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Mapping the Pathways to Staphylococcal Pathogenesis by Comparative Secretomics

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INTRODUCTION

The gram-positive bacterium *Staphylococcus aureus* is a frequent component of the human microbial flora that can turn into a dangerous pathogen. As such, this organism is capable of infecting almost every tissue and organ system in the human body. It does so by exporting a variety of virulence factors to the cell surface and extracellular milieu of the human host. Like all living organisms (201), *S. aureus* contains several protein transport pathways, among which the general secretory (Sec) pathway is the most well known and best described. Proteins that need to be transported to an extracytoplasmic location generally contain an N-terminal signal peptide that is needed to target the newly synthesized protein from the ribosome to the translocation machinery in the cytoplasmic membrane. Next, the protein is threaded through the Sec translocon in an unfolded state. During this translocation step, or shortly thereafter, the signal peptide is removed by a so-called signal peptidase (SPase). Upon complete membrane translocation, the protein has to fold into its correct conformation and will then be retained in an extracytoplasmic compartment of the cell or secreted into the extracellular milieu. In the case of gram-positive cocci, such as *S. aureus* (Fig. 1), we distinguish three extracytoplasmic subcellular compartments, namely, the membrane, the membrane-cell wall interface, and the cell wall. Since surface-exposed and secreted proteins of *S. aureus* play pivotal roles in the colonization and subversion of the human host, it is of major importance to obtain a clear understanding of the protein transport pathways that are active in this organism (103). Knowledge about the protein sorting mechanism has become all the more relevant with the emergence of staphylococcal resistance against last-defense antibiotics, such as vancomycin. The scope of this review is to provide a state-of-the-art roadmap of staphylococcal secretomes, which include both protein transport pathways and the extracytoplasmic proteins of staphylococcal organisms. The focus is on *S. aureus*, but comparisons with *Staphylococcus epidermidis* and the best-characterized gram-positive bacterium, *Bacillus subtilis*, are included where appropriate. Importantly, the present review aims to integrate the results of genomic and proteomic studies on *S. aureus* secretomes, representing the first documented "comparative secretomics" study. Specifically, this review deals with known and predicted exported virulence factors, pathways for protein transport, signals for subcellular protein sorting or secretion, and the exoproteomes of different *S. aureus* isolates, as defined by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and mass spectrometry (Fig. 2 and 3). The exoproteome is defined by all *S. aureus* proteins that can be identified in the extracellular milieu of this organism and thus includes proteins actively secreted by living cells and the remains of dead cells. For a clear appreciation of the present review, it is important to bear in mind that the proteins exported from the cytoplasm could be directly involved in staphylococcal virulence, whereas the respective protein export systems represent the "pathways to pathogenesis."

EXPORTED STAPHYLOCOCCAL VIRULENCE FACTORS

S. aureus and *S. epidermidis* are organisms that occur naturally in and on the human body. While *S. epidermidis* is mostly present on human skin, *S. aureus* can be found on mucosal surfaces. *S. aureus* is carried by 30 to 40% of the population (143) and can be identified readily in the nose, but the organism can also be detected in other moist regions of the human body, such as the axillae, perineum, vagina, and rectum, which thereby form a major reservoir for infections. Although most staphylococcal infections are nosocomial (i.e., hospital acquired), an increase in the

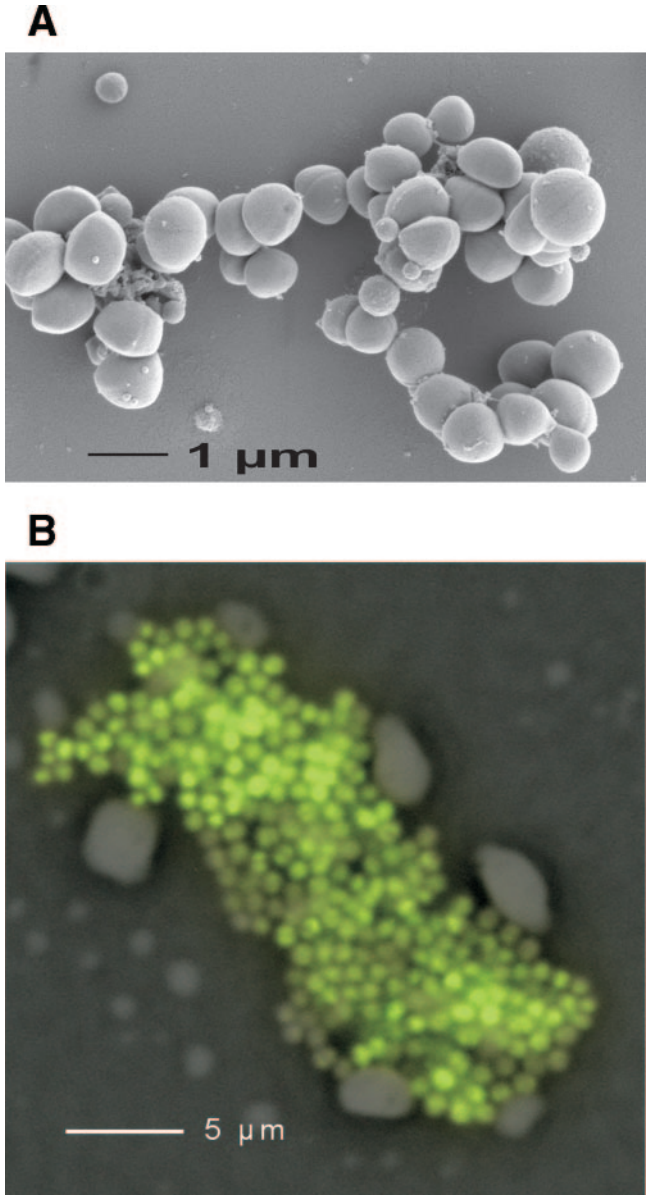

FIG. 1. Imaging of *S. aureus* RN6390. (A) For scanning electron microscopy, a drop of washed culture of bacteria was fixated for 30 min with 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.38. Next, the fixated bacteria were placed on a piece (1 cm^2) of cleaved 0.1% poly-L-lysine-coated mica sheet and washed in 0.1 M cacodylate buffer. This specimen was dehydrated in an ethanol series consisting of 30%, 50%, 70%, 96%, and anhydrous 100% (3×) solutions for 10 min each, critical point dried with CO_2, and sputter coated with 2 to 3 nm Au/Pd (Balzers coater). The specimen was fixed on a scanning electron microscope stub holder and observed in a JEOL FE-SEM 6301F microscope. (B) Micrograph of a cluster of *S. aureus* cells grown in blood culture medium. The cells were fixed with ethanol and hybridized with the fluorescein-labeled peptide nucleic acid (PNA) probe PNA-Stau. The image was generated by merging an epifluorescence image with the negative of a phase-contrast image.

