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Characterization of the Accessory Sec System of *Staphylococcus aureus*▽

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The SraP adhesin of *Staphylococcus aureus* is a member of a highly conserved family of serine-rich surface glycoproteins of gram-positive bacteria. For streptococci, export of the SraP homologs requires a specialized transport pathway (the accessory Sec system). Compared to streptococci, however, SraP is predicted to differ in its signal peptide and glycosylation, which may affect its dependence on a specialized system for transport. In addition, two genes (*asp4* and *asp5*) essential for export in *Streptococcus gordonii* are missing in *S. aureus*. Thus, the selectivity of the accessory Sec system in *S. aureus* may also differ compared to streptococci. To address these issues, the five genes encoding the putative accessory Sec system (*secY2*, *secA2*, and *asp1-3*) were disrupted individually in *S. aureus* ISP479C, and the resultant mutants were examined for SraP export. Disruption of *secA2* resulted in the near complete loss of SraP surface expression. Similar results were seen with disruption of *secY2* and *asp1*, *asp2*, or *asp3*. To assess whether the accessory Sec system transported other substrates, we compared secreted proteomes of ISP479C and a *secA2* isogenic mutant, by two-dimensional fluorescence difference gel electrophoresis. Although two consistent differences in proteome content were noted between the strains, neither protein appeared to be a likely substrate for accessory Sec export. Thus, the accessory Sec system of *S. aureus* is required for the export of SraP, and it appears to be dedicated to the transport of this substrate exclusively.

The binding of bacteria to platelets is a postulated central event in the pathogenesis of infective endocarditis (23). Initial colonization of damaged endothelium on the valve surface may be mediated by the attachment of blood-borne bacteria to platelets bound at the site of injury (9, 24, 31). Platelets may subsequently be recruited to the site of infection through direct adhesion to immobilized bacteria (6). These events, in combination with bacterial proliferation, are thought to produce the hallmark lesion of infective endocarditis, the macroscopic vegetation.

Staphylococcus aureus has been shown to bind to human platelets through a variety of adhesins. Many of these surface components bind platelets through their interaction with bridging molecules, such as fibrinogen or fibronectin (7, 13, 15, 17, 22). In addition, our laboratory has identified a large surface glycoprotein of *S. aureus*, the serine-rich adhesin for platelets (SraP) (Fig. 1) that also mediates binding to human platelets (21). Although the receptor for SraP binding has not been identified, it appears that SraP can bind directly to the platelet surface (21). Loss of SraP expression is associated with reduced virulence in an animal model of endocarditis, indicating that this interaction is important for the pathogenesis of endovascular infection (21).

SraP shares similarity with a group of cell wall-associated glycoproteins found in a number of organisms including, *Streptococcus sanguinis*, *Streptococcus pneumoniae*, and *Streptococcus agalactiae* (4, 12, 16, 19, 20, 25, 30). Among the best-characterized members of the family is GspB of *Streptococcus gordonii*. This large, extensively glycosylated surface protein has a multidomain structure that includes two serine-rich domains, an N-terminal receptor binding domain, and an atypically long signal peptide (Fig. 1). Export of GspB occurs exclusively through an accessory Sec system, whose sole function appears to be the transport of this substrate to the bacterial surface. This pathway is comprised of SecY2 and SecA2 (homologs of SecY and SecA, respectively, of the general Sec system) and five accessory Sec proteins (Asp1 to Asp5) that lack homology to any proteins of known function (4, 28).

The components for GspB glycosylation and export are encoded in a locus located immediately downstream of the gene encoding the adhesin (Fig. 1). Although the organization of this locus is well conserved across species, the SraP accessory Sec locus of *S. aureus* has a number of distinctive features. First, it contains only two glycosylation-related genes (*gtfA* and *gtfB*) compared to four such genes in *S. gordonii* (*gly*, *nss*, *gtfA*, and *gtfB*) (Fig. 1) (26), suggesting that SraP may be less extensively glycosylated compared to GspB. Since glycosylation is a major structural feature of GspB that precludes its export by the canonical Sec system, these findings suggest that SraP may be less stringent in its export requirements. In addition, the signal peptide of SraP differs from that of GspB in that its signal peptide contains fewer glycine residues in the hydrophobic region, which block the entry of the protein into the canonical Sec pathway (3). Third, two essential components of the accessory Sec system in *S. gordonii* (*asp4* and *asp5*) (28) are absent in *S. aureus* (Fig. 1). The lack of Asp4 and Asp5 in *S. aureus* indicates that the accessory Sec system of *S. aureus* may function differently from its ortholog in *S. gordonii*. Taken together, these differences in the export substrate and *sec* locus indicate that the accessory Sec system in *S. aureus* may have altered substrate specificity compared to *S. gordonii.* To address these issues, we examined the effects of disrupting the

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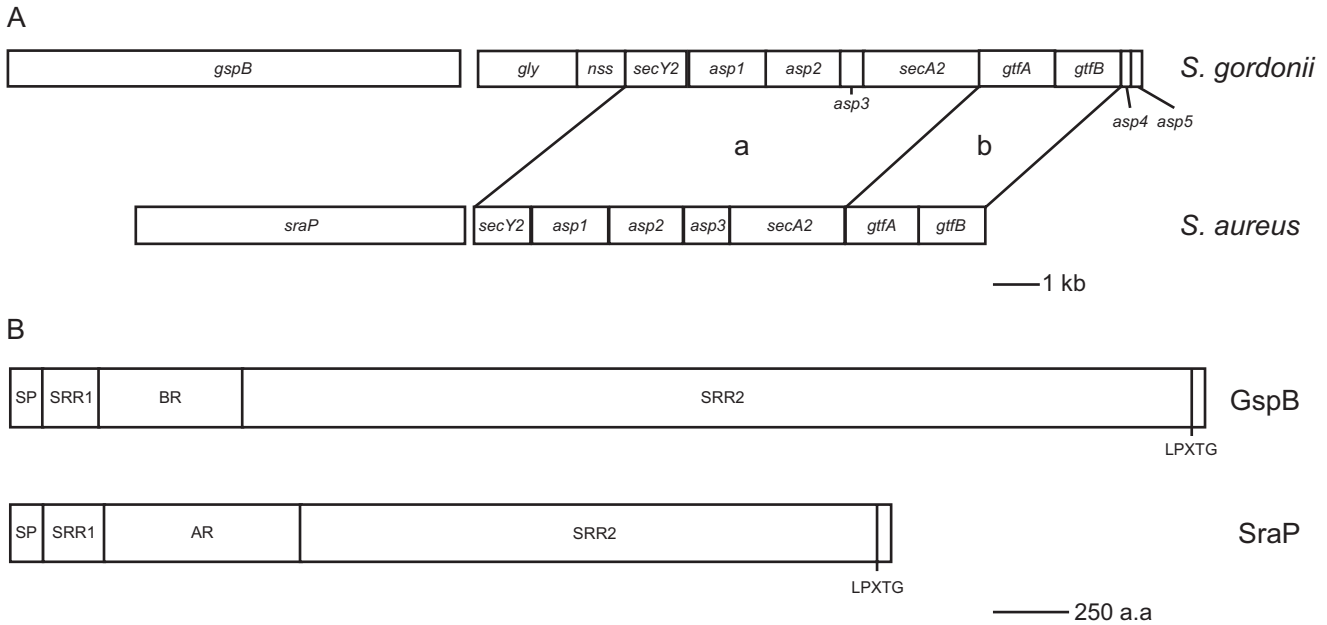

