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Exoproteome of *Staphylococcus aureus* reveals putative determinants of nasal carriage

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Summary

Due to the increasing prevalence of nosocomial and community-acquired antibiotic resistant *Staphylococcus aureus* (SA), understanding the determinants of SA nasal carriage has become a major imperative. Previous research has revealed many host and bacterial factors that contribute to SA nasal carriage. To assess bacterial factors that facilitate nasal carriage, we compared the exoproteome of a nasal carrier strain of SA to a genetically similar non-carrier strain. Additionally, the carrier strain biofilm exoproteome was also compared against its planktonic counterpart. Using high throughput proteomics, it was observed that the carrier strain of SA secretes a greater number of proteins that may promote successful colonization of the human nose, including cell attachment and immunoevasive proteins, than the non-carrier strain. Similarly, SA carrier strain biofilm exoproteome contains a greater number of immunoevasive proteins than its planktonic counterpart. Analysis of the most abundant immunoevasive proteins revealed that Staphylococcal protein A was present at significantly higher levels in carrier than in non-carrier strains of SA, suggesting an association with nasal carriage. While further analyses of specific differences between carrier and non-carrier strains of SA are required, many of the differentially expressed proteins identified can be considered to be putative determinants of nasal carriage.

Keywords

Innate immunity; bacteria/bacterial immunity; comparative proteomics; nasal colonization; exoproteome

Introduction

Staphylococcus aureus is one of the most common causes of community-acquired and nosocomial infections throughout the world.¹ These infections have become even more pertinent with the global spread of community-acquired Methicillin-resistant SA (CA-MRSA)² and the emergence of Vancomycin-resistant SA (VRSA).³ In the US alone, the mortality rate from SA infections surpasses those attributed to HIV/AIDS.² Many

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community-acquired and nosocomial *SA* infections are disseminated through nasal carriage, which occurs in approximately one quarter of the population,¹ thus identifying determinants of nasal carriage is a priority for successful amelioration of this condition.

Although nasal carrier strains of *SA* have evolved diverse strategies to ensure their survival and carriage in nasal passages, colonization can also be attributed to amenable hosts that carry *SA* persistently or intermittently.⁴⁻⁶ In an immunologically robust host, nasal secretions contain a plethora of defensive antibacterial proteins and peptides such as lysozyme, lactoferrin, secretory leukoprotease inhibitor (SLPI), cathelicidins, α -defensins and β -defensins including human β -defensin 3 (HBD-3), the most effective of the β -defensins against *SA* infections.^{4,7-9} However, a key determinant for the nasal carriage of *SA* is the failure of these secretions to prevent colonization. Carrier strains, and not non-carrier strains of *SA*, have been reported to persist and replicate within nasal fluids from carrier donors and on the surface of organotypic nasal epithelia, indicating that carriers have factors that aid in nasal colonization of *SA*.^{10,11} Likewise, investigations by several groups have indicated that colonization of the human nose by *SA* is influenced by bacterial determinants including sortase A (SrtA),^{12,13} clumping factor B (ClfB),^{14,15} tagX,¹⁶ and enterotoxins.¹⁷⁻¹⁹ However, this may not represent the complete repertoire of bacterial factors necessary for nasal colonization in humans.^{20,21}

Our previous studies on a nasal carrier strain of *SA* indicate that it possesses several colonization advantages in comparison to its genetically similar non-carrier counterpart.¹¹ These advantages included downregulation of host HBD-2, HBD-3 and interleukin-1 (IL-1), along with the ability to form biofilms.^{11,22} Biofilm-producing strains of *SA* exhibit higher survival rates against not only antibiotic drugs, but also against natural AMPs present in the host's nasal mucosa.^{22,23} Interestingly, the formation of protective biofilms was not evident in the non-carrier strain.²² Collectively, these findings led us to postulate that the origin of factors, which facilitated nasal colonization by *SA* carrier strains, are present at the primary interface between the host and the pathogen, namely the bacterial surface and freely secreted proteins collectively termed the exoproteome.

Using high throughput gel-free proteomics in concert with 2D-PAGE, we determined the repertoire of proteins contained within the exoproteome of a successful nasal carrier strain of *SA* in comparison to its genetically similar non-carrier counterpart. Analysis of these differences revealed putative determinants of nasal carriage. For the first time, we also compared the exoproteome of the biofilm form of the carrier strain of *SA* with its planktonic counterpart to assess its contribution to successful nasal carriage. Exoproteome analysis by 2D-PAGE revealed a marked difference in the distribution of proteins between carrier and non-carrier *SA* strains. Subsequent isobaric tagging for relative and absolute quantification (iTRAQ)²⁴ confirmed these findings, revealing that the carrier strain of *SA* expressed a greater number of proteins involved in cell attachment and immunoevasion than the non-carrier strain. On closer examination of the most abundant immunomodulatory proteins, we found that Staphylococcal protein A (SPA), known to be involved in *SA* virulence and biofilm formation,²⁵⁻²⁷ was secreted in significantly greater amounts by *SA* nasal carrier strains compared to the non-carrier strain. This may indicate a relationship between SPA and the carriage status of *SA*. By comparing the exoproteomes between a successful carrier strain of *SA* and a non-carrier strain, we have identified individual proteins and functional classes of proteins that may determine the nasal carriage status of *SA*.

Materials and Methods

Bacterial strains and culture conditions

SA strain D30 that has been extensively characterized,^{5,11,21} was originally isolated from the anterior nares of a healthy donor, and served as the carrier strain for the experiments herein. SA strain 930918-3, (from Ian Holder, Shriners Burn Hospital, Cincinnati, Ohio, USA), which is genetically similar to the carrier strain D30, and has been extensively characterized,²¹ served as the non-carrier strain of SA in these experiments. Additionally, persistent SA carrier strains D20, D98, D39 and intermittent carrier strain D37 were used in this study.⁵ SA was cultivated on Tryptic Soy Agar (TSA; Bacto™, Becton, Dickinson and Company, MD, USA) and subcultured in Trypticase Soy Broth (TSB; Bacto™, Becton, Dickinson and Company, MD, USA), from which stocks were prepared. For all experiments, snap-frozen (-80°C) stocks of SA were thawed rapidly and cultured at 37°C. Levels of inocula were estimated by measuring the absorbance of a washed bacterial suspension in PBS (Mediatech Inc., VA, USA) at 625 nm. An OD at 625 nm of 0.1 approximated 2.0×10^7 CFU/ml. Inocula were quantitated by spreading 10µl aliquots of the liquid culture on TSA and enumerating CFU following 18 hrs incubation at 37°C.^{11,22}