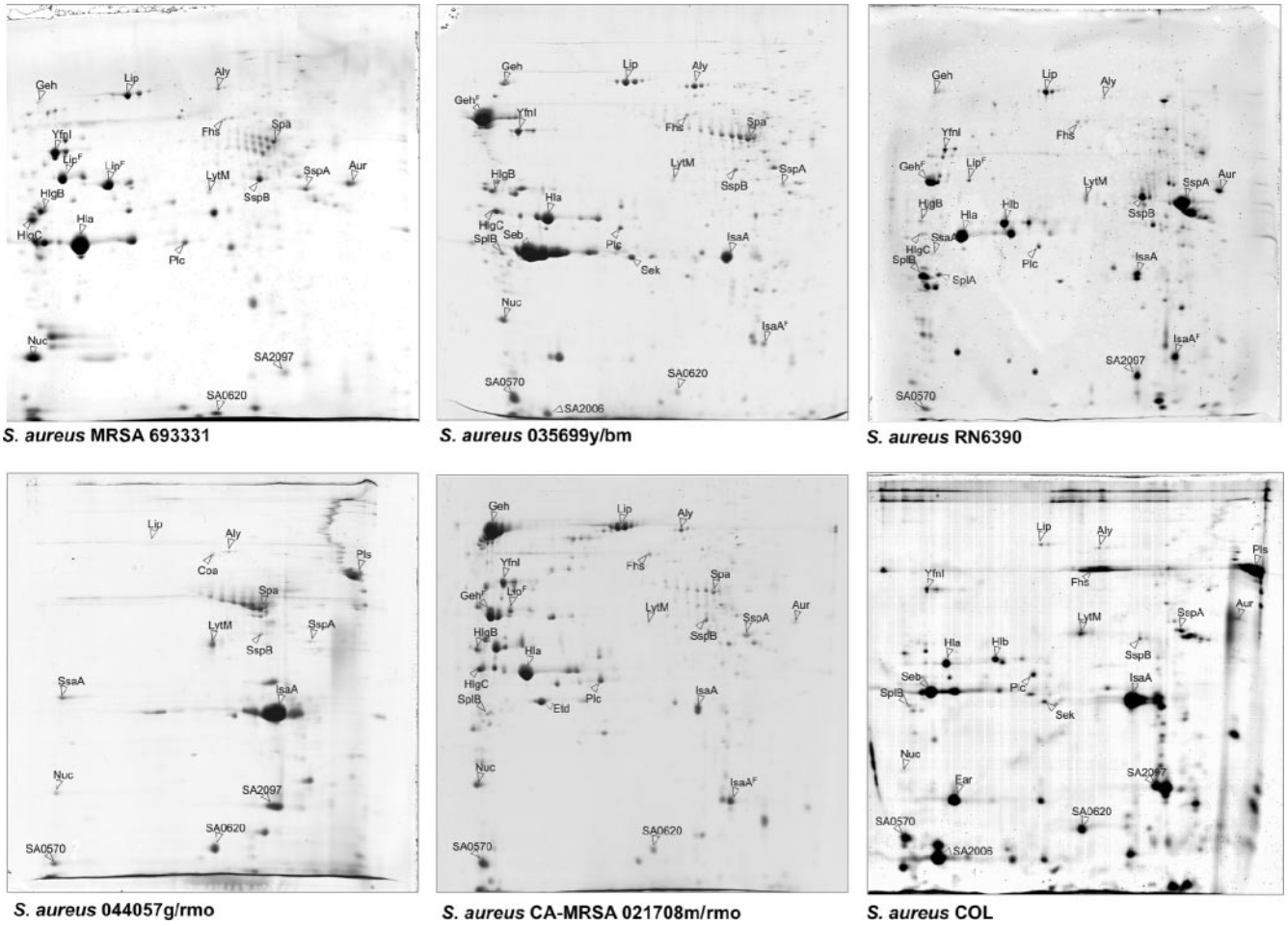

FIG. 2. Extracellular proteomes of different *S. aureus* strains. Proteins in the growth medium fractions of different staphylococcal isolates, grown in TSB medium (37°C) to an optical density at 540 nm (OD_{540}) of 10, were separated by 2D-PAGE using immobilized pH gradient strips in the pH range of 3 to 10 (Amersham Pharmacia Biotech, Piscataway, N.J.). Each gel was loaded with 350 μg protein extracts and, after electrophoresis, stained with colloidal Coomassie blue. Proteins were identified by matrix-assisted laser desorption ionization–time of flight mass spectrometry. The corresponding protein spots are labeled with protein names according to the *S. aureus* N315 database or NCBI entries for proteins not present in N315. The *S. aureus* strains that were used in these experiments are RN6390 and COL and four clinical isolates from the University Medical Center Groningen, named MRSA693331, 035699y/bm, 0440579/rmo, and CA-MRSA021708m/rmo.

number of cases of community-acquired, antibiotic (methicillin)-resistant infections is currently being observed worldwide (27, 67, 185). The risk of intravascular and systemic infection by *S. aureus* rises when the epithelial barrier is disrupted by intravascular catheters, implants, mucosal damage, or trauma. Interestingly, after infection, cells of *S. aureus* can persist unnoticed in the human body for a long time (years), after which they can suddenly cause another infection. *S. aureus* is primarily an extracellular pathogen whose colonization and invasion of human tissues and organs can lead to severe cytotoxic effects. Nevertheless, *S. aureus* can also be internalized by various cells, including nonphagocytic cells, which seems to induce apoptosis (43, 72, 120). Although *S. aureus* has the potential to form biofilms (64), *S. epidermidis* infections are particularly notorious for the formation of thick multilayered biofilms on indwelling catheters and other implanted devices. The formation of such a biofilm takes place in several steps, during which the bacteria first adhere rapidly to the surface of a polymer material that has been coated with a film of proteinaceous and nonproteinaceous organic host molecules (56). Bacteria that adhere to this film produce extracellular polymeric substances, mostly polysaccharides and proteins, in turn resulting in a strong attachment to the polymer surface and other bacteria in the growing biofilm. Ultimately, the biofilm is composed of multiple layers of cells, cellular debris, polysaccharides, and proteins. *S. epidermidis* factors that are essential for biofilm formation include the polysaccharide intercellular adhesin (107), the accumulation-associated protein (157), and the biofilm-associated protein (184). Polysaccharide intercellular adhesin is most likely identical to the polysaccharide adhesion protein.

Virulence of *S. aureus*

The pathogenicity of *S. aureus* is caused by the expression of an arsenal of virulence factors (Table 1), which can lead to superficial skin lesions, such as styes, furunculosis, and paronychia, or to more serious infections, such as pneumonia, mastitis, urinary tract infections, osteomyelitis, endocarditis, and even sepsis. In very rare cases, *S. aureus* causes meningitis. The

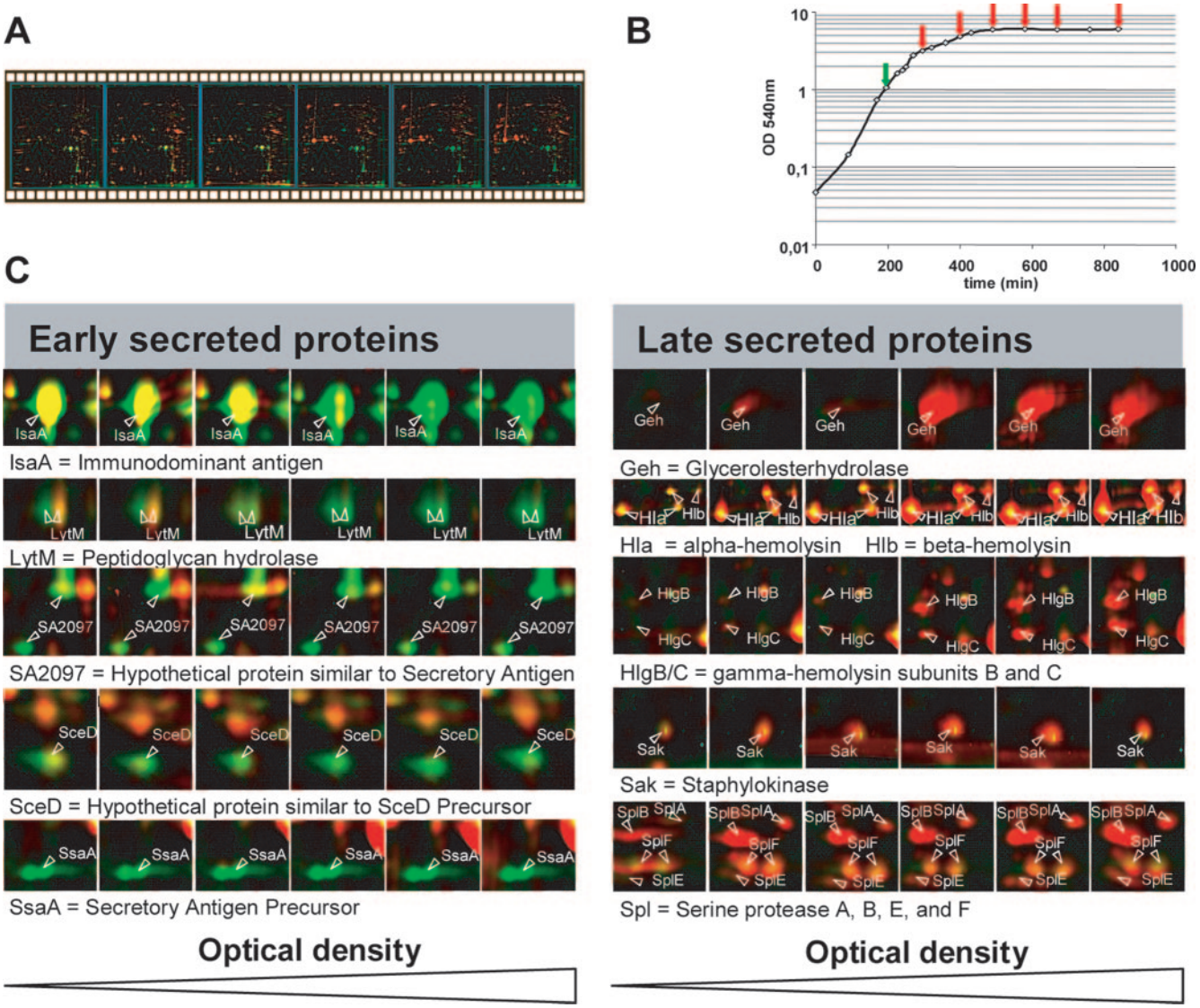

FIG. 3. Dynamics of the amount of extracellular proteins during growth of *S. aureus* RN6390 in TSB medium. (A) Individual dual-channel 2D patterns of extracellular proteins during the different phases of the growth curve for cells grown in TSB medium were assembled into a movie. The protein pattern at an OD_{540} of 1 (labeled in green) was compared with the protein patterns at higher optical densities (labeled in red). As a consequence of dual channel labeling, spots where the intensities do not differ between the compared gels are yellow, and spots with different intensities are either green or red (15). (B) Growth curve for *S. aureus* RN6390 grown in TSB medium, as determined by OD_{540} readings. The sampling points for proteomics analyses are indicated by arrows. (C) Proteomic signatures of selected proteins representing different regulatory groups, as revealed by dual-channel imaging. The amounts of the respective proteins at an OD_{540} of 1 (spots labeled in green) for cells grown in TSB medium were compared with the relative amounts of these proteins at higher optical densities (spots labeled in red). Proteins were stained with Sypro ruby.