FIG. 1. Schematic diagram of the accessory Sec loci of *S.aureus* and *S. gordonii*. (A) The export pathways include two sets of common genes: (a) the export-related genes *secY2*, *asp1*, *asp2*, *asp3*, and *secA2* and (b) the glycosyltransferase genes *gtfA* and *gtfB* that are predicted to modify the adhesins. Note that the streptococcal accessory secretion system includes two additional secretory components encoded by the genes *asp4* and *asp5* and two additional glycosylating enzymes that are encoded by the *gly* and *nss*. (B) Domain structure of the two adhesins, SraP and GspB. SP, atypically long signal sequence; SRR1 and SRR2, serine-rich domains; BR and AR, putative N-terminal binding regions.

accessory Sec system on SraP-specific and general protein transport by *S. aureus*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and primers. The bacterial strains, plasmids, and primers used in the present study are described in Table 1. *S. aureus* ISP479C is a commonly used laboratory strain (18). PS767 is an isogenic variant of ISP479C, in which *sraP* has been disrupted by the insertion of *ermB* (21).

Media and antibiotics. Tryptic soy broth (TSB) and tryptic soy agar, blood agar, and RPMI were used as culture media for *S. aureus*. *Escherichia coli* was grown in Luria-Bertani (LB) broth or on LB agar. Erythromycin was used at a concentration of 15 μg/ml for selection of *S. aureus* and at 400 μg/ml for selection of *E. coli*. Spectinomycin was used at a concentration of 50 μg/ml for selection of *E. coli* and 1 mg/ml for selection of *S. aureus*.

Mutagenesis of accessory *sec* genes by allelic replacement. Recombinant plasmids were constructed to partially disrupt by allelic replacement the genes encoding members of the putative accessory Sec pathway. Two specific flanking regions of each gene were amplified by PCR. The primer pairs (Table 1) were designed to encode unique restriction sites at the ends of the amplification products. The products were digested with the appropriate enzymes and purified after agarose gel electrophoresis. The knockout vectors were then constructed in a multistep process. The purified PCR products were ligated on either side of the *spec* gene in the *E. coli* vector pS326. To generate a vector suitable for replication in *S. aureus*, the pS326-based constructs were linearized and then ligated to temperature-sensitive staphylococcal vector pTSermC (8). The ligation product was electroporated into *E. coli* DH5α, and transformants were selected on LB agar supplemented with erythromycin. The newly generated shuttle vectors were used for transformation of *S. aureus* RN4220 by electroporation. The plasmids were then transferred to *S. aureus* ISP479C by transduction using phage 80α. Transductants were cultured sequentially at nonpermissive and permissive temperatures in the presence of spectinomycin. Spectinomycin-resistant, erythromycin-sensitive colonies were selected for testing by PCR and Southern blotting to confirm that the targeted genes had been disrupted. Selected mutations were then transduced back into WT ISP479C, which had not undergone growth at high temperature, by phage 80α.

Microarray analysis. Portions (1 ml) of the overnight cultures, grown in LB, were diluted into 100 ml, and the cultures were grown at 37°C with shaking at 200 rpm to an optical density at 600 nm of 0.7. A 2-ml portion of each culture (10^9 CFU) was transferred to 4 ml of RNA Protect (Qiagen, Germantown, MD) and mixed, and the cells were pelleted by centrifugation for 4 min at 10,000 × *g* at 25°C, resuspended in 1 ml of RNA Protect, and then frozen at −80°C.

Total RNA was isolated from the cell pellets by using the Fastprep cell homogenizer (Qbiogene, Irvine, CA) and the RNeasy system (Qiagen, Valencia, CA), with on-column DNase digestion, according the manufacturer's instructions, with the exception of an additional wash with 350 μl of RW1 prior to DNase digestion. RNA was eluted in 40 μl of RNase free distilled H_2O and quantified by UV spectroscopy, and the quality of the sample was assessed by capillary electrophoresis using an Agilent 2100 Bioanalyzer (Agilent Biosystems, Santa Clara, CA).

RNA from three independent isolations was pooled and labeled with biotin-16-UTP (Roche Applied Science, Indianapolis, IN) representing 40% of the total UTP in each reaction, using the MessageAmp II bacterial aRNA amplification kit (Ambion, Austin TX) according to the manufacturer's instructions. After labeling, the aRNA was fragmented (5× fragmentation buffer, product 9003371; Affymetrix, Santa Clara, CA) and hybridized to wyeSaur2a microarrays (Wyeth, King of Prussia, PA, and Affymetrix) (5). Six genomes are represented on the chip: N315, Mu50, MRSA252, MSSA476, NCTC 8325, and COL. Microarray probe intensities were analyzed by using GeneSpring GX 7.3.1 (Agilent Biosystems). Only probes flagged "present" by the Affymetrix microarray processing software (3,017 of 7,792 total probes) were used in the analysis. Significance differences between probe intensities for strains ISP479C and ISP479C *sec2A*::*spec* were evaluated by using analysis of variance with a cross-gene error model, parametric test with all available error estimates selected, and the Benjamini and Hochberg false discovery rate correction for multiple comparisons.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), Western blotting, and glycan detection. Proteins from the culture media and cellular compartments were isolated and prepared for electrophoresis as described previously (1, 21). In brief, overnight cultures of *S. aureus* were diluted 1:50 in TSB and grown for 3 h at 37°C with shaking at 270 rpm. The optical density at 600 nm of the cultures was adjusted to 1, and equal volumes of each sample were centrifuged (3,200 × *g*) to pellet the cells. The media were filtered using nonsterile 0.22 μM Millex-GV polyvinylidene difluoride filters (Millipore). Proteins in the filtered media were precipitated with trichloroacetic acid (1), and the pellets were washed with acetone and then dried. The washed proteins were prepared for electrophoresis by suspension in 1× loading buffer. Cell wall pro-