Preparation of carrier strain biofilms

SA carrier strain snap-frozen culture was incubated in TSB media and allowed to grow until stationary phase (6-8 h). The stationary culture was transferred to flasks containing 50 ml TSB at a 1:100 dilution and incubated without shaking at 25°C for 3 days to promote biofilm formation. Biofilms were subsequently washed three times with 0.85% NaCl and incubated overnight in ambient PBS. The supernatant was then centrifuged and used for exoproteome purification.²²

Preparation of protein extracts and 2D-PAGE

2D-PAGE was used to analyze exoproteomes from the carrier strain D30 and non-carrier strain 930918-3. Based on the growth kinetics of our SA strains, a modified approach from Dreisbach and colleagues was utilized for SA exoproteome preparation.²⁸ Briefly, one liter of bacterial culture was grown for 24 hrs at 250 rpm, 37°C, centrifuged and resuspended in 40 ml of PBS. Protease and phosphatase inhibitors (Halt™ protease and phosphatase single-use inhibitor, Pierce, IL, USA) were added to the culture and shaken overnight and the resulting supernatant was collected and filter (0.2µm) sterilized. The extract was further purified by a C18 SepPak (Waters Corporation, MA, USA) column according to the manufacturer's instructions, except that the final elution was in 80% acetonitrile/ 20% HPLC grade water. The sample was dehydrated to 10µl and diluted with 100µl of HPLC grade water. The total protein concentration of the sample was estimated using Micro BCA™ Protein Assay Kit (Thermo Scientific, Pierce, IL, USA) according to the manufacturer's instructions.²⁹⁻³² Approximately 400µg of total protein was subjected to 2D-PAGE analysis as per the manufacturer's protocol (BioRad, CA, USA). The sample was prepared in rehydration sample buffer I (8 M urea, 2% CHAPS, 50 mM DTT (dithiothreitol) 1x ReadyStrip buffer 0.1–0.4% (w/v) Bio-Lyte ampholytes) and absorbed onto a ReadyStrip IPG gel strip, pH 4-7 (BioRad, CA, USA). The strip was then subjected to isoelectric focusing (IEF) at 50 VµA per strip, initially at 250V for 15 minutes and then ramping to 10,000V in 3 hours followed by an additional focusing for 12–16 hrs at 20°C until 60,000 volt-hours was reached. Strips were rinsed in glycine gel running buffer and soaked in Equilibration Buffer I (6 M urea, 0.375 M Tris, pH 8.8, 2% SDS, 20% glycerol, 2% (w/v) DTT) for 5 minutes followed by Equilibration Buffer II (6 M urea, 0.375 M Tris, pH 8.8, 2% SDS, 20% glycerol, and 2.5% (w/v) iodoacetamide) for 5 minutes. The gel strips were then rinsed in SDS gel buffer and resolved by a 2nd dimension using a precast BioRad

Ready Gel Tris-HCl, 4–20% linear gradient at 40 mA for 5 hrs. The gels were subsequently stained using silver nitrate.

Protein digestion, labeling with iTRAQ reagents and On-Line 2D NanoLC-MS/MS

Approximately 120 µg of the exoproteome sample from the planktonic SA carrier strain D30, the planktonic non-carrier strain 930918-3 and the carrier strain D30 biofilm were subjected to overnight acetone precipitation at –20°C. Samples were resuspended in dissolution buffer, alkylated, trypsin-digested at 37°C overnight and labeled with iTRAQ 4plex Reagents kit as per the manufacturer's instruction (AB Sciex Inc., CA, USA). iTRAQ tags were applied as follows: iTRAQ 114 = Carrier strain D30 biofilm, iTRAQ 115 = planktonic non-carrier strain 930919-3, iTRAQ 116 = planktonic carrier strain D30, iTRAQ 117 = planktonic carrier strain D30. The four iTRAQ samples were mixed, lyophilized, resuspended in Strong Cation Exchange (SCX) solvent, and subjected to LC-MS/MS analysis of each peak fraction by QSTAR ESI quadrupole time-of-flight tandem MS system (Applied Biosystems, CA, USA).^{24,33,34} Complete iTRAQ analyses were performed three independent times for each sample tested.

iTRAQ Data Analysis

Raw MS data processing, protein identification, quantification and subsequent statistical analyses were performed using the ParagonTM algorithm³⁵ of ProteinPilot version 2.0.1 software (AB Sciex Inc., CA, USA). Comprehensive searches against the National Center for Biotechnology Information (NCBI) bacterial database with biological modifications and amino acid substitutions were performed to identify the proteins. Additionally, parameters such as cysteine alkylation by MMTS, iTRAQ modification of N-terminal peptide residues, modifications of lysine and tyrosine residues were considered during the analyses, as well as other default parameters were considered by the software. Further classification and analyses of the identified proteins were performed using the ProGroupTM algorithm. This algorithm enabled confident protein identification using the least set of identified peptides based on total protein scores that is generated from peptide confidence scores. Scores higher than a 95% confidence level were used for identifying proteins. Mean, Standard Deviation (S.D) and *p* values generated by the ProGroup algorithm were used for relative protein quantification between the samples. iTRAQ fold-ratio >1.2 and <0.8 combined with a *p* value < 0.05 was used to determine differential protein expression between the samples. These cutoff values for variation in the expression, recommended by the ParagonTM algorithm, are a widely accepted fold-ratio.^{34,36-42} Proteins identified with one distinct contributing peptide were subjected to manual validation by the assessment and confirmation of their MS/MS spectra (Supplemental Fig S1).^{24,33,35,43}

Anti-SPA ELISA

The expression of protein A (SPA) was quantified using the Assay DesignsTM protein A Enzyme Immunometric Assay (EIA) kit (Enzo Life Sciences International, PA, USA) as per the manufacturer's instructions with minor modifications. Briefly, exoproteome samples from 1L of stationary phase cultures ($\sim 2.0 \times 10^{10}$ CFU/ml) of nasal carrier, non-carrier and epidemic strains were prepared as mentioned previously and equal volumes (25 µl) at dilutions $1/1 \times 10^5$, $1/2 \times 10^5$, $1/4 \times 10^5$ and $1/16 \times 10^5$ were subjected to assay analysis. The concentration of SPA was calculated against standards according to manufacturer's instructions and represented as µg/ml of total protein. A one-tailed Student's *t*-test was used to measure the significance of SPA expression between SA strains.