virulence factors that *S. aureus* employs to cause these diseases are displayed at the surface of the staphylococcal cell or secreted into the host milieu (57). Specifically, these virulence factors include (i) surface proteins that promote adhesion to and colonization of host tissues, (ii) invasins that are exported to an extracytoplasmic location and promote bacterial spread in tissues (leukocidin, kinases, and hyaluronidase), (iii) surface factors that inhibit phagocytic engulfment (capsule and protein A), (iv) biochemical properties that enhance staphylococcal survival in phagocytes (carotenoid and catalase production), (v) immunological disguises (protein A, coagulase, and clotting factor), (vi) membrane-damaging toxins that disrupt eukaryotic cell membranes (hemolysins and leukotoxin), (vii) superantigens that contribute to the symptoms of septic shock (SEA-G, toxic shock syndrome toxin [TSST], and ET), and (viii) determinants for inherent and acquired resistance to antimicrobial agents.

Most virulence factors are expressed in a coordinated fashion during the growth cycle of *S. aureus*. The best-characterized regulators of virulence factors are the accessory gene regulator (*agr*) (124, 144, 152) and the staphylococcal accessory regulator (SarA) (29, 30). Ziebandt et al. (208) showed that extracellular proteins can be divided into two groups based on the timing of their expression in cells grown in tryptic soy broth

TABLE 1. Virulence factors of *S. aureus*

Pathogenic action	Virulence factors	Proteins or other compounds	Functions	References
Colonization of host tissues	Surface proteins	ClfA, ClfB, FnbA, FnbB, IsdA, SdrC, SdrD, SdrE,	Adhesins, fibronectin- and fibrinogen-binding proteins	35, 68, 93, 111, 114, 149, 155, 198
Lysis of eukaryotic cell membranes and bacterial spread	Membrane-damaging toxins, invasins	Geh, Hla, Hld, HlgA-C, HysA, Lip, LukD, LukE, LukF, LukS, Nuc	Hemolysins, hyaluronidase, leukocidin, leukotoxin, lipases, nucleases	97, 108, 158
Inhibition of phagocytic engulfment	Surface factors	CapA-P, Efb, Spa	Capsule, protein A	102, 196
Survival in phagocytes	Biochemical compounds	KatA, staphyloxanthin	Carotenoids, catalase production	96, 109
Immunological disguise and modulation	Surface proteins	ClfA, ClfB, Coa, Spa	Clumping factor, coagulase, protein A	141, 142, 191
Contribution to symptoms of septic shock	Exotoxins	Eta, Etb, SEA-G, TSST-1	Enterotoxins SEA to SEG, exfoliative toxin, TSST	48, 80, 203
Acquired resistance to antimicrobial agents	Resistance proteins	BlaZ, MecA, VanA	Methicillin and vancomycin resistance	28, 85, 199

(TSB), i.e., proteins that are expressed only at low cell densities and proteins exclusively expressed at high cell densities. *agr* seems to be an important positive regulator of proteins that are expressed at higher optical densities (e.g., proteases, hemolysins, and lipases) and a negative regulator of proteins that are expressed during the exponential growth phase (e.g., immunodominant antigen A, secretory antigen precursor, and several proteins with unknown functions). In addition, Gill et al. (63) identified 15 other two-component regulatory systems in the genomes of *S. aureus* and *S. epidermidis* that are potentially involved in staphylococcal virulence. In this respect, it is interesting that the antibiotic cerulenin, which is known to inhibit protein secretion by *S. aureus* at sub-MIC levels, was recently reported to block the transcriptional activation of at least two regulatory determinants, *agr* and *sae*. Thus, it seems that cerulenin inhibits the transcription of genes for secretory proteins rather than the secretion process of these proteins (1). In contrast, it was previously believed that cerulenin would interfere with membrane function through an inhibition of normal fatty acid synthesis.

Notably, to date, relatively little information is available on the molecular nature of the stimuli that are perceived by the major regulators of the expression of virulence factors. Overall, it should be clear that strain-specific differences in gene regulation by *agr*, *sae*, *sarA*, or other regulators may dramatically influence the repertoire of produced virulence factors, thereby having a profound impact on the disease-causing potential of different strains. Since the interplay of different regulators and cell-to-cell communication can impact differently on the expression of different virulence factors, even the disease-causing potential of individual *S. aureus* cells within a genetically identical population may vary.

Resistance of *S. aureus* to Antibiotics

Resistance of *S. aureus* to antibiotics was observed very soon after the introduction of penicillin about 60 years ago. In the following years, the amazing ability of staphylococci to develop resistance to antibiotics has resulted in the emergence of methicillin-resistant *S. aureus* (MRSA) and *S. epidermidis* strains. In fact, methicillin resistance was observed already in 1961 in nosocomial isolates of *S. aureus*, 1 year after the introduction of methicillin (85). Resistance towards methicillin is a result of the production of an altered penicillin binding protein, PBP2a (or PBP2′), which has less affinity for most β-lactam antibiotics. The PBP2a protein, which is located at the membrane-cell wall interface, is of major importance for cell wall biogenesis by mediating the cross-linking of peptidoglycans. PBP2a is encoded by the *mecA* gene, which is located on a mobile genetic element known as the staphylococcal cassette chromosome *mec* element (SCC*mec*) (28, 84). SCC*mec* is a basic mobile genetic element that serves as a vehicle for gene exchange among staphylococcal species (49). In addition to the *mecA* gene, SCC*mec* carries the *mecA* regulatory genes *mecI* and *mecR*, an insertion sequence element (IS*431mec*), and a unique cassette of recombinase genes (*ccr*), which are responsible for SCC*mec* chromosomal integration and excision. Five different types of SCC*mec* elements, types I to V, have been identified so far, based on the classes of *mecA* gene and *ccr* gene complexes (84). The type I SCC*mec* contains the *mecA* gene as the only resistance element, while the type II and III elements contain, besides *mecA*, multiple determinants for resistance against non-β-lactam antibiotics. Accordingly, type II and III SCC*mec* elements are responsible for multidrug resistance in nosocomial MRSA isolates. Type IV SCC*mec* elements, like type I elements, contain no resistance genes other than *mecA*, and they are significantly smaller than the type II and III elements. This might serve as an evolutionary advantage, making it easier for these mobile genetic elements to spread across bacterial populations. Type V SCC*mec* elements are also small compared to the other elements and differ in their set of recombinase genes (84). Whereas the type I to IV SSC*mec* elements contain the two recombinase genes *ccrA* and *ccrB*, the type V elements contain a single copy of a gene, *ccrC*, homologous to a cassette chromosome recombinase gene. In addition, two open reading frames, *hsdS* and *hsdM*, which encode a restriction-modification system, are unique to these elements. Phylogenetic analyses of these genes showed a distant relationship with their homologues in other *S. aureus* genomes and suggested a foreign origin for these genes.