TABLE 1. Strains, plasmids, and primers

Bacterial strain, plasmid, or primer	Genotype, sequence (5′→3′), or description[a]	Source or reference[b]
Strains		
S. aureus		
ISP479C	8325-4 *pig-131*	18
RN4220	Restriction-deficient strain that can be transformed with DNA from *E. coli*	14
PS767	ISP479C *sraP*::*ermB*	21
PS1166	ISP479C *secA2*::*spec*	This study
PS1167	ISP479C *secY2*::*spec*	This study
PS1257	ISP479C *asp1*::*spec*	This study
PS1280	ISP479C *asp2*::*spec*	This study
PS1249	ISP479C *asp3*::*spec*	This study
E. coli DH5α	F^- ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 endA1 recA1 hsdR17*(r_K^- m_K^+) *deoR thi-1 supE44* λ^- *gyrA96 relA1*	11
Plasmids		
pS326	High-copy *E. coli* vector with MCS-Spec^r	27
pTSermC	PTV1ts derivative with MCS and Erm^r	8, 10
Primers		
SY2LU	AGTCTCTCGAGATTAGTGATGAACCCTTAGTC	-*secY2* left flank For
SY2LD	AGTCTAAGCTTGCGGAAACGAACATTAAATCG	-secY2 left flank Rev
SY2RU	AGTCTGAATTCTAAATTATCAAGCACGACGCG	-*secY2* right flank For
SY2RD	AGTCTGGATCCTTTTCTTCAATTAGTTGAGCC	-*secY2* right flank Rev
A1LU	AGTCTCTCGAGGGAACGATGTTATTAGTTTGG	-*asp1* left flank For
A1LD	AGTCTAAGCTTTCATTTTCAAGGTGCATTCCC	-*asp1* left flank Rev
A1RU	AGTCTGAATTCAGGCACAATCGACAAATAGCC	-*asp1* right flank For
A1RD	AGTCTGGATCCAGGTTCTAAATCGTCTCCTCC	-*asp1* right flank Rev
A2LU	AGTCTCTCGAGAATTGGAATTATGCGTACGCC	-*asp2* left flank For
A2LD	AGTCTAAGCTTCCATCGTTTGTGTATAGGTCC	-*asp2* left flank Rev
A2RU	AGTCTGAATTCTATGGGTCAATTTTTACTCGG	-*asp2* right flank For
A2RD	AGTCTGGATCCTGAAAATGATACTTTAGAGCC	-*asp2* right flank Rev
A3LU	AGTCTCTCGAGAGTGATGAATCGCAGTATTCC	-*asp3* left flank For
A3LD	AGTCTAAGCTTACAGTTCAAAAAATCATTCG	-*asp3* left flank Rev
A3RU	AGTCTGAATTCGGATGGAAGACGATATCAAGG	-*asp3* right flank For
A3RD	AGTCTGGATCCCGTTGCTGTTAATGTTTTACC	-*asp3* right flank Rev
SA2LU	AGTCTCTCGAGATATCCTGATGAAGCCTATGC	-*secA2* left flank For
SA2LD	TGCATAAGCTTCAGGTAACAATGTATCTAGTG	-*secA2* left flank Rev
SA2RU	AGTCTGAATTCGATTATTTAGTTAAGCGATGG	-*secA2* right flank For
SA2RD	AGTCTGGATCCATATTCAACACCGCTACTCGC	-*secA2* right flank Rev

[a] Erm^r, erythromycin resistance; Spec^r, spectinomycin resistance. Restriction sites are indicated by underscoring in the sequences.
[b] For the primers, the position and orientation (forward [For] or reverse [Rev]) are specified.

teins were isolated from whole bacteria by treatment with lysostaphin (21). The pelleted cells were washed three times with phosphate-buffered saline (PBS) and then suspended in protoplasting buffer (10 mM Tris-HCl [pH 7.5], 10 mM $MgCl_2$, 30% raffinose, EDTA-free Complete protease inhibitors [Roche]) supplemented with 50 μg of lysostaphin/ml. The suspension was incubated for 30 min at 37°C and then centrifuged (16,000 × *g*) to pellet the protoplasts. The supernatant was removed, filtered as described above, and then prepared for electrophoresis by the addition of the appropriate volume of loading buffer. The protoplasts were washed in protoplasting buffer and then suspended in molecular-grade water supplemented with DNase (500 U/ml). The protoplast suspension was incubated for 15 min at 37°C and then centrifuged (16,000 × *g*) to separate the cytoplasmic fraction from the membrane-enriched insoluble material. The soluble cytoplasmic material was diluted 1:2 and prepared for electrophoresis by the addition of the appropriate volume of loading buffer. The membrane-enriched pellet was washed with molecular-grade water and then suspended in 1× loading buffer. Each of the subcellular protein fractions was separated by electrophoresis, using 4 to 12% gradient polyacrylamide gels and the Novex Tris-acetate gel system (Invitrogen) according to the manufacturer's instructions. One gel of each set was stained with GelCode (Pierce), and the proteins in the other gels were then transferred electrophoretically to BioTrace NT membranes (Pall Corp.) in Towbin buffer. The membranes were treated overnight with 1× blocking reagent (Roche) and then incubated with polyclonal rabbit anti-SraP serum (diluted 1:1,000 in block solution) (21), polyclonal rabbit anti-SecA serum (*S. gordonii*), or polyclonal rabbit anti-alpha-toxin serum (diluted 1:10,000 in block solution) (Sigma). Goat anti-rabbit immunoglobulin G (IgG) coupled to horseradish peroxidase and SuperSignal PicoWest (Pierce) was then used to detect SraP and alpha-toxin. When Western blotting was performed to quantify SraP transport, all membrane-blocking steps were performed with 3% gelatin in PBS. In addition, Cy5-coupled goat anti-rabbit IgG was used as a secondary antibody. Fluorescent signals were detected by using a Typhoon scanner (GE Healthcare) and quantified by using ImageQuant (version 5.2; GE Healthcare).

