to have particularly high affinity for the bacterial cell wall, and they are currently considered to be novel anchorless surface proteins.[53] These proteins have been detected frequently on the cell surface of Gram-positive bacteria,[18,20,67−70] and some of these are reported to be immunogenic in the SA strains associated with human and bovine (subclinical mastitis) infections.[71−73] However, the majority of the identifications related to ribosomal and other translation-associated proteins in the present exoproteome

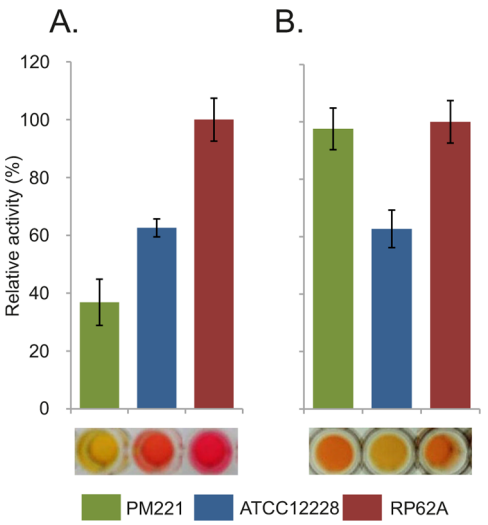

Figure 4. Urease and β-lactamase activity of PM221, ATCC12228, and RP62A. Relative β-lactamase (A) and urease (B) activities (%) in culture supernatants of PM221, ATCC12228, and RP62A were assessed by incubating equal amounts of filtered sample with the indicated substrates/indicators for 20 h at 37 °C. The color change was detected with a microtiter plate as illustrated below the diagrams. Both tests were conducted with four biological replicates. Results are presented as mean relative activities, with error bars indicating SD.

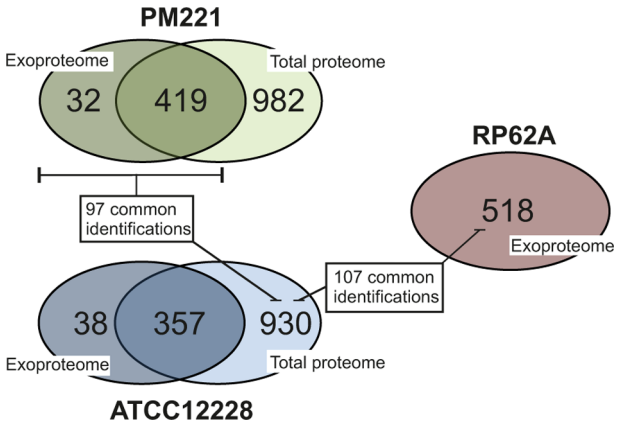

Figure 5. Comparison of the exoproteome and total proteome identifications. There were 97 proteins that were identified from the exoproteome of PM221 and from the total proteome of ATCC12228 but not from the ATCC12228 exoproteome. Additionally, 107 proteins were identified from the RP62A exoproteome and from the ATCC12228 total proteome but not from the ATCC12228 exoproteome.

study have been previously identified in MVs purified from SA.[57,58] Thus, MV production provides another hypothesis to explain the extracellular localization of these proteins in addition to moonlighting and cell lysis.

MVs are naturally produced into the extracellular milieu by bacteria, delivering bacterial proteins that affect immune activation and suppression, attachment, stress response, internalization, and virulence.[74,75] In addition, MVs contribute to bacterial survival, nutrient acquisition, quorum signaling, biofilm development, and horizontal gene transfer, and they allow proteins (e.g., enzymes or toxins) to reach their specific targets, such as host cells or other bacterial cells, in a concentrated and protected form.[75−77] Our results suggest that the MV type of nonclassical secretion mechanism is likely to be exploited by SE

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to allow the export of cytoplasmic proteins. At present, this protein export mechanism has been overlooked in Gram-positive bacteria, with only few reports published for SA to date.[57,58,74,75,78] The total number of proteins identified from MVs is reported to be nearly 100, of which most are predicted to be involved in coagulation, adhesion, glycolysis, transcription, and translation.[57] Studies have also demonstrated that SA MVs are composed of proteins such as alpha toxin that is able to induce death of host cells[58,75] and increase drug resistance.[78] In the present study, one of the exoproteins identified from all three strains, but with a markedly higher identification score from PM221 and RP62A as compared to that from ATCC12228, was β-lactamase (Bla), an enzyme that confers resistance to the β-lactam group of antibiotics. Our in vitro enzyme assays also indicated that the more virulent human and bovine strains were more efficient in exporting this enzyme. Bla has been shown to be exported via MVs in SA to enable the adaptation of ampicillin-susceptible bacteria,[78] and it could be that this enzyme expressed by staphylococci is protected by MVs in order to improve viability in hostile antibiotic-containing environments. Overall, these findings suggest that the virulent strains may exploit nonclassical protein export mechanisms involving protein moonlighting and MVs to promote full virulence. In addition, several cell wall/membrane-associated proteins were identified in the extracellular space of each of the three strains, which may indicate that this subgroup of exoproteins is associated only loosely with the cell surface. We also detected several TMD-containing proteins and proteins with LysM or LPXTG motifs, which may have been liberated into the culture media after proteolysis, such as the HtrA-mediated proteolysis recently described in *Bacillus subtilis*.[79]

The comparison of the secreted exoprotein compositions indicated that the virulent RP62A strain exported more virulence factors (e.g., hemolysin, antigens, and urease) compared those from PM221 and ATCC12228, which is in accordance with the clinical origin of this strain. Urease is a multicomplex pathogenicity-related enzyme encoded by the *ureABC* and *ureDEFG* operons,[55] from which the UreA, UreB, and UreG subunits were specifically identified from RP62A. The in vitro analysis of urease activity revealed that the human strains secreted more urease than the bovine strain, which could reflect the natural environments of RP62A and ATCC12228 on human skin or on indwelling medical devices, where they may encounter urea in body fluids.

In conclusion, the present study is the first in-depth exoproteome analysis of SE highlighting strain-specific factors able to contribute to virulence and adaptation. We show that RP62A is the most efficient of these three SE strains in the export of adaptation/virulence-associated proteins and that the two infection-associated strains, PM221 and RP62A, share the highest number of identifications. Our findings also suggest that several cytoplasmic proteins with likely adhesive and immunogenic functions are exported via nonconventional secretion mechanisms in SE and that they are likely to play important roles in establishing productive infection.

■ ASSOCIATED CONTENT

Supporting Information

Growth curves for PM221, ATCC12228, and RP62A; Coomassie Blue stained gels used for protein identification; Mascot and Paragon search results from biological replicates for PM221, ATCC12228, and RP62A; list of all predicted proteins and identified exoproteins from the bovine mastitis strain PM221; list of all predicted proteins and identified exoproteins from the commensal-type human skin strain ATCC12228; list of all predicted proteins and identified exoproteins from the commensal-type human skin strain RP62A; MS/MS data from single peptide identifications; protein identifications shared by two strains; and comparison of PM221, ATCC12228, and RP62A protein identifications between the current study and a previous total proteome study. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

CoNS; coagulase negative; IMI; intramammary infection; SE; *Staphylococcus epidermidis*; SA; *Staphylococcus aureus*; 1DE; one-dimensional gel electrophoresis; GeLC−MS/MS; 1DE coupled with LC−MS/MS; TS; tryptic soy; TCA; trichloroacetic acid; TFA; trifluoroacetic acid; COG; orthologous groups of proteins; GO; gene ontology; IMP; integral membrane protein; TMD; transmembrane domain; MV; membrane vesicle

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