Results

Comparative analyses of SA exoproteomes reveal differences in the distribution pattern of proteins between nasal carrier and non-carrier strains

Since the first interface between host and pathogen is the bacterial exoproteome, we hypothesized that a comparison between a carrier and a genetically similar non-carrier SA strain exoproteome would reveal key differences that could be important in nasal colonization of SA. To screen initially for these differences we used 2D-PAGE and observed significant differences in the distribution of secreted proteins between nasal carrier and non-carrier strains as typified by the distribution of MS/MS-verified SPA, (49.5 kDa). Additionally, multiple isoforms of SPA were exclusively detected in the carrier strain (Fig 1).

Although 2D-PAGE analysis displayed a majority of the proteins in carrier (D30) and non-carrier (930918-3) exoproteomes and their distribution, a more thorough quantitative approach was required in order to identify low abundance and hydrophobic proteins. To this end, we employed a gel-free system known as iTRAQ coupled with mass spectrometry to provide quantitative as well as qualitative analysis of the differences between the carrier and non-carrier strain of SA.

iTRAQ-coupled LC-MS/MS analyses of identified a total of 488 proteins in the aggregate SA exoproteome

An experimental workflow for the analysis of SA exoproteomes by iTRAQ is described in Fig 2A. Briefly, purified proteins were subjected to trypsin-digestion and labeled with iTRAQ reagents. Peptides from SA carrier strain (D30) exoproteome were labeled with iTRAQ reagents 116 and 117 whilst peptides from the non-carrier strain (930918-3) and carrier strain (D30) biofilm exoproteomes were labeled with iTRAQ reagents 115 and 114, respectively. These iTRAQ-labeled samples were then mixed, lyophilized and fractionated using Strong Cation Exchange (SCX) chromatography. Subsequently, SCX fractions were subjected to LC-MS/MS analysis (Fig 2A). The entire set of experiments from the collection of the exoproteome to LC-MS/MS analysis was performed 3 independent times (N=3). LC-MS/MS analysis of 21 SCX fractions identified a total of 488 proteins (95% confidence, unused score >1.3) from 5970 distinct peptides (supplemental **Table S2 and S3**). In addition, the identified proteins' expression levels were significantly different between carrier strain, non-carrier strain and carrier strain biofilm ($p<0.05$) conditions as depicted by the volcano plot illustration of iTRAQ data (Fig 2B and 2C).

Representative iTRAQ ion spectra and MS/MS spectra showing protein identification and quantification of selected proteins from carrier and non-carrier strains are illustrated in Fig 3. Fig 3A reveals representative MS/MS and iTRAQ ion spectra of a uniquely identified peptide (99% confidence) from immunoglobulin G binding protein A precursor. iTRAQ analysis revealed a >2.2 fold increase in expression of this protein between carrier D30 (iTRAQ label 116) and non-carrier 930918-3 (iTRAQ label 115) strains. Fig 3B and Fig 3C illustrate the iTRAQ ion spectra and MS/MS spectra for the proteins ABC transporter substrate-binding protein and autolysin revealing lower and equal expression levels, respectively, between carrier strain D30 (iTRAQ label 116) and non-carrier strain 930918-3 (iTRAQ label 115). The corresponding amino acid sequences of the peptides depicted in Fig 3 are given in supplemental Table S1.

The nasal carrier strain of *SA* expresses a greater number of proteins implicated in colonization than its non-carrier counterpart

The proteins identified in the exoproteomes were categorized with reference to their potential contribution to nasal carriage as conceived by Burian and colleagues.⁴⁴ These included proteins involved in metabolism, protein synthesis and trafficking, stress, pathogenesis and immunomodulation, cell adhesion, cell division and cycle, transport and unknown functions (supplemental **Table S1**). iTRAQ analyses revealed that the carrier strain of *SA* expressed a markedly different repertoire of proteins in comparison to the non-carrier strain (Fig 4A and 4B). We observed that 131 proteins were differentially expressed between carrier and non-carrier strain exoproteomes. Of these 131 proteins, 66 were expressed in higher amounts in the carrier strain of *SA* compared to the non-carrier strain, and 25 of these proteins exhibited a >2-fold increase in expression levels (see Table 1 for a partial list of proteins and supplemental Table S2 for the complete list).

i.) CELL ADHESION—Many adhesive proteins such as clumping factor B (ClfB), fibronectin-binding protein A (FnBPA), and iron-regulated surface determinant A (IsdA) are involved in binding of the bacteria to host matrix molecules and hence are thought to play key roles in nasal colonization of *SA*.⁴⁵ Our exoproteomic analyses revealed that additional adhesive proteins might play critical roles in colonizing the human nasal mucosa (Table 1). We observed that the *SA* carrier exoproteome contained a markedly larger number of adhesive proteins than the non-carrier strain (Fig 4C). One of the major proteins involved in host attachment, the cell wall surface anchor family protein (SasD), demonstrated nearly 4-fold higher expression in the carrier compared to the non-carrier strain (Table 1). iTRAQ analysis also detected significantly higher levels of two proteins in the carrier strain that have previously been implicated in nasal colonization: serine-aspartate repeat family protein (SrdH) and sortase (Srt) (Table 1). SasD and SrdH are two cell wall-anchored proteins containing host attachment domains (MSCRAMMs).^{46,47} These MSCRAMM proteins are covalently anchored to the host adhesive matrix molecules via the LPXTG motif recognized by sortase.¹³ SasD contains a slightly modified LPXAG anchor to host matrix molecules.

ii.) IMMUNOMODULATORY PROTEINS AND TOXINS—Immunomodulatory proteins play critical roles in *SA* infection of the host and subsequent nasal carriage.⁴⁸ Based on a comprehensive literature survey of the functions of these proteins, our results indicated that the carrier strains of *SA* contained a greater number of proteins which downregulate immunity, whereas the non-carrier strains contained a greater number of proteins which upregulate host immunity. Some of the most noticeable immunomodulatory proteins detected in greater abundance in the carrier strain were SPA and surface elastin binding protein (EbpS) (Table I). On the other hand, penicillin binding protein (PBP) 2, and cold shock protein (Csp) that makes *SA* susceptible to antibiotics and antimicrobial peptide human cathepsin G respectively,^{49,50} were found in higher levels in the non-carrier strain.