Glycosylation of SraP was assessed by lectin blotting with succinyl wheat germ agglutinin (sWGA). This lectin is able to detect glycosylated SraP with approximately an eightfold-greater sensitivity than anti-SraP serum as assessed by dot blot analysis (data not shown). Protein extracts were prepared as described above, separated by electrophoresis, transferred to membranes, and blocked for 2 h with 1% gelatin in PBS. The membranes were then incubated with biotinylated sWGA, washed, and probed with streptavidin-horseradish peroxidase to detect glycoproteins by chemiluminescence.

Two-dimensional fluorescence difference gel electrophoresis (2D-DIGE). (i) Sample preparation and labeling. Overnight cultures of bacteria were diluted 1:100 in TSB or RPMI and grown in a rotary shaker (270 rpm) at 37°C for 3 h. The cultures were centrifuged to pellet the bacteria, and the supernatants were clarified with a 0.45-μm-pore-size filter and supplemented with Complete protease inhibitors (Roche). The media were then concentrated by ultrafiltration (≥10-kDa retention cutoff; Millipore), and the protein concentration was determined. The proteins were then precipitated with trichloroacetic acid and suspended at a concentration of 3 mg/ml in 30 mM Tris-HCl (pH 8.8), 7 M urea, 2 M thiourea, and 4% CHAPS {3-[(3-cholamidopropyl)-dimethylammonio]-1-pro-

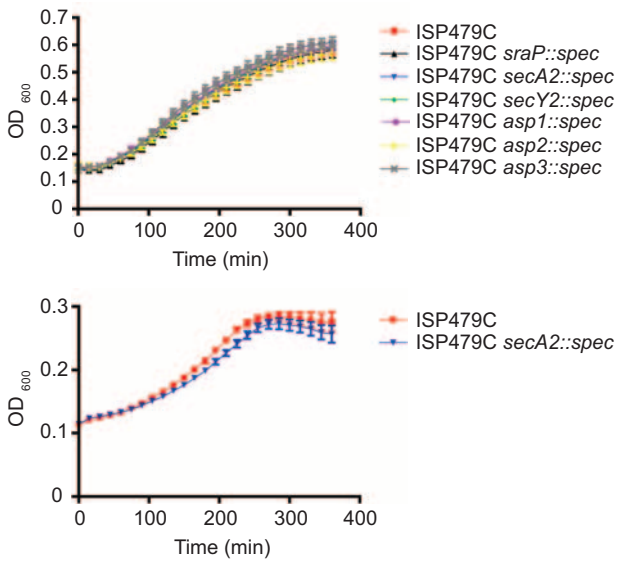

FIG. 2. Growth of *S. aureus* ISP479C and isogenic mutants of the accessory Sec system. Overnight cultures of *S. aureus* were subcultured in TSB (top panel) or RPMI (bottom panel), and the growth of the organisms was monitored by determining the optical density.

panesulfonate}. A total of 30 μg of each sample preparation was labeled with 1.0 μl of 20 μM CyDye, incubated in the dark on ice for 30 min, and then quenched by adding 1.0 μl of 10 mM lysine. The Cy3- and Cy5-labeled samples were diluted to 250 μl by the addition of 2× 2D sample buffer (8 M urea, 4% CHAPS, 20 mg of dithiothreitol/ml, 2% pharmalytes, and a trace amount of bromophenol blue), 100 μl of destreak solution, and rehydration buffer (GE Biosciences).

(ii) Isoelectric focusing and SDS-PAGE. Labeled samples were loaded into an immobilized pH gradient strip (pH range 3 to 10), and isoelectric focusing was performed in three steps (1 h at 500 V, followed by 1 h at 1,000 V and 2 h at 8,000 V) at 20°C in the dark. After focusing, the immobilized pH gradient strip was equilibrated according to the manufacturer's instructions, transferred onto an SDS gel (10.5% SDS gel prepared using low-fluorescence glass plates), and sealed with 0.5% (wt/vol) agarose solution (in SDS gel running buffer). The gel was run at 10 mA for 30 min, followed by 40 mA for 2.5 h.

(iii) Spot detection and analysis. The gels were scanned analyzed by ImageQuant software (version 5.0; GE Healthcare), and the images were then subjected to in-gel analysis using DeCyder software (version 6.0; GE Healthcare). Protein spots of interest were chosen based on the intensity of the individual CyDye signals and the quality of the three-dimensional peak image. A 1.5-fold difference in normalized fluorescence intensity was considered significant and was the minimum intensity difference for spot selection. The selected protein spots were subjected to in-gel trypsin digestion, peptide extraction, and desalting, followed by MALDI-ToF/ToF analysis (Applied Biosystems). Peptides were analyzed by using MASCOT and the NCBInr database to identify the selected protein spots. 2D-DIGE and mass spectrometry identification of proteins of interest were performed by Applied Biomics, Inc. (Fremont, CA).

RESULTS

Mutagenesis of the accessory *sec* locus. Isogenic variants of ISP479C containing disruptions in the *secA2*, *secY2*, or *asp* genes were generated by allelic replacement. As shown in Fig. 2, disruption of the accessory Sec pathway had no effect on growth of the mutants in vitro compared to the parent strain. To ensure that disruptions of the accessory Sec pathway did not alter secretion via the canonical Sec pathway, culture supernatants of the parent and mutant strains were compared by