Vancomycin resistance was first reported for *Enterococcus faecium* (101), and transfer of vancomycin resistance from enterococci, such as *Enterococcus faecalis*, to *S. aureus* has been shown to occur (137). Vancomycin has long been a last-resort antibiotic for multiple-drug-resistant *S. aureus* strains, but already in 1996 a strain was isolated which showed reduced sensitivity towards vancomycin (78). Shortly afterwards, additional strains were isolated in different countries and were designated vancomycin intermediately resistant *S. aureus* (VISA). These strains show a significantly thickened cell wall,

TABLE 2. Sequenced and annotated genomes of *S. aureus* strains

Strain	Origin[a]	Genome size (kbp)		No. of protein-encoding genes	
		Chromosome	Plasmid	Chromosome	Plasmid
COL	Hospital-acquired MRSA	2,809	4	2,615	3
MRSA252	Hospital-acquired MRSA	2,903		2,656	
MSSA476	Community-acquired MSSA	2,800	21	2,579	19
Mu50	Hospital-acquired VISA	2,879	25	2,697	34
MW2	Community-acquired MRSA	2,820		2,632	
N315	Hospital-acquired MRSA	2,815	25	2,588	31
NCTC8325	Hospital-acquired MSSA	2,821		2,892	
USA300	Community-acquired MRSA	2,873	45	2,560	44
RF122	Bovine mastitis isolate	2,743		2,515	

[a] MSSA, methicillin-sensitive *S. aureus*.

which allows them to sequester more vancomycin than non-VISA strains, thereby preventing the detrimental effects of this antibiotic (42). A search for the genetic basis of the lowered vancomycin sensitivity of the *S. aureus* Mu50 strain revealed that important genes for cell wall biosynthesis and intermediary metabolism have mutations compared to those in MRSA strains, which might lead to altered expression of genes involved in cell wall metabolism and a thickened cell wall (4). The first highly-vancomycin-resistant strain was isolated in 2002 (199). This strain was shown to carry a plasmid which contains, among other resistance genes, the *vanA* gene plus several additional genes required for vancomycin resistance. The proteins encoded by these genes are responsible for replacing the C-terminal D-alanyl–D-alanine (D-Ala–D-Ala) of the disaccharide pentapeptide cell wall precursor with a depsipeptide, D-alanyl–D-lactate (D-Ala–D-Lac), thereby lowering the cell wall affinity for vancomycin (24).

Export of Virulence Factors from the Cytoplasm

Since most proteinaceous virulence factors are displayed at the surface of the staphylococcal cell or released into the medium, it is important for our understanding of the pathogenic potential of these organisms to map their pathways for protein transport. While specific questions relating to surface display or secretion of particular virulence factors have been addressed for several years, more holistic studies on the genomics and proteomics of these processes have been documented in the scientific literature only very recently. Moreover, no systematic analysis of pathways and cellular machinery for protein transport has thus far been performed for staphylococci. This review is aimed at filling this knowledge gap. To do so, we have taken full advantage of the availability of six completely sequenced and annotated *S. aureus* genomes and one of the two sequenced *S. epidermidis* strains as well as recently published data on the analysis of staphylococcal cell wall proteomes and exoproteomes. Additionally, we have combined published information with bioinformatics-derived data on all potential signals for protein export from the cytoplasm and secretion into the extracellular milieu or retention in the membrane or cell wall. Since polytopic membrane proteins do not appear to have major direct roles in virulence other than causing drug resistance, such membrane proteins remain beyond the scope of this review. Furthermore, since the secretome of *B. subtilis* has been characterized extensively, at the level of both the protein export machinery and the exoproteome, we have compared the staphylococcal secretomes with that of *B. subtilis*. To our knowledge, this has resulted in the first "comparative secretomics" study.

S. AUREUS STRAINS SUITABLE FOR COMPARATIVE SECRETOMICS

Nine sequenced and fully annotated genomes of *S. aureus* are available in public databases (Table 2) (http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi; http://www.tigr.org), and six of these genomes were used in the present study. These include the genome of one of the first hospital-acquired MRSA isolates, *S. aureus* COL (63), which has been used widely in research on staphylococcal methicillin and vancomycin resistance. The sequenced MRSA252 strain (79) is a hospital-acquired epidemic strain, which was isolated from a patient who died as a consequence of septicemia. The sequenced MSSA476 strain (79) is a community-acquired invasive strain that is penicillin and fusidic acid resistant but susceptible to most commonly used antibiotics. *S. aureus* Mu50 and N315 (100) are hospital-acquired MRSA strains isolated from Japanese patients. In addition, the Mu50 strain displays intermediate vancomycin sensitivity. Finally, the community-acquired isolate *S. aureus* MW2 (7) is a highly virulent MRSA strain isolated from a 16-month-old girl from the United States. Notably, the most widely used laboratory strain, NCTC8325, has been sequenced, but the nucleotide sequence and corresponding annotation were not available for the present analyses. This was also the case for the community-acquired MRSA strain USA300 (46). Furthermore, the sequence of *S. aureus* RF122, a strain associated with mastitis in cattle, is now also available in the NCBI database (unpublished), but it was not included in the present review, which is primarily focused on staphylococcal pathogenicity towards humans. Using multilocus sequence typing with seven housekeeping genes of the different *S. aureus* strains, Holden et al. (79) showed that the MRSA252 strain is phylogenetically most distantly related to the other sequenced strains, while the Mu50 and N315 strains are indistinguishable by multilocus sequence typing, as are the MSSA476 and MW2 strains. The COL and NCTC8325 strains are relatively closely related to each other.

Sequenced and annotated genomes of other staphylococcal species, such as *S. epidermidis*, *Staphylococcus haemolyticus*, and *Staphylococcus carnosus*, are also publicly available. How-

TABLE 3. Secretion machinery of *S. aureus*, *S. epidermidis*, and *B. subtilis*[a]

Pathway and component	Protein(s)	Presence of protein		
		S. aureus	*S. epidermidis*	*B. subtilis*
Sec pathway				
Chaperone	Ffh	+	+	+
	FtsY	+	+	+
	FlhF	−	−	+
	CsaA	−	−	+
Translocation motor	SecA1	+	+	+
	SecA2	+	+	−
Translocation channel	SecY1	+	+	+
	SecY2	+	+	−
	SecE	+	+	+
	SecG	+	+	+
	SecDF	+	+	+
	YajC (YrbF)	+	+	+
Lipid modification	Lgt	+	+	+
Signal peptidase	SpsA (inactive)	+	+	−
	SpsB (SipSTUV)	+	+[b]	+
	SipW (ER-type)	−	−	+
	LspA	+	+[c]	+
Folding catalyst	PrsA	+	+	+
	BdbC	−	−	+
	DsbA (BdbD)	+	+	+
Tat pathway				
Translocase	TatA	+	−	+
	TatC	+	−	+
Pseudopilin pathway	ComGA	+	+	+
	ComGB	+	+	+
	ComC	+	+	+
Bacteriocins	Bacteriocin-specific ABC transporters	Unknown	Unknown	+
Holins	CidA (holin)	+	+	+
	LrgA (anitholin)	+	+	+
ESAT-6 pathway	EsaA	+	−	+
	EsaB	+	−	+
	EsaC[d]	+	−	−
	EssA	+	−	−
	EssB	+	−	+
	EssC	+	−	+

[a] Based on BLAST searches with the corresponding proteins of *B. subtilis* in the finished genome database (http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi).
[b] Two potentially active type I SPases are present in this strain and share homology with *B. subtilis* SipS and SipU.
[c] Two LspA proteins are present in this strain.
[d] This protein is missing in the *S. aureus* MRSA252 strain.

ever, with the exception of *S. epidermidis* strain ATCC 12228 (207), these are not included in the present review, which focuses primarily on *S. aureus*. A comparative genomic analysis of *S. aureus* COL, Mu50, MW2, and N315 and the sequenced *S. epidermidis* strains RP62a and ATCC 12228 revealed that these species and strains have a set of 1,681 genes in common (63). In contrast, 454 genes are present in the *S. aureus* strains but not in *S. epidermidis*, whereas 286 genes are present in *S. epidermidis* but not in *S. aureus*. Most of the strain-specific and species-specific genes can be related to the presence or absence of particular prophages and genomic islands.

PATHWAYS FOR STAPHYLOCOCCAL PROTEIN TRANSPORT

The bacterial machinery for protein transport is currently best described for *Escherichia coli* (gram negative) and *B. subtilis* (gram positive) (for reviews, see references 44, 174, and 175). Many of the known components that are involved in the different routes for protein export from the cytoplasm and in posttranslocational modification of exported proteins in these organisms are also conserved in *S. aureus* and *S. epidermidis* (Table 3). In general, proteins that are exported are synthesized with an N-terminal signal peptide, which directs them to a particular transport pathway. Consequently, the presently known signal peptides are classified according to the export pathway into which they direct the corresponding proteins or the type of signal peptidase that is responsible for their removal (processing) upon membrane translocation. The staphylococcal protein export pathways that have been characterized experimentally or that can be deduced from sequenced genomes are shown schematically in Fig. 4 and are discussed below. Since these pathways are likely used for the export of virulence factors to the cell surface and the milieu of the host,