Immuno-evasive proteins including cell surface elastin binding protein (EbpS), SPA, PBP1, immunodominant staphylococcal antigen A precursor and α -hemolysin precursor identified in our study have also been independently observed in other studies.^{28,43,51-53} However, their roles with relevance to nasal colonization have not been elucidated. Notably, we observed that the expression levels of immunomodulatory toxins such as α -hemolysin precursor that are responsible for bacterial invasion of the host, were elevated in the carrier strain. Interestingly, leukocidin/hemolysin toxin family protein that helps in the formation of pores during *SA* pathogenesis of host was dominant in the non-carrier strain (Table 1).

iii.) TRANSPORT PROTEINS—*S. aureus* carrier strains can resist the host's antimicrobial agents by active efflux of the agents using translocation machineries.⁵⁴ This

phenomenon is mirrored in our results in which the nasal carrier strain produced substantially more transport proteins compared to its non-carrier counterpart (Fig 4C, Table 1). These included several proteins from the ATP-binding cassette (ABC) transporter super family and one from the common protein translocation machinery, Sec translocon (Table 1). Sec translocon proteins are implicated in bacterial pathogenesis and in the secretion of virulence proteins.⁵³ We observed that a key member of this translocon, protein-export membrane protein SecF that is part of the bifunctional translocase SecDF, was found in higher levels in the carrier strain indicating the likely involvement of these transport proteins in nasal colonization.

iv.) STRESS—*S. aureus*, when subjected to osmotic shock, heat, oxidative stress, starvation, alkaline shock etc, triggers a stress response.⁵⁵ Our iTRAQ data revealed that the carrier strain of *SA* secreted fewer stress response proteins in comparison to its non-carrier counterpart. Specifically, stress proteins such as CsbD-like superfamily and alkaline shock protein 23 (ASP23) were expressed in lower levels in the carrier strain (Table I). However, not all stress proteins were down-regulated in the carrier strain. Some stress proteins that are normally expressed when *SA* is exposed to environmental stress such as superoxide dismutase (SOD) and thioredoxin-disulphide reductase were expressed at similar levels by both strains (Table I).

The *SA* nasal carrier biofilm exoproteome contains greater number of stress and immunoevasion proteins than its planktonic counterpart

We previously reported that the carrier strain of *SA* (D30) adopted a biofilm mode of growth under ambient laboratory conditions.²² We hypothesized that biofilm formation may facilitate nasal carriage, given the importance of this form of growth to colonization and defensive capabilities of *SA*.⁵⁶ We therefore compared the biofilm exoproteome of a carrier strain of *SA* with its planktonic counterpart. To our knowledge, this was the first time such a comparison has been reported for the respective exoproteomes. We observed that 84 proteins were differentially expressed between planktonic and biofilm growth forms of the carrier strain of *SA*. Of the 84 proteins, 46 were expressed at higher amounts in the biofilm exoproteome compared to its planktonic counterpart and 35 exhibited >2-fold expression (See Table 1 for partial list of proteins and supplemental Table S3 for the complete list).

It was observed that the exoproteome of the biofilm form of the nasal carrier strain of *SA* was markedly different in terms of stress and immunomodulatory proteins compared to its planktonic counterpart (Fig 5A and 5B). Specifically, iTRAQ analyses revealed that the carrier strain biofilm had 4-fold fewer adhesive proteins as compared to the planktonic form (Fig 5C). Specifically, D-alanine-activating enzyme (DltD) protein, involved in D-alanylation of wall teichoic acid (WTA) during cell wall synthesis, was significantly down-regulated in the biofilm exoproteome.⁵⁷ Another protein involved in cell wall peptidoglycan synthesis, N-acetylmuramoyl-L-alanine amidase, was also significantly down-regulated in the biofilm form, perhaps indicating a lower cell wall turnover in the *SA* carrier strain when it adapts a biofilm mode of growth.

Adhesive proteins, together with immunomodulatory proteins, enable biofilms to resist the action of antibiotics and other antimicrobial agents.^{58,59} Not surprisingly, our iTRAQ analyses revealed that the biofilm growth form of the carrier strain of *SA* secreted more immunomodulatory proteins than the planktonic form (Fig 5C). Curiously, penicillin binding proteins (PBPs), including PBP1 and PBP4 that enable *SA* to resist β -lactam antibiotics, were expressed in lower levels in the biofilm growth mode. Another mechanism that allows bacterial biofilms to evade antimicrobial agents is the use of efflux pumps and

transporters.⁶⁰ Unexpectedly, the carrier strain biofilm secreted 3-fold fewer number of transport proteins compared to the planktonic form (Fig 5C).

Although the number of immunomodulatory proteins was only marginally greater in the carrier strain than the non-carrier strain based on the iTRAQ findings, these results did not concur with other analyses we conducted. We had observed that the resistance of the carrier strain to innate immune defense molecules (Reactive Oxygen Intermediates) is twice as much as the non-carrier strain (data not shown). Additionally, the resistance of biofilms to innate host molecules is 10-15 times greater than the planktonic variety (data not shown). Perhaps, the effectiveness of immune evasion by *SA* is not determined by the number of immunomodulatory proteins, but by the potency and quantity of individual proteins. To this end, we further analyzed one of the most abundant immunological *SA* proteins, Staphylococcal Protein A (SPA).

SPA from *SA* carrier strains is found in higher concentrations than the non-carrier strain

SPA which can be found to be either cell-wall associated or secreted, is well known for its binding, immunological, and biofilm promoting properties.^{48,26,25} Not surprisingly, we noticed throughout the *SA* exoproteomic analyses that levels of SPA were consistently higher in the carrier strain than the non-carrier strain (Fig 1). Using an SPA ELISA, we confirmed that the carrier strain had nearly 8-fold higher SPA levels than the non-carrier strain (Fig 6). This was verified in several other persistent carrier strains such as (D20, D39 and D98) and an intermittent carrier strain (D37). The levels of SPA were much higher in persistent carriers and comparatively lower in the intermittent carrier, although all carriers had significantly higher SPA levels compared to the non-carrier strain (Fig 6). These results suggest a possible correlation between increased SPA levels and nasal carriage.

Peptide mass fingerprinting of SPA from the carrier strain revealed that one of the isoforms of SPA was 49.5 kDa. Subsequent MS/MS and in silico analyses revealed that the protein contains three IgG binding domains as found in most species of *Staphylococcus* and also a cell wall localization motif LysM.⁶¹ This motif is responsible for SPA interaction with cell wall components of the bacteria but is not consistently present in all species of *SA*.^{56,61} Furthermore, MS/MS analysis also revealed the repeated occurrence of the LysM motif in other proteins such as cell wall binding autolysin, and cell surface elastin binding protein (data not shown). This repeated occurrence of LysM motif in important cell attachment proteins may indicate its possible involvement in *SA* nasal colonization.

Discussion

Through a comprehensive analysis of the exoproteomes of a nasal carrier strain of *SA* and a genetically similar non-carrier strain, we have endeavored to analyze proteins or groups of proteins, which may contribute to nasal carriage. In tandem, we compared the exoproteomes of biofilm and planktonic carrier *SA* cultures in order to evaluate possible determinants of nasal carriage. Interestingly, our results indicated that the exoproteome of the carrier strain of *SA* (D30) contains a greater number of proteins related to adhesion, protein transport and immunoevasion, and fewer stress proteins than its genetically similar non-carrier counterpart (930918-3). Similarly, an analysis of the exoproteome of the biofilm growth form of *SA* carrier strain revealed a greater number of immunoevasive proteins in comparison to its planktonic counterpart but fewer stress, adherence, and transport proteins.