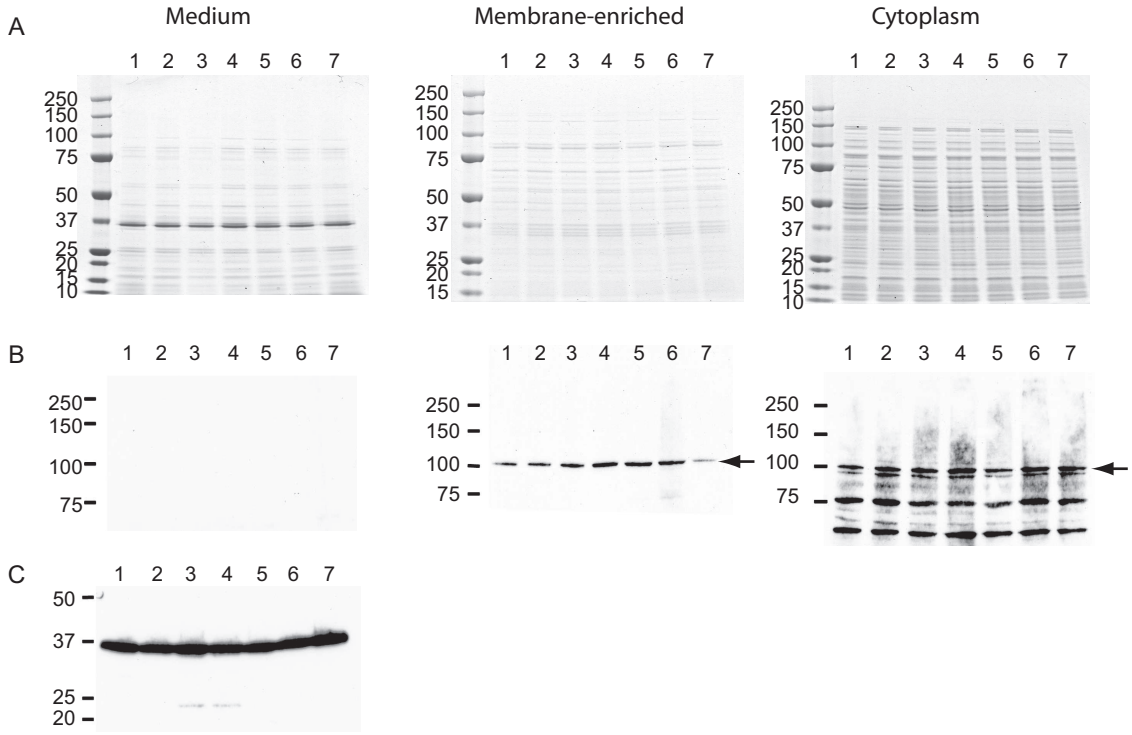

FIG. 3. Characterization of culture medium, membrane-associated, and cytoplasmic fractions. Lane 1, ISP479C; lane 2, PS767 (SraP$^-$); lane 3, PS1167 (SecY2$^-$); lane 4, PS1166 (SecA2$^-$); lane 5, PS1257 (Asp1$^-$), lane 6, PS1280 (Asp2$^-$); lane 7, PS1249 (Asp3$^-$). (A) GelCode stained SDS-PAGE; (B) Western blotting with anti-SecA sera; (C) Western blotting with anti-Hla sera. Arrows indicate the position of SecA in on the blots. Note that equivalent amounts of alpha-toxin are present in all culture samples.

TABLE 2. Genes with differential expression in *S. aureus* wild type versus Δ*secA2* mutant

Open reading frame	Fold change[a]	Description of putative gene product function
N315-tRNA	6.081	tRNA
SAtRNA16	2.997	tRNA-Ser
SAtRNA19	2.993	tRNA-Ser
SAtRNA27	2.345	tRNA-Leu
COL-SA0178	2.089	Sucrose-specific PTS transporter protein
COL-SA0179	2.255	Transcriptional regulator (RpiR family)
COL-SA0651	3.090	Conserved hypothetical
COL-SA0930	2.652	Conserved hypothetical
COL-SA2173	2.397	Alkaline shock protein
COL-SA2323	2.043	Imidazolonepropionase
COL-SA2324	2.395	Urocanate hydratase
COL-SA2694	2.133	Lipase
COL-SA0205	–2.04	Pyruvate-formate lyase-activating enzyme
COL-SA1840	–2.19	Conserved hypothetical
COL-SA2039	–2.01	Putative acetyltransferase
COL-SA2655	–2.28	Arginine/ornithine antiporter

[a] Wild type versus Δ*secA2* mutant.

Western blotting for the levels of alpha-toxin secreted into the growth media. This protein was chosen because it has a typical signal sequence, indicating that it is exported via the canonical Sec system, and because it is predominantly secreted into the culture medium, making it a convenient indicator of Sec function. As shown in Fig. 3C, culture media from ISP479C and its isogenic mutants contained equivalent amounts of the toxin, indicating that these mutations do not affect export by the canonical Sec system. To ensure that there was no contamination of the culture media due to cellular leakage, the cytoplasm, membrane, and media from the wild type and mutants were tested for the presence of SecA. As shown in Fig. 3B, SecA was present in the cytoplasm and membrane fraction of all samples. However, it is completely absent from the medium fractions, indicating that the cells were not releasing cytoplasmic proteins due to leakage or cellular breakdown.

Effects of accessory *sec* locus mutations on transcription in *S. aureus*. To determine whether disruption of the accessory Sec pathway resulted in pleiotropic effects on *S. aureus* transcription, microarray analysis was performed on RNA extracted from ISP479C and the SecA2$^-$ variant (PS1166) during mid-logarithmic growth. Changes that might occur as a result of disrupting the Sec system include alteration of regulatory pathways (e.g., Agr and Sar), induction of stress responses, or other effects related to the normal physiological state (such as metabolic or uptake pathways) of the organism. Changes in transcription of ≥2-fold and an intensity level greater than 1 were considered relevant. Only 12 genes met these criteria (Table 2). Eight of the twelve were genes whose transcription was greater in the wild type relative to the SecA2 mutant

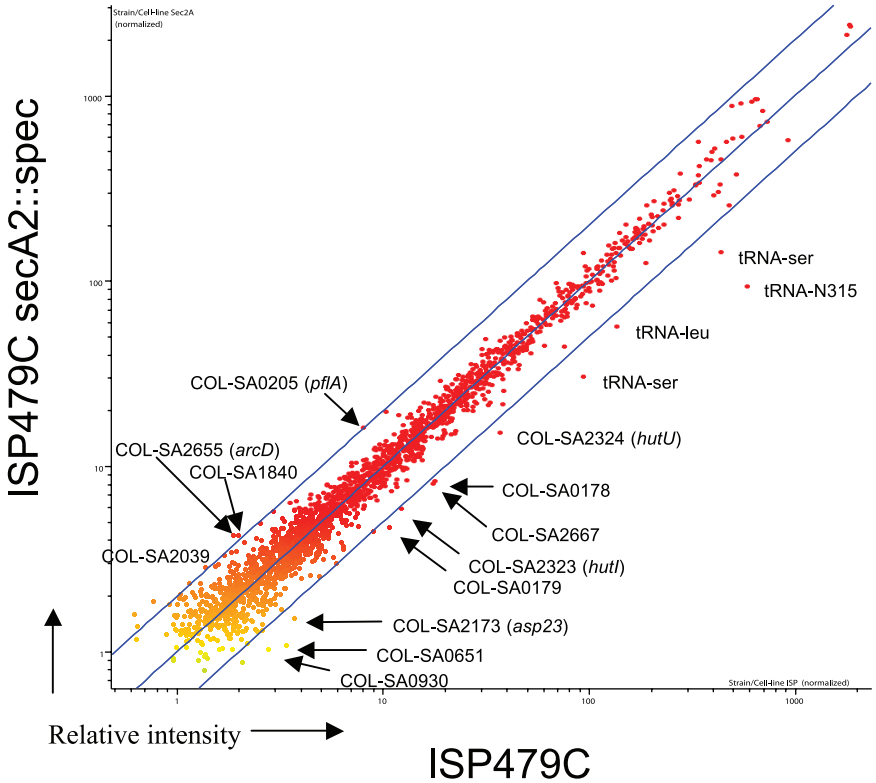

FIG. 4. Microarray analysis of ISP479C and an isogenic *secA2* mutant. Scatter plot of probe intensities from Affymetrics wyeSaur2a microarrays for wild-type strain ISP479C and *sec2A* mutant PS1166. Points outside of the two outer diagonal lines represent a >2-fold difference in the probe intensities between the two strains. The identities of selected probes are indicated by their respective *S. aureus* COL gene designations and common names if applicable. No significant differences ($P < 0.05$) in gene expression were found between strains by analysis of variance using the Benjamini and Hochberg false discovery rate correction for multiple comparisons.