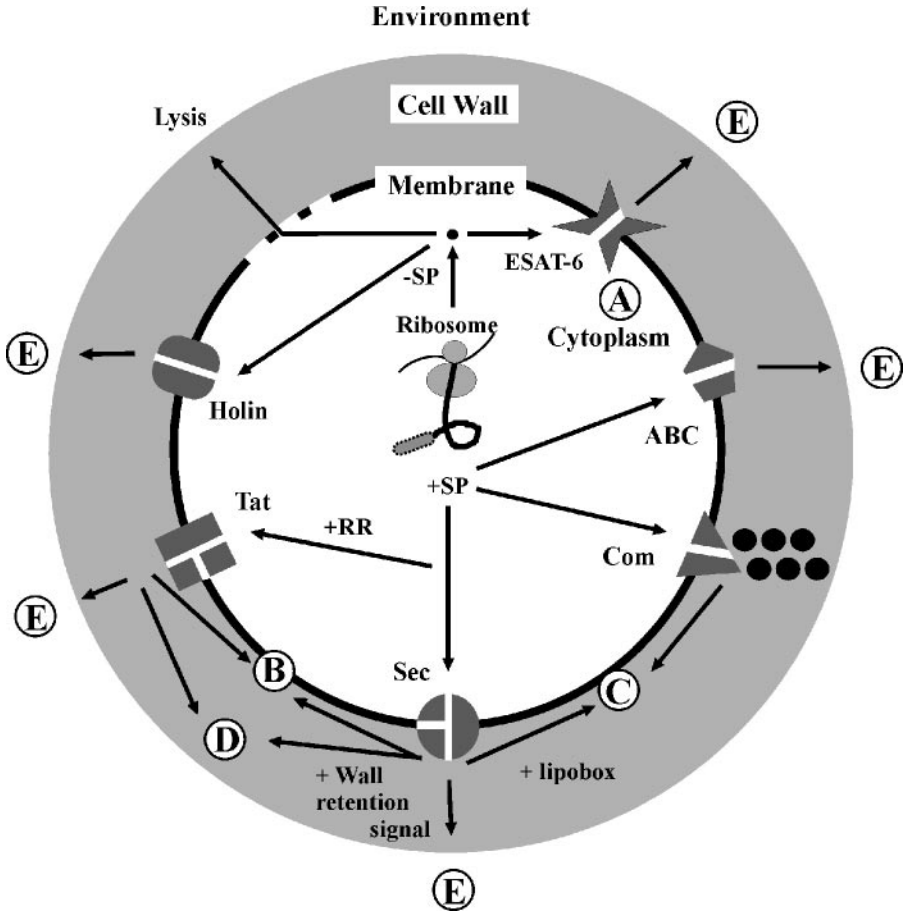

FIG. 4. Staphylococcal pathways to pathogenesis. The figure shows a schematic representation of a staphylococcal cell with potential pathways for protein sorting and secretion. (A) Proteins without signal peptides reside in the cytoplasm. (B) Proteins with one or more transmembrane-spanning domains can be inserted into the membrane via the Sec, Tat, or Com pathway. (C) Lipoproteins are exported via the Sec pathway and are anchored to the membrane after lipid modification. (D) Proteins with cell wall retention signals are exported via the Sec, Tat, or Com pathway and retained in the cell wall via covalent or high-affinity binding to cell wall components. (E) Exported proteins with a signal peptide and without a membrane or cell wall retention signal can be secreted into the extracellular milieu via the various indicated pathways.

Fig. 4 can be regarded as a subcellular road map to staphylococcal pathogenesis.

Components of the Sec Pathway

The most commonly used pathway for bacterial protein transport is the general secretory (Sec) pathway. Specifically, this pathway is responsible for the secretion of the majority of the proteins found in the exoproteome of *B. subtilis*, which is probably also the case for most other gram-positive bacteria, including *S. aureus* (174). Unfortunately, there are very few published data available concerning the Sec pathway of *S. aureus*, and therefore we have filled in the current knowledge gaps with data obtained from studies of *B. subtilis* or *E. coli*. Proteins that are exported via the Sec pathway contain signal peptides with recognition sites for so-called type I or type II SPases. Notably, type II SPase recognition sites overlap with the recognition sites for the diacylglyceryl transferase Lgt. Precursor proteins with a type II SPase recognition sequence are lipid modified prior to being processed, and the resulting mature proteins are retained as lipopoteins in the cytoplasmic membrane via their diacylglyceryl moieties. Furthermore, the Sec-dependent export of proteins can be divided into the following three stages: (i) targeting to the membrane translocation machinery by export-specific or general chaperones, (ii) translocation across the membrane by the Sec machinery, and (iii) posttranslocational folding and modification. If the translocated proteins of gram-positive bacteria lack specific retention signals for the membrane or cell wall, they are secreted into the growth medium.

Preprotein targeting to the membrane. In *B. subtilis*, the only known secretion-specific chaperone is the signal recognition particle (SRP), which consists of small cytoplasmic RNA (scRNA), the histone-like protein HBsU, and the Ffh protein. Ffh and HBsU bind to different moieties of the scRNA. Studies with *E. coli* have shown that upon emergence from the ribosome, the signal peptide of a nascent secretory protein can be recognized by several cytoplasmic chaperones and/or targeting factors, such as Ffh or trigger factor (TF) (55). In contrast to Ffh, which is required for cotranslational protein export in *E. coli*, the cytoplasmic chaperone SecB has mainly been implicated in posttranslational protein targeting. Notably,

however, SecB is absent from the sequenced gram-positive bacteria, including *S. aureus* and *B. subtilis*. Most likely, ribosome-nascent chain complexes of *S. aureus* are thus targeted to the membrane by SRP, which, by analogy to the case in *B. subtilis* and *E. coli*, will probably involve the SRP receptor FtsY. At the membrane, the nascent preprotein will be directed to the translocation machinery. This process is likely stimulated by negatively charged phospholipids (45), the Sec translocon (17, 45), and/or the SecA protein (25). In this respect, SecA may function not only as the translocation motor (see below) but also as a chaperone for preprotein targeting (75). While it has been shown that Ffh is essential for growth and viability in *E. coli* and *B. subtilis*, this does not seem to be the case for all bacteria. For example, Ffh, FtsY, and scRNA are not essential in *Streptococcus mutans*. In this organism, the SRP is merely required for growth under stressful conditions, such as low pH (<pH 5), high salt (3.5% NaCl), or the presence of H_2O_2 (0.3 mM). This suggests that SRP has an important role in the export of proteins to the membrane or cell wall to protect *S. mutans* against environmental insults (71).

For *B. subtilis*, it has been proposed that the general chaperone CsaA may have a role in preprotein targeting to the membrane, similar to SecB of *E. coli*. This view is supported by the observation that the *B. subtilis* CsaA protein has binding affinity for SecA and preproteins (126). However, CsaA is not conserved in *S. aureus*. Therefore, it remains to be investigated whether other chaperones with a preprotein targeting function are present in *S. aureus*.

Translocation across the membrane. As deduced from known genome sequences, the translocation machinery of *S. aureus* consists of several Sec proteins. The mode of action of these proteins has been studied in great detail in *E. coli* (44, 197). After binding of a preprotein to a SecA dimer, the SecA molecules will bind ATP, resulting in conformational changes that promote their insertion together with the preprotein into the membrane-embedded translocation channel. Subsequent hydrolysis of ATP causes SecA to release the preprotein, return to its original conformation, and deinsert from the translocation channel. Repeated cycles of ATP binding and hydrolysis by SecA, together with the proton motive force, drive further translocation of the preprotein across the membrane. The translocation channel is essentially formed by the SecE and SecY proteins, which are conserved in all bacteria (189). An additional nonessential channel component is SecG, which serves to increase the translocation efficiency. While the SecY proteins of different bacteria show a relatively high degree of sequence similarity, the SecE and SecG proteins, though present in all bacteria, are less well conserved. Specifically, the SecE and SecG proteins in *B. subtilis*, *S. aureus*, and *S. epidermidis* are considerably shorter than the equivalent proteins of *E. coli*. Although SecA and SecY of *S. aureus* (referred to here as SecA1 and SecY1) have not yet been characterized functionally, they are of major importance for the growth of *S. aureus*. This was demonstrated with the help of specific antisense RNAs (86). Upon *secA* antisense induction, a strong growth defect was observed, and *secY* antisense induction turned out to be lethal.

Remarkably, the genome of *S. aureus* contains a second set of *secA* and *secY* genes, referred to as *secA2* and *secY2*, respectively. In contrast to the SecA1 and SecY1 proteins, their homologues are not essential for growth and viability. It is presently unknown whether SecA2 and SecY2 transport specific proteins across the membrane of *S. aureus*. However, it has been shown for other pathogenic gram-positive bacteria which also possess a second set of SecA and SecY proteins that these proteins are required for the transport of certain proteins related to virulence. In *Streptococcus gordonii*, the export of GspB, a large cell surface glycoprotein that contributes to platelet binding, seems to be dependent on the presence of SecA2 and SecY2 (13). This protein contains large serine-rich repeats, an LPXTG motif for cell wall anchoring (see below), and a very large signal peptide of 90 amino acids. In *Streptococcus parasanguis*, two other proteins, FimA and Fap1, are known to be secreted via SecA2-dependent membrane translocation. FimA is a (predicted) lipoprotein which is a major virulence factor implicated in streptococcal endocarditis. The FimA homologue in *S. aureus* is a manganese-binding lipoprotein (MntA) associated with an ATP-binding cassette (ABC) transporter. Fap1 of *S. parasanguis* is involved in adhesion to the surfaces of teeth. Like FimA, Fap1 has a long signal peptide of 50 amino acids, serine-rich repeats, and an LPXTG motif for cell wall anchoring. To date, it is not known what determines the difference in specificity of the SecA1/SecY1 and SecA2/SecY2 translocases. It is also not known whether the SecA2/SecY2 translocase shares SecE and/or SecG with the SecA1/SecY1 translocase, whether these translocases function completely independently from each other, or whether mixed translocases can occur. Clearly, the *secE* and *secG* genes are not duplicated in *S. aureus*. The SecE and SecG functions in the SecA2/SecY2 translocase may, however, be performed by the *S. aureus* homologues of the Asp4 and Asp5 proteins of *S. gordonii*, for which SecE- and SecG-like functions have been proposed (172).