Other researchers have examined various aspects of the *SA* proteome, with reference to proteins that promote adherence,^{45,62} immunoevasive proteins,^{48,63} total proteome,⁵² secretome,⁵¹ exoproteome⁶⁴ and surfacome²⁸ in addition to mRNA levels during nasal colonization.^{65,44} However, our studies have focused on the difference between proteins in

the exoproteome of a carrier strain of *SA* and a non-carrier strain in order to elucidate putative determinants that might influence *SA*'s ability to colonize nasal mucosa.

Several proteomics studies have indicated that *SA* proteins present in the extracellular milieu such as secreted and cell surface exposed proteins are highly diverse.^{53,66,28} However, no correlation between nasal carriage and heterogeneity of these proteins was discussed. In contrast, our study not only identified similar heterogeneity in cell surface and secreted proteins, but also deduced a marked difference in profile of cell surface and secreted proteins between nasal carrier and non-carrier strains.

The adherence of the *SA* carrier strain to host cells is an integral process in nasal carriage. Although several adhesive proteins have already been identified as important factors for nasal carriage,^{15,45,16} our exoproteome analysis has revealed that the carrier strain of *SA* contains twice as many adhesive proteins as a non-carrier strain. The results have highlighted the importance of SasD and SrdH, which have hitherto not been considered important in nasal carriage. Together with high expression levels of Srt, this seems to be indicative of high processing levels of membrane bound proteins, which is an important indicator in nasal carriage as considered by Burian and colleagues.⁴⁴

In contrast, *SA* carrier biofilm exoproteome secreted fewer adhesive proteins compared to the planktonic exoproteome. This may be expected as adhesion is already established once the carrier has formed a biofilm and subsequently the main function of the bacteria is survival and possibly ongoing detachment to aid in biofilm dissemination. Specifically, the adhesive D-alanine-activating enzyme (DltD) (responsible for D-alanylation of WTA during cell wall synthesis) was detected in the biofilm exoproteome.⁵⁷ It has been noted that inactivating the *dlt* operon in *SA* leads to increased susceptibility of *SA* to antimicrobial peptides including defensins and protegrins.⁶⁷ Perhaps the presence of *dlt* operon in *SA* biofilms confers a multifunctional role of resistance to antimicrobial peptides, adhesion and biofilm formation.

Together with adhesion, immunomodulation is one of the key factors in the nasal carriage of *SA*, since it allows for long-term survival of the bacteria in the host.^{48,62} Previously, we observed the ability of the carrier strain of *SA* (D30) to down-regulate defensins and pathogen receptors,¹¹ indicating the involvement of bacterial immunomodulatory proteins in *SA* nasal carriage. In support of this concept, the present results revealed that the nasal carrier isolates of *SA* contain a greater number of proteins that downregulate host immunity, whereas the non-carrier isolates contain a greater number of proteins that upregulate host immunity. This result is verified in Burian and colleagues' observations on nasal colonization.⁴⁴ A large proportion of these immunomodulatory proteins have also been corroborated in the surfacomes of the *SA* COL, Newman, RN6390 and USA300.²⁸ Additionally, our proteomics approach revealed that the carrier strain biofilm revealed a similar trend with greater number of immunomodulatory proteins being found in the biofilm when contrasted with the planktonic growth form.

The high abundance of SPA, a versatile immunoevasive and binding molecule, may be linked to the carrier strains' overall immunoevasive strategy. Interestingly, differential SPA expression patterns even within persistent and intermittent carriers could advantageously be used as a diagnostic tool to differentiate them. Certainly, greater amounts of surface bound molecules with high levels of sortase could be associated with the observation that proteins involved in protein modification, secretion and trafficking are found in greater numbers in the carrier strain of *SA* in comparison to the non-carrier strain.

The rates of cell wall turnover found in *Staphylococcus* in general are quite high.⁶⁸ This constant turnover of the cell wall provides ample decoy material for *SA* to engage host

innate defenses and is proportional to successful colonization of the human nasal passages.⁴⁴ This strategy can be seen in other pathogen/host dynamics such as *Schistosoma spp* and is referred to as sloughing.⁶⁹

Recently, researchers discovered that low concentrations of host chemokines, including CXCL9 and CXCL10, induce the release of SPA, while high concentrations of chemokines can also be antibacterial.^{70,71} This echoes findings from our previous research on the effects of the cytokine IL-1 α . We demonstrated that the carrier strain of *SA* downregulated the production of IL-1 α during infection and IL-1 α decreased the growth of *SA* carrier strain on nasal epithelial cells.²² At this stage we have not assessed if the cytokine would also induce SPA. Since we detected multiple isoforms of SPA in the carrier strain of *SA* but not in the non-carrier, we suspect that it may be posttranslationally modified in sequential stages such as the glycosylation patterns observed in the Golgi apparatus.⁷² Glycosylation of exoproteins such as SPA might play crucial roles in bacterial pathogenesis and immunoevasion.⁷³ We hypothesize that SPA is sequentially glycosylated in nasal carrier strains of *SA* and may be linked to nasal carriage.

One of the more surprising observations from our carrier strain exoproteome analysis was the low expression of stress proteins in comparison to the non-carrier strain. Perhaps, as observed by other authors, nasal colonization does not constitute a full blown infection but rather a persistent sub-clinical infection.^{5,44} Interestingly, transcript analysis of some key stress proteins also revealed that stress response stimuli are absent in the nasal milieu.⁴⁴

It has been postulated that *SA* develops resistance to nasal fluids by decreasing the uptake of antimicrobials or actively translocating them from the cell,⁵⁴ and several bacterial transport proteins play vital roles in these processes. A greater number of transport proteins in the exoproteome of nasal carrier strain in comparison to the non-carrier strain could make it more resistant to antimicrobial agents in the nasal milieu. Specifically, several proteins from the ATP-binding cassette (ABC) transporter super family and the protein-export membrane protein SecF (part of the bifunctional translocase SecDF) were found in much higher levels in the carrier strain. Further investigation into the implications of these results and the role of these transport proteins in the nasal carriage of *SA* is currently being pursued.