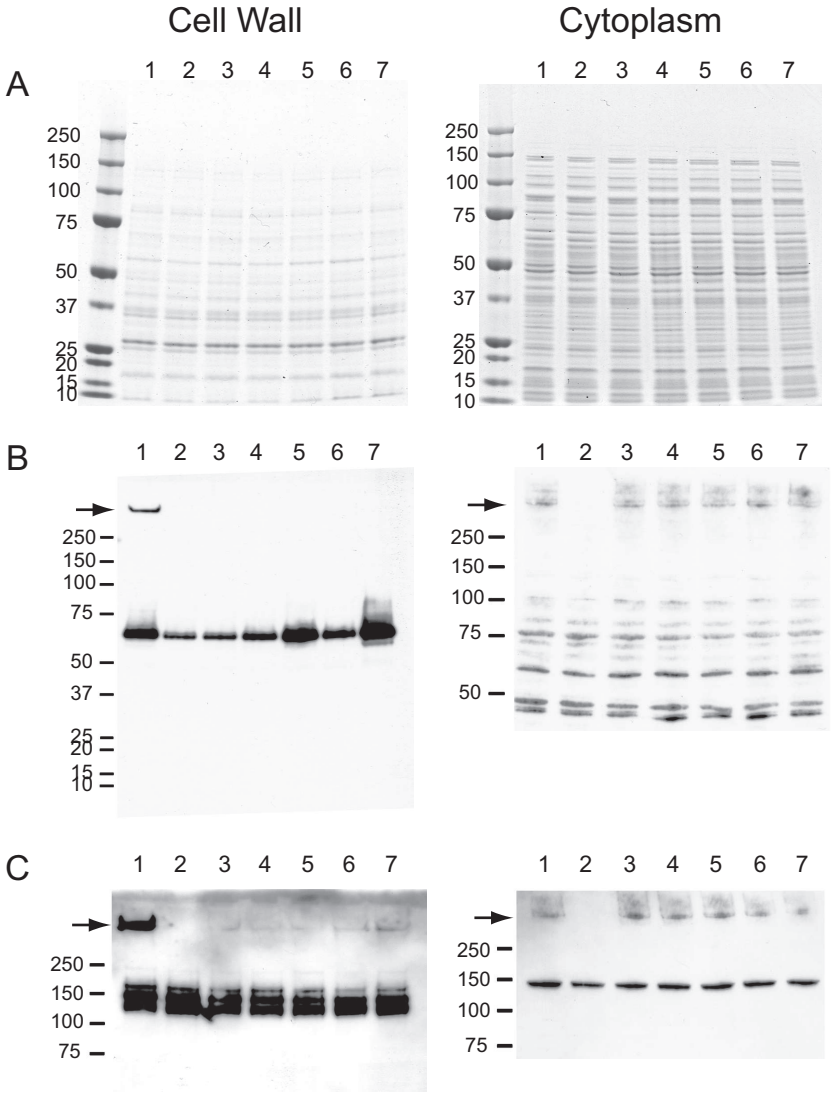

FIG. 5. SraP from wild-type *S. aureus* ISP479C and isogenic accessory Sec pathway mutants. Lane 1, ISP479C; lane 2, PS767 (SraP$^-$); lane 3, PS1167 (SecY2$^-$); lane 4, PS1166 (SecA2$^-$); lane 5, PS1257 (Asp1$^-$); lane 6, PS1280 (Asp2$^-$); lane 7, PS1249 (Asp3$^-$). GelCode-stained SDS-polyacrylamide gel. (B) Western blot analysis of *S. aureus* cell wall and cytoplasmic protein extracts. SraP was detected using anti-SraP sera. (C) Lectin blot analysis of *S. aureus* cell wall and cytoplasmic protein extracts. Carbohydrate residues on SraP were detected using sWGA. Arrows indicate the position of SraP in on the blots.

PS1116, while four genes were transcribed preferentially in the mutant. Of note, no significant changes were seen in expression of genes encoding components of the Agr or Sar pathways, indicating that no major regulatory systems were perturbed by disruption of *secA2* (Fig. 4). Moreover, no significant changes in transcription were observed that would indicate that mutation of the accessory Sec pathway adversely affects the cellular physiological state. Specifically, no stress responses (including DnaK related pathways) were observed, nor were any metabolic pathways (glycolytic, etc.) affected. However, disruption of *secA2* was associated with a significant reduction (>2-fold) in the expression of two Ser-tRNAs.

Secretion of SraP through the accessory Sec pathway. To examine the role of the accessory Sec system in the transport of SraP, we examined the impact of individually disrupting the genes encoding this pathway on the presence of SraP in the cell wall. As shown in Fig. 5B (lanes 3 to 7), disruption of *secA2*, *secY2 asp1*, *asp2*, or *asp3* resulted in a >90% decrease in SraP expression on the cell wall, as measured by quantitative Western blotting. To confirm that disruption of these genes nearly abolished SraP export and to assess the impact of these mutations on the glycosylation of SraP, we analyzed the cell wall extracts by lectin blotting with sWGA. Our previous studies have shown that this lectin can detect *N*-acetylglucosamine on members of this family of glycoproteins with high sensitivity (2). Dot blot analysis of cell wall extract from ISP479C showed that sWGA was eightfold more sensitive in detecting SraP than was anti-SraP serum (data not shown). When assessed by this method, disruption of *secA2*, *secY2*, or *asp1-3* was again found to markedly reduce (but not abolish) SraP export (Fig. 5C). Of note, exported SraP still bound sWGA, and was unchanged in its apparent molecular weight, indicating that glycosylation of