In *E. coli*, the heterotrimeric SecYEG complex is associated with another heterotrimeric complex composed of the SecD, SecF, and YajC proteins (138). This complex has been shown to be involved in the cycling of SecA (51) and the release of the translocated protein from the translocation channel (113). SecD and SecF are separate but structurally related proteins in most bacteria, including *E. coli*. Interestingly, in *B. subtilis* and *S. aureus*, natural gene fusions between the *secD* and *secF* genes are observed. Accordingly, the corresponding SecDF proteins can be regarded as molecular "Siamese twins" (20). Unlike SecA, SecY, and SecE, the SecDF protein of *B. subtilis* is not essential for growth and viability, and its role in protein secretion is presently poorly understood (20). *B. subtilis secDF* mutants showed only a mild secretion defect under conditions of high-level synthesis of secretory proteins. The known SecDF proteins have 12 (predicted) transmembrane domains with two large extracytoplasmic loops, between the first and second transmembrane segments and between the seventh and eighth transmembrane segments. For *E. coli* SecD, it has been shown that small deletions in the large extracytoplasmic loop result in malfunctioning of the protein, while the stability of the SecDF-YajC complex is not affected (138). It has therefore been proposed that this loop in SecD might provide a protective structure in which translocated proteins can fold more efficiently. The large extracytoplasmic loop in SecF has been proposed to interact with SecY, thereby stabilizing the translocation channel formed by SecYEG. Homologues of the *E. coli*

YajC protein are present in many bacteria, including *S. aureus* and *B. subtilis* (YrbF), but their role in protein secretion has not yet been established. It is presently not known whether the *S. aureus* SecDF-YajC complex associates specifically with the SecA1/SecY1 translocase, the SecA2/SecY2 translocase, or both.

Type I signal peptidases. Signal peptides of preproteins are cleaved during or shortly after translocation by an SPase I or SPase II, depending on the nature of the signal peptide (180, 187). The *B. subtilis* chromosome encodes five type I SPases, named SipS, SipT, SipU, SipV, and SipW (176, 178, 186). Two of these, SipS and SipT, are of major importance for the processing of secretory preproteins, growth, and viability. In *S. aureus*, only two SPase I homologues are present, namely, SpsA and SpsB. The catalytically active SPase I in *S. aureus* is SpsB, which is probably essential for growth and viability (38). This SPase can be used to complement an *E. coli* strain that is temperature sensitive for preprotein processing. In general, type I SPases recognize residues at the −1 and −3 positions relative to the cleavage site (187). For *B. subtilis*, it has been shown that all secretory proteins identified by proteomics have Ala at the −1 position and that 71% of these secretory proteins have Ala at the −3 position (174). In contrast, various residues are tolerated at the −2 position, including Ser, Lys, Glu, His, Tyr, Gln, Gly, Phe, Leu, Ala, Asp, Asn, Trp, and Pro. Interestingly, Bruton et al. (23) studied the cleavage sites in substrates of *S. aureus* SpsB and showed that this enzyme has a preference for basic residues at the −2 position and tolerance for hydrophobic residues at this position. However, an acidic residue at the −2 position resulted in a significantly reduced rate of processing. The second SPase I homologue of *S. aureus* (SpsA) appears to be inactive, since it lacks the catalytic Ser and Lys residues, which are replaced with Asp and Ser residues, respectively. The presence of an apparently catalytically inactive SpsA homologue is a conserved feature of all staphylococci with sequenced genomes. Notably, in addition to an inactive SpsA homologue, *S. epidermidis* contains two SpsB homologues, which show the greatest similarity to SipS and SipU of *B. subtilis*. To date, it is not known whether the inactive SpsA homologues contribute somehow to protein secretion in these organisms.

Lipid modification of lipoproteins. In *E. coli*, lipid modification of prolipoproteins involves three sequential steps that are catalyzed by cytoplasmic membrane-bound proteins. The first step involves the transfer of a diacylglyceryl group from phosphatidylglycerol to the sulfhydryl group of the invariant Cys residue present at the +1 position of the signal peptide cleavage site in lipoprotein precursors. This step is catalyzed by a phosphatidyl glycerol diacylglyceryl transferase (Lgt), as shown for *E. coli* by Sankaran and Wu (161). The recognition sequence for Lgt, which includes the Cys residue that becomes modified with diacylglyceryl, is known as the lipobox. Lipid modification of the lipobox Cys residue is necessary for the lipoprotein-specific type II signal peptidase (LspA) to recognize and cleave the signal peptide of a prolipoprotein, which represents the second step in lipoprotein modification. The third step involves the transfer of an *N*-acyl group by an *N*-acyl transferase (Lnt), resulting in the formation of *N*-acyl diacylglycerylcysteine at the N terminus of the mature lipoprotein. Although Lgt and LspA are present in most, if not all, bacteria, Lnt is present only in gram-negative bacteria (180). Like the case for other gram-positive bacteria, no homologue of Lnt could be detected in the genomes of *S. aureus* or *S. epidermidis* (169), which suggests that the lipoproteins of these organisms are not N acylated.

S. aureus Lgt is a protein of 279 amino acids that contains a highly conserved HGGLIG motif (residues 97 to 102). Although the His residue in this motif was shown to be essential for the catalytic activity of *E. coli* Lgt (160), it is not strictly conserved in all known Lgt proteins. On the other hand, the strictly conserved Gly at position 103 of *E. coli* Lgt, which is equivalent to Gly98 of *S. aureus* Lgt, is required for the activity of this protein. Stoll et al. (169) showed that an *S. aureus lgt* mutation has no effect on growth in broth, as also observed for *B. subtilis* (104). Nevertheless, the absence of Lgt has a considerable effect on the induction of an inflammatory response. Importantly, lipid modification serves to retain exported proteins at the membrane-cell wall interface. This is particularly relevant for gram-positive bacteria, which lack an outer membrane that represents a retention barrier for exported proteins. In the absence of Lgt, *B. subtilis* cells release a variety of lipoproteins into the extracellular milieu, in the form of both unmodified precursor proteins and alternatively processed mature proteins that lack the N-terminal Cys residue (3). Similarly, the *S. aureus lgt* mutation resulted in the shedding of certain abundant lipoproteins, such as OppA, PrsA, and SitC, into the broth. These lipoproteins are normally retained in the membrane or cell wall of *S. aureus*.

Type II signal peptidase. As described above, lipoprotein signal peptides of prolipoproteins are cleaved by type II SPases after the Cys residue in the lipobox is modified by Lgt. Although *B. subtilis* and many other bacteria contain only one copy of the *lspA* gene, some organisms, such as *S. epidermidis*, *Bacillus licheniformis*, and *Listeria monocytogenes*, contain a second copy. LspA is a membrane protein that spans the membrane four times, and both its N and C termini face the cytoplasmic side of the membrane (178, 188). Six amino acid residues are important for SPase II activity, of which two Asp residues form the active site (178). While processing of lipoproteins by LspA is essential for growth and viability for *E. coli* and other gram-negative bacteria (202), it is not essential for *B. subtilis* (177) and other gram-positive bacteria, such as *Lactococcus lactis* (190). This suggests that processing of prolipoproteins is not essential for their functionality. This view is supported by the fact that PrsA, a lipoprotein required for correct folding of translocated proteins, is essential for viability of *B. subtilis* (99). In the absence of LspA, some of the lipoproteins of *B. subtilis* are processed in an alternative way by unidentified proteases, and the activity of unprocessed lipoproteins in *lspA* mutants is reduced. Also, in these *B. subtilis* mutants, secretion of the nonlipoprotein AmyQ was severely reduced (177). This reduction might be the consequence of a malfunction of unmodified PrsA in AmyQ folding. Although most *lspA* mutants have been studied in gram-negative bacteria and a few nonpathogenic gram-positive bacteria (177, 190), Sander et al. (159) showed a severely attenuated phenotype of *lspA* mutants of the pathogen *Mycobacterium tuberculosis*, which implies an important role for lipoprotein processing by LspA during infection with *M. tuberculosis*. In *S. aureus*, both the *lspA* and *lgt* genes are present as single copies in the genomes of all six

sequenced strains. Interestingly, one of the two LspA homologues in *S. epidermidis* (125 amino acids) is considerably shorter than other known LspA proteins, including its large paralogue (177 amino acids). This is mainly the result of an additional N-terminal transmembrane domain in the large LspA proteins. As a result, the short *S. epidermidis* LspA protein is predicted to have three membrane-spanning domains, with the N terminus located on the outside of the cell, the C terminus located on the inside of the cell, and the (putative) active-site Asp residues located on the outer surface of the cytoplasmic membrane.