Increasing evidence suggests that the phenomenon of *SA* nasal carriage is a complex host-pathogen interaction for which the bacteria has evolved numerous immunoevasion strategies for successful colonization of the host.^{5,11,22} Our current exoproteomic study suggests that the *SA* nasal carrier strain is able to adapt itself to the human nasal passages by secreting a distinct repertoire of proteins in comparison to the non-carrier strain. Indeed, we have elicited the identities of several underreported proteins, which may be important in the nasal carriage of *SA*. Additionally, while it is not known if every carrier strain of *SA* adopts a biofilm mode of growth in the nose, it has become increasingly apparent that a biofilm mode of growth may be more representative of the *in vivo* condition.^{74,75} This comparison can be a foundation for future studies to identify fully the state of *SA* in the nose and its contributing factors. In conclusion, this exoproteome analysis has elucidated important strategies adopted by the *SA* carrier strain in its dissemination through nasal carriage that further augments our understanding of nasal carriage of *SA*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

SA	Staphylococcus aureus
MRSA	Methicillin resistant <i>Staphylococcus aureus</i>
HBD-3	Human β -defensin 3
2D-PAGE	Two-dimensional gel electrophoresis
AMPs	Antimicrobial peptides
ITRAQ	Isobaric tags for relative and absolute quantitation
ACN	Acetonitrile
SCX	Strong cation exchange
MMTS	Methyl methane-thiosulphonate
EF	Error factor
2D-LC	Two-dimensional liquid chromatography
TSB	Tryptic Soy Broth
SPA	Staphylococcal protein A

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FIG 1. Comparative exoproteome analysis of carrier (D30) and non-carrier (930918-3) strains of SA reveals significant differences in protein distribution

The distribution pattern of the exoproteomes of genetically similar strains of SA differed from the carrier to the non-carrier strain as evidenced by the distribution of Staphylococcal protein A (SPA) 49.5 KDa (circled). SPA spots were confirmed by ELISA and Mass spectrometry.

FIG 2. Integrated experimental workflow for the analysis of SA exoproteome by iTRAQ and volcano plot illustration of iTRAQ data

(A) The exoproteome of the carrier strain, the non-carrier strain and a carrier strain biofilm (growth media free) were extracted, purified and concentrated with the aid of C18 solid phase extraction cartridges. Proteins were subject to trypsin-digestion and labeled with iTRAQ reagents. Digests from the carrier strain (D30) exoproteome were labeled with iTRAQ reagents 116 and 117 whilst digests from the non-carrier strain (930918-3) exoproteome and carrier strain (D30) biofilm were labeled with iTRAQ reagents 115 and 114 respectively. Labeled samples were mixed, lyophilized and fractionated using Strong Cation Exchange (SCX) chromatography. The HPLC fractions were analyzed by LC-MS/MS on a QSTAR ESI quadrupole time-of-flight tandem MS system. The entire set of experiments from the collection of the exoproteome to LC MS/MS analysis was performed 3 times (N=3) and the raw MS/MS data were collated together for further analysis. (B) Protein expression fold change in SA carrier strain as compared to non-carrier strain. (C) Protein expression fold change in SA carrier strain under planktonic and biofilm growth conditions. Fold differences of protein expression are plotted against their respective *p* values. All proteins identified by at least one peptide with greater than 95% confidence are represented here. Horizontal dashed lines identify fold changes with *p* values of 0.05. Vertical dashed lines delineate a 2-fold change in the ratio of protein expression.

FIG 3. Representative iTRAQ MS/MS spectra showing protein identification in carrier and non-carrier strains from selected proteins

Representative MS/MS spectra of uniquely identified peptides (confidence, 99%) from (A) Immunoglobulin G binding protein A precursor (B) ABC transporter, substrate-binding protein and (C) Autolysin are represented here. The inset reveals the iTRAQ reagent peaks for relative quantitation in the *SA* strains. Illustrations for higher, lower and equal expression levels between carrier strain D30 (iTRAQ label 116) and non-carrier strain 930918-3 (iTRAQ label 115) have been provided.

FIG 4. The nasal carrier strain of *SA* expresses a greater number of adhesion/ binding proteins in its exoproteome than its non-carrier counterpart

A comparison of *SA* exoproteomes using iTRAQ has revealed that (A) the nasal carrier strain of *SA* (D30) expresses a different repertoire of proteins than (B) a genetically similar non-carrier strain (930918-3). (C) Percentage of proteins that were significantly up-regulated in that category in comparison to the other tested strain. The carrier strain of *SA* contains a greater proportion of proteins related to binding and adhesion but less stress proteins. Proteins were identified with high confidence ($p < 0.05$) in three independent experiments ($N=3$).

FIG 5. The biofilm growth form of nasal carrier strain of *SA*, in comparison to its planktonic counterpart contains marked differences in its exoproteome related to stress and immunoevasion The expression of proteins from the exoproteome of (A) the planktonic form of *SA* nasal carrier strain and (B) the biofilm form of the *SA* nasal carrier strain differ significantly in (C) proteins pertaining to stress, adhesion, immunomodulation and transport. Proteins were identified with high confidence ($p < 0.05$) in three independent experiments ($N=3$).

FIG 6. Immunomodulatory Staphylococcal protein A (SPA) is significantly up regulated in nasal carrier strains of SA compared to its non-carrier counterpart

The expression of SPA was measured in the exoproteomes of one non-carrier and 4 persistent carrier strains (PC) D20 (** $p = 0.001$), D30 (** $p = 0.006$), D39 (** $p = 0.007$), D98 (** $p = 0.009$) and 1 intermittent carrier (IC) D37 (** $p = 0.008$), strain using anti-SPA ELISA and compared to non-carrier strain 930918-3. Equal volumes of total exoproteome from nasal carrier and non-carrier strains were used for ELISA in three independent experiments (N=3) and the result was expressed as percentage of SPA concentration to total protein concentration.