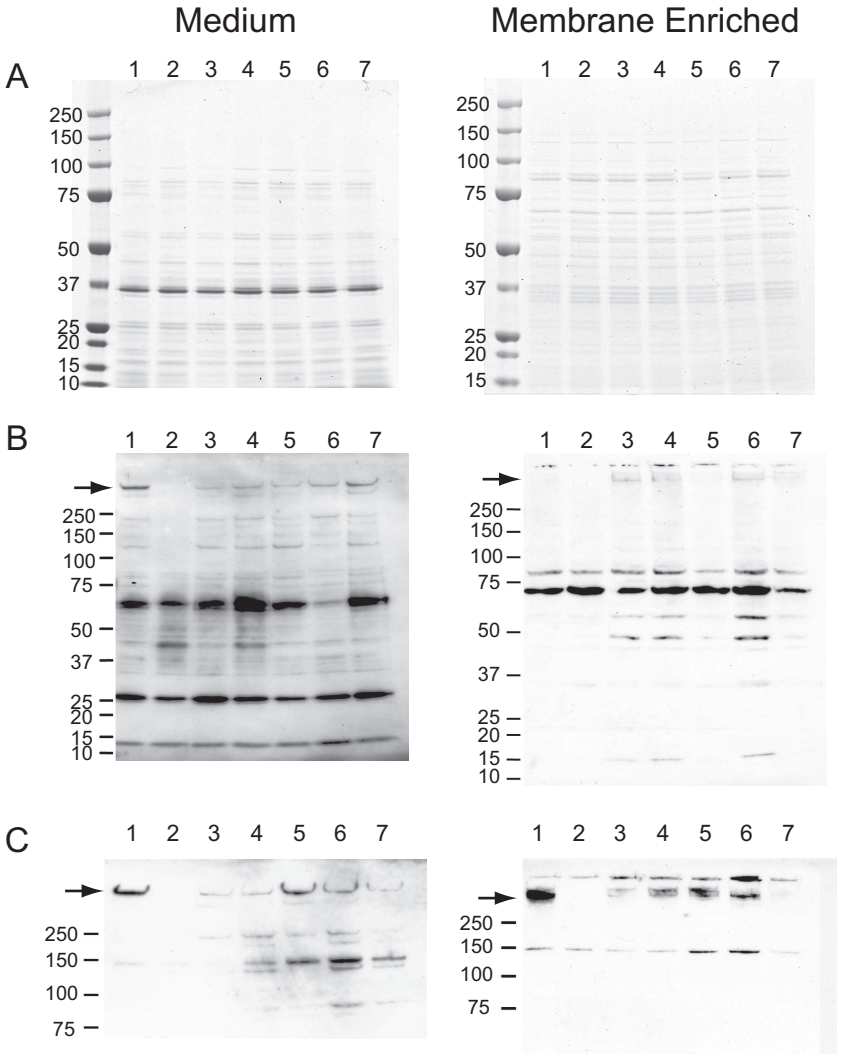

FIG. 6. SraP from wild-type *S. aureus* ISP479C and isogenic accessory Sec pathway mutants. Lane 1, ISP479C; lane 2, PS767 (SraP$^-$); lane 3, PS1167 (SecY2$^-$); lane 4, PS1166 (SecA2$^-$); lane 5, PS1257 (Asp1$^-$); lane 6, PS1280 (Asp2$^-$); lane 7, PS1249 (Asp3$^-$). GelCode-stained polyacrylamide gel. (B) Western blot analysis of proteins from the spent media and membrane fraction of *S. aureus*. SraP was detected using anti-SraP sera. (C) Lectin blot analysis of *S. aureus* media and membrane protein extracts. Carbohydrate residues on SraP were detected using sWGA. Arrows indicate the position of SraP in the blots.

the preprotein was unaltered in the isogenic strains (and that these mutations were not polar). We also tested the other cellular fractions from the wild type and mutants for the presence of SraP, by blotting with either anti-SraP serum or sWGA (Fig. 6). As expected, no SraP was detected in the SraP$^-$ mutant (PS767). In all of the accessory Sec mutants, SraP was present in the cytoplasmic and membrane fractions. Small amounts of SraP could also be detected in the culture medium, presumably reflecting cell wall turnover. Thus, it appears that components of the accessory Sec system are essential for the efficient transport of glycosylated SraP from the cytoplasm to the bacterial cell surface.

Secretion of extracellular proteins through the accessory Sec pathway. To assess whether the accessory Sec system of *S. aureus* has broad substrate specificity, we examined the secreted proteome of ISP479C and its SecA2$^-$ variant, PS1166, using 2D-DIGE. This method is able to reveal differences in complex protein mixtures with a high degree of sensitivity based on separation of differentially labeled protein sample on a single 2D gel (29). The supernatants from three independent log-phase cultures of each strain were evaluated in parallel. Two were prepared from culture media of bacteria grown in TSB (Fig. 7A, panels 1 and 2). Since samples generated from cultures grown in TSB can have high background fluorescence (P. Dunman, unpublished data), a third pair of samples was obtained from bacteria grown in RPMI (Fig. 7A, panel 3). In all, 41 proteins in at least one of the three replicate samples showed detectable differences in levels of expression (1.5-fold change in normalized fluorescence intensity), when media from ISP479C and PS1166 were compared. However, only nine of these proteins were detected in more than one sample pair. Of the nine proteins that were identified in multiple samples, only two proteins consistently differed in their levels of expression. Supernatants from ISP479C contained higher levels of a predicted lipase (accession gi 49245892) compared to PS1166