Signal peptide peptidase. After translocation and processing of the preproteins by signal peptidases, the signal peptides are rapidly degraded by signal peptide peptidases (SPPases). In *B. subtilis*, two SPPases, TepA and SppA, are known to be involved in translocation and processing of preproteins (21). While TepA is required for translocation and processing of preproteins, SppA is required only for efficient processing of preproteins. Remarkably, no homologues of SppA or TepA were detectable by BLAST searches in the sequenced genomes of *S. aureus* and *S. epidermidis*. As reported by Meima and van Dijl (119), *L. lactis* contains a protein that shows limited similarity to TepA of *B. subtilis* and ClpP of *Caenorhabditis elegans*, suggesting that this protein might be an SPPase analog in *L. lactis*. In *S. aureus* and *S. epidermidis*, this protein homologue also seems to be present and is predicted to be a cytoplasmic membrane protein (our unpublished observations).

Folding catalysts (PrsA and BdbD). Proteins that are transported across the membrane in a Sec-dependent manner emerge at the extracytoplasmic membrane surface in an unfolded state. These proteins need to be rapidly and correctly folded into their native and protease-resistant conformation before they are degraded by proteases in the cell wall or extracellular environment (162). An important folding catalyst in *B. subtilis* is PrsA, which shows homology to peptidyl-prolyl *cis*/*trans*-isomerases. PrsA is a lipoprotein (see "Lipoproteins" below) that is essential for efficient protein secretion and cell viability in *B. subtilis* (99, 162). Studies on the effects of PrsA depletion showed that the relative amounts of extracellular proteins from PrsA-depleted cells were significantly reduced (192). No data have been published on *S. aureus* mutants lacking PrsA, and it will be interesting to investigate whether PrsA is also essential for the viability and virulence of this organism. It has already been shown that *S. aureus* lacking Lgt releases an increased amount of PrsA into the extracellular milieu (169), which might indicate that (most) pre-PrsA is not fully functional but is sufficient for viability. The observation by Stoll et al. (169) also shows that, like the case in *B. subtilis* (3), unmodified pre-PrsA is not effectively retained in the cytoplasmic membrane.

Other proteins that are involved in proper folding of extracellular proteins in *B. subtilis* are the membrane proteins BdbC and BdbD, which are involved in the formation of disulfide bonds. Both proteins have been shown to be necessary for stabilization of the membrane- and cell wall-associated pseudopilin ComGC (118). This protein, which is required for DNA binding and uptake during natural competence, contains an intramolecular disulfide bond (31). Both BdbC and BdbD are also important for the folding of heterologously produced *E. coli* PhoA, which contains two disulfide bonds, into an active and protease-resistant conformation (21, 118). Although a homologue of BdbD (named DsbA) is present in *S. aureus*, there is no homologue of BdbC in this organism. The same appears to be true for *S. epidermidis*. Nevertheless, measurements of the redox potential of purified DsbA indicated that this protein can act as an oxidase, and this view was confirmed by complementation studies with a *dsbA* mutant strain of *E. coli* (53). The absence of a BdbC homologue in the staphylococci is remarkable, since *B. subtilis* BdbC and BdbD are jointly required for the folding of ComGC and *E. coli* PhoA. Notably, all sequenced *S. aureus* genomes encode homologues of ComGC, including the Cys residues that form the disulfide bond in *B. subtilis* ComGC. This raises the question of whether ComGC of *S. aureus* does indeed contain a disulfide bond and, if so, which protein(s) is involved in the formation of this disulfide bond. Notably, *S. aureus* DsbA was recently shown to be a lipoprotein that does not seem to contribute to the virulence of this organism, as tested in mouse and *Caenorhabditis elegans* models (53). Furthermore, DsbA was shown to be dispensable for β-hemolysin activity, despite the fact that this protein contains a disulfide bond, which is required for activity (54). Therefore, the biological function of DsbA in staphylococci remains to be elucidated.

Tat Pathway

The twin-arginine translocation (Tat) pathway exists in many bacteria, archaea, and chloroplasts. This pathway was named after the consensus double (twin) Arg residues that are present in the signal peptide. The twin Arg residues are part of a motif that directs proteins specifically into the Tat pathway. In contrast to the Sec machinery, where only unfolded proteins are translocated across the membrane, the Tat machinery is capable of translocating folded proteins. In gram-negative bacteria, streptomycetes, mycobacteria, and chloroplasts, an active Tat pathway seems to require three core components, named TatA, TatB, and TatC (14, 47, 125, 154, 204). In all gram-positive bacteria except streptomycetes and *Mycobacterium smegmatis*, the Tat pathway involves only TatA and TatC (47, 204). Recent studies with *E. coli* and chloroplasts have resulted in a model that proposes key roles for TatB-TatC complexes in signal peptide reception and for TatA-TatB-TatC complexes in preprotein translocation (2, 36). Interestingly, certain mutations in *E. coli* TatA have been shown to allow this protein to compensate for the absence of TatB (18). This demonstrates that TatA is intrinsically bifunctional, which is consistent with the fact that most gram-positive bacteria lack TatB but have TatA (90). In *B. subtilis*, two minimal TatA-TatC translocases with distinct specificities are active (88). While the constitutively expressed TatAy-TatCy translocase of *B. subtilis* is required for secretion of a protein with unknown function, YwbN, the TatAd-TatCd translocase seems to be expressed only under conditions of phosphate starvation for secretion of the phosphodiesterase PhoD (175, 188). Most other gram-positive bacteria that have *tatA* and *tatC* genes, including *S. aureus*, appear to have only one TatA-TatC translocase. The functionality of the *S. aureus* Tat translocase remains to be demonstrated. In contrast to *S. aureus*, *S. epidermidis* seems to lack a Tat pathway.

Pseudopilin Export (Com) Pathway

In *B. subtilis*, four proteins, ComGC, ComGD, ComGE, and ComGG, have been identified as having an N-terminal pseudopilin-like signal peptide (174, 175). All four of these proteins are involved in DNA binding and uptake and are localized in the membrane and cell wall. It is thought that these proteins form a pilus-like structure in the cell wall or modify the cell wall to provide a passage for DNA uptake. Translocation to the extracytoplasmic membrane surface is possible only when these proteins are processed by the pseudopilin-specific SPase ComC in *B. subtilis* (52). SPases of this type are bifunctional and catalyze not only signal peptide cleavage but also methylation of the N terminus of the mature protein (170). Furthermore, export and functionality of the four ComG proteins depend on the integral membrane protein ComGB and the traffic ATPase ComGA, which is located at the cytoplasmic side of the membrane (32, 69). Homologues of ComC, ComGA, ComGB, and ComGC, but not ComGD, ComGE, and ComGG, are present in the six sequenced *S. aureus* strains. This suggests that the Com system of *S. aureus* is not involved in DNA uptake but is part of another solute transport process.

ABC Transporters

Bacteriocins are peptides or proteins that inhibit the growth of other bacteria. Most of the characterized bacteriocins can be divided into several classes, depending on specific posttranslational modifications, the presence and processing of particular leader peptides, and the machinery for export from the cytoplasm. A well-described class of bacteriocins is formed by the lantibiotics. Members of this class are composed of short peptides that contain posttranslationally modified amino acids, such as lanthionine and β-methyllanthionine (117). The production of bacteriocins in *S. aureus* has been described for various strains. *S. aureus* C55 produces the two lantibiotics C55α and C55β (129). These lantibiotics are both encoded by a 32-kb plasmid, which is readily lost upon growth at elevated temperatures. C55α and C55β showed antimicrobial activity towards other *S. aureus* strains and *Micrococcus luteus* but not towards *S. epidermidis*. Furthermore, the nonlantibiotics BacR1 (40), aureocin A53 (134), and aureocin A70 (132, 133) have been identified as bacteriocins with activity against a broad range of bacteria. The genes for both aureocins are located on a plasmid that is present in *S. aureus* strains isolated from milk. By analogy with the well-described bacteriocin export machineries of other organisms (73, 145), it can be anticipated that all of the aforementioned bacteriocins are exported to the external staphylococcal milieu by dedicated ABC transporters. However, no experimental evidence for this assumption has been published for *S. aureus*. Notably, it has been demonstrated that secretion of the lantibiotics epidermin and gallidermin of *S. epidermidis* Tü3298 and *Staphylococcus gallinarum*, respectively, is facilitated by so-called one-component ABC transporters. Specifically, the ABC transporter GdmT has been implicated in the transport of these lantibiotics (145).