TABLE 1

Proteins from the exoproteome of the nasal carrier strain of SA and the non-carrier strains are assigned into functional categories as identified and quantified by iTRAQ

Accession No.	Protein name	Number of peptides ^a	Fold ratio ^b		
			carrier/non-carrier	p value	Planktonic carrier/Biofilm carrier
Stress related proteins					
gi 87162409	CsbD-like superfamily	34	0.4565 ^c	0	1.3708 ^d 0.0058
gi 87162159	Hypothetical protein SAUSA300_1582, similar to CsbD-like family protein	13	0.6747	0	2.0123 0.0135
gi 87162200	Alkyl hydroperoxide reductase subunit C	27	1.1686 ^d	0.0002	1.6239 0.0128
gi 87161642	Alkyl hydroperoxide reductase subunit F	1	0.4971	0.0957	0.3723 0.188
gi 87160786	Hypothetical protein SAUSA300_1652, putative universal stress protein	24	0.862	0.0006	0.8978 0.2323
gi 87162087	Universal stress protein family	18	2.9027	0.0004	3.0664 0
gi 70726220 ^e	Hypothetical protein SH1219, putative universal stress protein	7			
gi 894289	Alkaline shock protein 23 (ASP23)	20	0.5771	0	1.1024 0.6569
gi 87160079	Peptide methionine sulfoxide reductase regulator (MsrR)	10	0.8648	0.1957	1.7545 0.0863
gi 87161086	Methionine-R-sulfoxide reductase	1	1.238	0.8192	15.0177 0.4261
gi 87161236	Thioredoxin [*]	12	1.3978	0.0003	0.7305 0.0387
gi 87161001	Thioredoxin-disulfide reductase	9	0.792	0.169	1.1776 0.6298
gi 87161687	Thiol peroxidase	8	0.756	0	1.4239 0.2404
gi 21282513	Hypothetical protein MW0784, similar to thioredoxin-fold containing protein family	5	0.8983	0.0001	1.2352 0.3626
gi 87160477	Putative thioredoxin	5	1.218	0.2208	1.2503 0.6608
gi 87160405	Hypothetical protein SAUSA300_1909, similar to thioredoxin family of proteins	4	0.987	0.9858	0.7007 0.628
gi 87160511	Catalase	5	0.5021	0.027	4.7999 0.0241
gi 87161707	Superoxide dismutase (Mn/Fe family)	5	0.9286	0.4111	0.7623 0.7832
gi 88195790	Putative ferritin	4	0.7199	0.5798	0.4058 0.1265
gi 87162273	Osmc/Ohr family protein	1	2.1538	0.6357	2.8528 0.3618

Accession No.	Protein name	Number of peptides ^a	Fold ratio ^b		
			carrier/non-carrier	p value	Planktonic carrier/Biofilm carrier
gi 87160980 ^e	Hypothetical protein SAUSA300_0725, similar to putative Oxidoreductase	1	0.5794		1.3866
Pathogenesis and immunomodulatory proteins					
gi 87160749	Cell surface elastin binding protein	200	1.7517 ^d	0	1.3029 0.0961
gi 133853458	Immunoglobulin G binding protein A precursor	138	2.2282	0	1.0756 0.3757
gi 56749001	Immunodominant staphylococcal antigen A precursor	57	1.4061	0	0.9476 0.656
gi 15926764	Penicillin-binding protein 1	43	1.3495	0.0004	1.4429 0.0048
gi 87162077	Penicillin binding protein 2	32	<i>0.6419</i>	<i>0.0001</i>	0.6655 0.1167
gi 87161157	Penicillin-binding protein 4	6	1.6283	0.1152	2.7796 0.013
gi 70726765	Beta-lactamase	10	0.0849	0	0.1207 0.0022
gi 87161577	Cold shock protein, CSD family	36	<i>0.7651</i> ^c	<i>0</i>	<i>0.7602</i> <i>0.0001</i>
gi 87160015	Staphylococcal tandem lipoprotein	14	<i>0.4398</i>	<i>0.0002</i>	1.1702 0.4995
gi 47169194 ^e	Chain A, staphylococcal protein A, B-domain, Y15W mutant, Nmr, 25 structures	12	0.9803		
gi 87160380	Alpha-hemolysin precursor	10	2.6865	0	1.1949 0.4484
gi 87160982	Leukocidin/hemolysin toxin family protein	10	<i>0.3131</i>	<i>0</i>	0.7128 0.2953
gi 87161881	Antibacterial protein	7	<i>0.5416</i>	<i>0</i>	0.8559 0.5626
gi 15927581	Hypothetical protein SA1813 similar to leukocidin-like protein 2	5	<i>0.3607</i>	<i>0</i>	1.1987 0.519
gi 87162162	Hypothetical protein SAUSA300_1018 similar to SCP/PR1-like extracellular protein	8	1.0669	0.5321	0.6697 0.3322
gi 87160365	Antibacterial protein	4	0.9121	0.1981	<i>0.2771</i> <i>0</i>
gi 87162347	Hypothetical protein SAUSA300_2164 similar to extracellular adherence protein	4	0.3271	0.1009	<i>0.1106</i> <i>0.0494</i>
gi 87160217	Secretory Antigen Precursor (SsaA)	4	0.9951	0.9764	0.5127 0.069
gi 88194063	Hypothetical protein SAOUHSC_00257 similar to EsxA virulent protein	4	0.384	0.0557	2.158 0.2965
gi 68565538	Protein esaA	2	0.2372	0.2204	0.197 0.2859
gi 87160905 ^e	Hypothetical protein SAUSA300_0282 similar to virulence protein EssB	1	0.2942		0.4179
gi 87162375	Hypothetical protein SAUSA300_1323 similar to	2	0.809	0.5491	0.1985 0.3431

Accession No.	Protein name	Number of peptides ^a	Fold ratio ^b			
			carrier/non-carrier	p value	Planktonic carrier/Biofilm carrier	p value
conserved virulence factor C						
gi 87160520	Acetyltransferase family protein in Oat A family	3	2.0482	0.0094	0.8063	0.7857
gi 87161173	Teicoplanin Resistance Associated Membrane Protein (TcaA)	3	0.8209	0.9069	1.4697	0.7023
gi 88195687	Hypothetical protein SAOUHSC_01999 similar to peroxiredoxin Q/BCP	3	2.9885	0.0002	1.9644	0.0359
gi 87162379 ^e	Ferredoxin	1	1.1786		1.8553	
gi 87161897	IgG-binding protein SBI	2	3.6719	0.1219	5.4157	0.0616
gi 87160565 ^e	Immunodominant antigen B	1	29.6467		2.0875	
gi 62391257 ^e	Secreted penicillin binding protein [<i>Corynebacterium glutamicum</i> ATCC 13032]	1	1.6876		2.2857	
Cell adhesion proteins						
gi 87160939	Cell wall surface anchor family protein	57	3.6116 ^d	0	1.2777	0.0838
gi 151222604	Hypothetical protein NWMN_2392 similar to cell wall surface anchor family protein	40	0.0911 ^c	0	0.2444	0
gi 87162315	Putative cell wall binding lipoprotein	16	1.2742	0.1446	1.1145	0.7887
gi 87162026	Autolysin	31	1.0742	0.6135	0.7695	0.2717
gi 87160697	D-alanine-activating enzyme/D-alanine-D-alanyl protein (dltD)	18	1.2272	0.2515	1.9912	0.0001
gi 87160121	D-alanine-activating enzyme/D-alanine-D-alanyl protein (dltC)	1	0.6864	0.533	16.1543	
gi 61213890	77 kda outer membrane protein precursor	11	0.2627	0	0.6572	0.1167
gi 87181509	UPF0365 protein SAV1573	8	1.8364	0.0005	2.9173	0.0019
gi 87160285	Rod shape-determining protein MreC	8	0.8103	0.0767	2.9577	0
gi 87160775	N-acetylmutamoyl-L-alanine amidase	8	1.5402	0.0651	2.0519	0.0096
gi 87161887	N-acetylmutamoyl-L-alanine amidase domain protein	7	1.893	0.3018	1.3838	0.4234
gi 88196468	Putative sortase	5	2.0829	0.001	1.2683	0.5337
gi 87161790	5'-nucleotidase family protein	3	3.6218	0.1664	1.5688	0.1773
gi 87160715	Fmt protein	2	1.1273	0.8064	1.1849	0.6709
gi 81673756	Phosphoglucosamine mutase	1	0.7583	0.739	3.3119	0.2556
gi 87160798	Serine-aspartate repeat family protein (SdrH)	1	1.49	0.0115	1.3251	0.4213