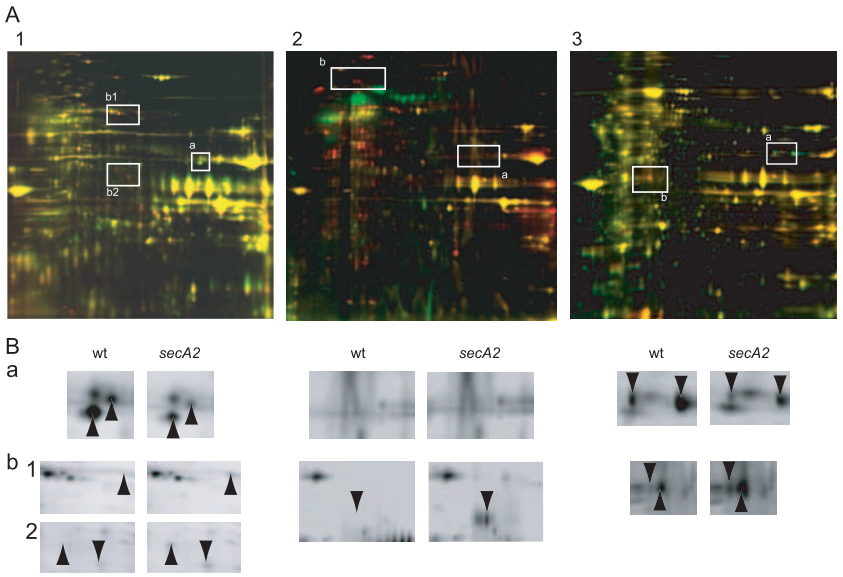

FIG. 7. Secreted proteins from *S. aureus* culture. (A) Wild-type *S. aureus* ISP479C and PS1166, an isogenic *secA2* mutant, were grown for 3 h in either TSB (panels 1 and 2) or RPMI (panel 3), and the culture media were analyzed by 2D-DIGE. Proteins from the wild-type culture media were labeled with Cy3 (green), while proteins from the mutant were labeled with Cy5 (red). Boxes indicate protein spots that are consistently enriched in one of the samples. (B) The boxed areas described in panel A are magnified, and the individual fluorescent channels are displayed. (Row a) A lipase was enriched in culture medium samples from wild-type *S. aureus*. (Row b) An *N*-acetylmuramoyl-L-alanine amidase domain protein was enriched in culture medium from the *secA2* mutant. Arrowheads indicate the relevant protein spots for comparison.

(Fig. 7B, row a). Interestingly, microarray analysis showed a 2.13-fold greater transcription of the gene encoding the lipase in the wild type relative to the *secA2* mutant. The second protein that consistently demonstrated differential expression was an *N*-acetylmuramoyl-L-alanine amidase domain protein (accession gi 57652384). It was enriched in the SecA2 mutant cultures (Fig. 7B, row b), and thus it is unlikely to be a direct substrate for the accessory Sec system. Thus, it appears that the accessory Sec pathway in *S. aureus* is dedicated to the transport of SraP exclusively.

DISCUSSION

Although the accessory Sec locus of *S. aureus* resembles that of *S. gordonii*, there are a number of key differences that may affect the function of this export pathway. First, the signal peptide of GspB contains three glycine residues in the hydrophobic region that impede the recognition of the protein by the canonical Sec pathway (3). The SraP signal peptide contains only two glycines, and their position is also changed relative to that of GspB, which could render SraP more amenable to export by the canonic pathway. Second, SraP has a pI that is acidic, while GspB is more basic in nature. The overall difference in charge may affect how the proteins interact with the accessory Sec system. Third, the staphylococcal locus lacks two genes, *gly* and *nss*, that are important for the glycosylation of GspB (27). When *gly* or *nss* were deleted in *S. gordonii*, GspB was shown to be differentially glycosylated by high-pH anion-exchange chromatography combined with pulsed amperometric detection. Since *S. aureus* lacks these two enzymes, it is likely that SraP is glycosylated differently or less extensively than GspB. Since glycosylation is one of the main barriers for GspB export by the canonical pathway, altered or reduced glycosylation of SraP may also render it a more suitable substrate for canonical transport. Finally, *S. aureus* also lacks Asp4 and Asp5, which may be integral membrane proteins and thus part of the accessory Sec translocon of *S. gordonii*. The absence of these proteins in *S. aureus* suggests that its accessory Sec translocon could have a different, and perhaps more permissive, substrate specificity compared to *S. gordonii*. For these reasons, we examined whether the key features of the accessory Sec system in *S. gordonii* were conserved in *S. aureus*.

Mutagenesis of any of the accessory *sec* genes generated strains that had no apparent growth defects, indicating that these genes were not essential for viability. The individual mutants also displayed an export defect that resulted in a loss of SraP expression on the cell surface when assessed by Western blotting. Of note, when export was assessed by lectin blotting, a small amount of SraP was present on the cell surface. Thus, it would appear that the accessory Sec system is largely essential for the export of SraP. However, since small quantities of the SraP can still be exported in the absence of a functional accessory Sec system, the glycoprotein may be transported with very poor efficiency by the canonical Sec system. Alternatively, some components of the canonical system may be able to substitute functionally for missing members of the accessory Sec system, thereby permitting limited secretion through the accessory Sec pathway. However, the minimal amount of SraP that is present on the surface of *S. aureus* in the accessory Sec mutants would indicate that such substitution is at best very inefficient.

As discussed above, the accessory Sec locus of *S. aureus* lacks four genes found in *S. gordonii*, suggesting that the staphylococcal accessory Sec system could be less restrictive in its substrate specificity. In fact, the accessory Sec-related secretomes in most gram-positive organisms have yet to be studied extensively. To assess this issue more definitely, we used 2D-DIGE to identify substrates of the accessory Sec system. This

method is able to reveal differences in complex protein mixtures with a high degree of sensitivity based on separation of differentially labeled protein samples on a single 2D gel. When the secreted proteome of wild-type *S. aureus* was compared to that of its isogenic SecA2 mutant, only two changes in the protein content were detected after repeated testing. The samples differed in the amount of a lipase and an amidase that were secreted by the two strains. However, the lipase also showed differential expression when assessed by microarray analysis, and thus various amounts in the secreted samples may be due to differences in transcription rather than transport. The amidase was enriched in the SecA2 mutant samples for unknown reasons, but it is unlikely to be a substrate for the accessory Sec system. Thus, it appears that this pathway is dedicated to a single substrate, SraP.

Since all of the conserved components of the accessory Sec system are essential for the efficient export of SraP, the differences between the SraP and GspB loci may provide a clearer explanation as to why small amounts of SraP are secreted when individual components of the accessory Sec locus are disrupted. As mentioned earlier, the absence of Gly and Nss in *S. aureus* may render SraP less extensively or differentially glycosylated than GspB. Since glycosylation is the key structural feature of mature GspB that hinders its export by the canonical Sec system, the less extensive glycosylation of SraP may make it more amenable to export by this pathway. These differences in glycosylation may also explain why Asp4 and Asp5 are not required for SraP export by the accessory Sec system. It is possible that SecY2 alone or in concert with the canonical SecE and SecG may be sufficient for the formation of the translocation channel. Thus, a combination of differential glycosylation and a distinct conformation of the translocon may define the selectivity of the accessory Sec pathway in *S. aureus*. Future studies assessing the importance of glycosylation and translocon structure in *S. aureus* may reveal how the SraP substrate is adapted for export by its accessory Sec system and provide further insight into the nature of secretion in bacteria.

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