Accession No.	Protein name	Number of peptides ^a	Fold ratio ^b		
			carrier/non-carrier	p value	Planktonic carrier/Biofilm carrier
gi 116694144 ^e	Flp pilus assembly protein TadC	1	1.3693		2.585
gi 81781921	Extracellular matrix protein-binding protein EMP precursor	1	0.5809	0.4841	1.4533
gi 91211353 ^e	AsmA suppressor of OmpF assembly mutants	1			0.5086
Transport proteins					
gi 87162197	Amino acid ABC transporter, amino acid-binding protein	34	0.9535	0.4842	1.3001
gi 87162140	Oligopeptide ABC transporter, substrate-binding protein	30	1.1547^d	0.0301	1.3918
gi 87161352	ABC transporter, substrate-binding protein	12	0.2931	0.0126	0.4137
gi 21282147	Hypothetical protein MW0418 similar to ABC transporter, substrate-binding protein	6	1.0242	0.8594	1.2602
gi 87161864	ABC transporter, substrate-binding protein	3	2.8266	0.0151	2.1079
gi 87160588	Molybdenum ABC transporter, molybdenum-binding protein (ModA)	17	1.5245	0.0005	1.8056
gi 87161641	Amino acid ABC transporter, permease/substrate-binding protein	7	6.1167	0	2.5162
gi 21284120	Oligopeptide transporter putative substrate binding domain	6	2.4913	0.0004	1.9632
gi 87161764	Putative iron compound ABC transporter, iron compound-binding protein	5	1.4063	0.0805	0.7301
gi 87160849	Iron compound ABC transporter, iron compound-binding protein	4	0.5869	0.0986	0.6344
gi 87161518	Glycine betaine/carnitine/choline ABC transporter	4	0.9366	0.6998	1.0605
gi 87162224	Osmoprotectant ABC transporter, permease	2	0.6537		3.9979
gi 126355053 ^e	ABC transporter-related protein [<i>Pseudomonas putida</i> GB-1]	1	37.5193		6.929
gi 149194563 ^e	ABC transporter-related protein [<i>Caminibacter mediatlanticus</i> TB-2]	1	1.6215		0.0083
gi 87162212 ^e	Amino acid ABC transporter, ATP-binding protein	1	5.1042		0.571
gi 87161315	Hypothetical protein SAUSA300_2378 similar to potassium efflux protein kefA	21	1.4953	0	1.6492
gi 87160965	Phosphocarrier protein (HPr)	11	1.1099	0.0026	0.4598
gi 87162382	PTS system, glucose-specific IIA component	11	1.0668	0.4928	2.9434
					0
					0.004

Accession No.	Protein name	Number of peptides ^a	Fold ratio ^b		
			carrier/non-carrier	p value	Planktonic carrier/Biofilm carrier
gi 87162442	Transferrin receptor	8	3.331	0	3.5596
gi 87160279	AcrB/AcrD/AcrF family protein	7	1.4716	0.1045	1.0728
gi 87162284	Putative ferrichrome ABC transporter	1	0.2468	0.0722	0.5068
gi 87161389	Putative iron compound ABC transporter, iron compound-binding protein	1	2.6052	0.134	1.4787
gi 87160515	Protein-export membrane protein SecF	6	1.4937	0.0097	0.7504
gi 87160369	Hypothetical protein SAUSA300_0833	3	0.5373	0.5051	0.4642
gi 15925912	RGD-containing lipoprotein	3	1.2004	0.4097	1.0422
gi 87161142	Ferric hydroxamate receptor	3	1.0332	0.8931	0.4321
gi 87160674	Putative lipoprotein	41	0.2959 ^c	0	0.7197
gi 87161872	Putative lipoprotein	2	4.2798	0.2519	2.4403
gi 87160414	Multidrug resistance protein A, drug resistance transporter	1	0.6934	0.2928	0.7756
gi 23005821 ^e	COG1131: ABC-type multidrug transport system, ATPase component	1			0.6174
gi 149201149	Nitrate transport ATP-binding subunits C and D	1	1.5776		7.9917
gi 151575108	Outer membrane efflux protein	1	1.1986	0.2951	3.8451
gi 87161139	Iron transport associated domain protein	1	1.6828		0.1596
gi 149910101	Hypothetical transport protein [<i>Moritella</i> sp. PE36]	1	2.2325	0	4.2379
gi 35211526 ^e	gi 0963 [<i>Gloeobacter violaceus</i> PCC 7421]	1			
gi 127512243 ^e	Efflux transporter, RND family, MFP subunit	1			
gi 146301866 ^e	RND efflux system, outer membrane lipoprotein, NodT family	1	0.9695	0.7073	0.4187
gi 17131745 ^e	all2652	1	1.7839		0.8275
gi 87162344	Phosphonate ABC transporter, phosphonate-binding protein	1	1.3653	0.3305	6.3883
gi 51595518	Molybdenum transport regulatory (repressor) protein (ModE)	1	1.3022	0.3562	1.1322
gi 152936446 ^e	Flagellar motor switch protein fltG	1			0.9636

^a The list contains quantitative information of the proteins (including the number of peptides) from the iTRAQ data set. These proteins have met the criteria (i.e., unused prot score >2.0, change in expression levels of at least 1.2-fold p-value <0.05 and EF <1.4 for all ratios) as defined in the Experimental Procedures.

^b Relative change in protein levels between secretomes

^c italicized numbers= down-regulation.

^d **bold numbers** =up-regulation.

^e iTRAQ identified unique proteins not quantified.

* Denotes a protein with multiple functions.