Holins

Holins are dedicated export systems for peptidoglycan-degrading endolysins that have been implicated in the programmed cell death of bacteria. These exporters, which are composed of homo-oligomeric complexes, can be subdivided into two classes, depending on the number of transmembrane segments. While class I holin subunits have three transmembrane segments, class II holin subunits have two transmembrane segments (206). In *S. aureus*, the *lrg* and *cid* operons are involved in murein hydrolase activity and antibiotic tolerance (66, 153). A disrupted *lrg* operon leads to an increase in murein hydrolase activity and a decrease in penicillin tolerance, and a disrupted *cid* operon leads to a decrease in murein hydrolase activity and an increase in penicillin tolerance. It is still unclear how the CidA and LrgA proteins are involved in these mechanisms, but these proteins display significant similarity to the bacteriaphage holin protein family, suggesting that they have a role in protein export. It has therefore been proposed that the CidA and LrgA proteins act on murein hydrolase activity and antibiotic tolerance in a manner analogous to that of holins and antiholins, respectively (11, 153). Sequence similarity searches showed that the genes for LrgA and CidA are conserved in the six sequenced *S. aureus* strains as well as in *S. epidermidis* and *B. subtilis*. Notably, none of the three holins of *B. subtilis* were shown to be involved in the secretion of proteins to the extracellular milieu (174, 200).

ESAT-6 Pathway

The ESAT-6 secretion pathway was first described for *M. tuberculosis*. It has been proposed that at least two virulence factors, ESAT-6 (early secreted antigen target, 6 kDa) and CFP-10 (culture filtrate protein, 10 kDa), are secreted via this pathway in a Sec-independent manner (16, 166). Since this pathway was discovered in mycobacteria, it is also known as the Snm pathway (secretion in mycobacteria) (37). The genes for ESAT-6 and CFP-10 are located in conserved gene clusters, which also encode proteins with domains that are conserved in FtsK- and SpoIIIE-like transporters. These conserved FtsK/SpoIIIE domains have been termed FSDs (26). In other gram-positive bacteria, including *S. aureus*, *B. subtilis*, *Bacillus anthracis*, *Clostridium acetobutylicum*, and *L. monocytogenes*, homologues of ESAT-6 have been identified (140). The genes for these ESAT-6 homologues are also found in gene clusters that contain at least one gene for a membrane protein with an FSD. In *S. aureus*, two proteins, named EsxA and EsxB, have been identified that seem to be secreted via the ESAT-6 pathway (26). The *esxA* and *esxB* genes are part of a cluster containing six other genes for proteins that have been implicated in the translocation of EsxA and EsxB. These include the EsaB and EsaC proteins, with a predicted cytoplasmic location, as well as the predicted membrane proteins EsaA, EssA, EssB, and EssC, among which EssC contains an FSD. Mutations in *essA*, *essB*, or *essC* result in a loss of EsxA and EsxB production, which may be related to inhibition of the synthesis of these proteins or their folding into a protease-resistant conformation. All sequenced *S. aureus* strains contain this cluster of *esa*, *ess*, and *esx* genes, but it seems to be absent from *S. epidermidis*. Interestingly, the genes for EsxB and EsaC appear

to be absent from the *S. aureus* MRSA252 strain. This implies that the ESAT-6 machinery of this strain may be required for the transport of only EsxA and perhaps a few other unidentified proteins. If so, EsaC would be dispensable for an active ESAT-6 pathway and might be specifically involved in the export of EsxB. Alternatively, the ESAT-6 pathway could be inactive in the *S. aureus* MRSA252 strain due to the absence of EsaC.

Lysis

Various studies have shown that certain proteins with typical cytoplasmic functions and without known signals for protein secretion can nevertheless be detected in the extracellular proteomes of different bacteria (174). Notably, many of these proteins, such as catalase, elongation factor G, enolase, glyceraldehyde-3-phosphate dehydrogenase, GroEL, and superoxide dismutase, are among the most highly abundant cytoplasmic proteins. This makes it likely that they are detectable in the extracellular proteome due to cell lysis. Perhaps such proteins are more resistant to extracytoplasmic degradation than are other proteins that are simultaneously released by lysis. However, the possibility that the extracellular localization of typical cytoplasmic proteins is due to the activity of as yet unidentified export pathways cannot be excluded. Clearly, until recently this possibility did still apply for the EsxA and EsxB proteins, which are now known to be exported via the ESAT-6 pathway. A clear indication that the presence of certain "cytoplasmic" proteins in the extracytoplasmic milieu of bacteria may relate to specific export processes was provided by Boël and coworkers (19), who showed that 2-phosphoglycerate-dependent automodification of enolase is necessary for its export from the cytoplasm.

PROPERTIES OF STAPHYLOCOCCAL SIGNAL PEPTIDES AND CELL RETENTION SIGNALS

Signal Peptides

All proteins that have to be transported from the cytoplasm across the membrane to the extracytoplasmic compartments of the cell, or the extracellular milieu, need to contain a specific sorting signal for their distinction from resident proteins of the cytoplasm. The known bacterial sorting signals for protein export from the cytoplasm are signal peptides (195). These signal peptides can be classified by the transport and modification pathways into which they direct proteins. Presently, four different bacterial signal peptides are recognized that share a common architecture but differ in the details (Fig. 5). Two of these direct proteins into the widely used Sec pathway, including the secretory (Sec-type) signal peptides and the lipoprotein signal peptides. Proteins with Sec-type or lipoprotein signal peptides are processed by different SPases (type I and type II SPases, respectively) and are targeted to different destinations. In *S. aureus*, proteins with Sec-type signal peptides are processed by the type I SPase SpsB and are targeted to the cell wall or extracellular milieu. Proteins with a lipoprotein signal peptide are lipid modified by Lgt prior to being processed by the type II SPase LspA. In principle, these lipoproteins are retained at the membrane-cell wall interface, but they can be liberated from this compartment by proteolytic removal of the N-terminal Cys that contains the diacylglyceryl moiety (3). Proteins with twin-arginine (RR) signal peptides appear to be processed by type I SPases, at least in *B. subtilis*, and are targeted to the cell wall or extracellular milieu (174). Proteins with a pseudopilin signal peptide are processed by the pseudopilin signal peptidase ComC and most likely are localized to the cytoplasmic membrane and the cell wall. Finally, bacteriocins contain a completely different sorting and modification signal that is usually called the leader peptide. The known leader peptides show no resemblance to the aforementioned signal peptides. The export of bacteriocins via ABC transporters results in their secretion into the extracellular milieu (121, 164).

Sec-type, lipoprotein, and RR signal peptides contain three distinguishable domains, the N, H, and C domains. The N-terminal domain contains positively charged amino acids, which are thought to interact with the secretion machinery and/or negatively charged phospholipids in the membrane. The H domain is formed by a stretch of hydrophobic amino acids which facilitate membrane insertion. Helix-breaking residues in the middle of the H domain may facilitate H domain looping during membrane insertion and translocation of the precursor protein. The subsequent unlooping of the H domain would display the SPase recognition and cleavage site at the extracytoplasmic membrane surface, where the catalytic domains of type I and type II SPases are localized (187). Helix-breaking residues just before the SPase recognition and cleavage site would facilitate precursor processing by SPase I or II. In fact, these helix-breaking residues and the SPase cleavage site, respectively, define the beginning and the end of the C domain. Notably, the C domains of pseudopilin signal peptides are located between the N and H domains (32, 33, 106, 148). Accordingly, processing by pseudopilin-specific SPases, such as ComC, takes place at the cytoplasmic side of the membrane and leaves the H domain attached to the translocated protein.

While many proteins that end up in the extracellular milieu or the cell walls of gram-positive bacteria have signal peptides, proteins without known export signals can also be found at these locations. The relative number of proteins without known signal peptides seems to vary for each organism. While these numbers are relatively low for *B. subtilis* and *S. aureus*, they are high for group A streptococcus and *M. tuberculosis* (174). As indicated above, some of the proteins without known export signals appear to be liberated from the cell by lysis, while others are actively exported, for example, via the ESAT-6 pathway. Although the precise export signal in proteins secreted via the ESAT-6 pathway has not yet been defined, a WXG motif is shared by these proteins and may serve a function in protein targeting (140). Furthermore, the signal for specific release of lysins via holins is presently not known.

Signal Peptide Predictions

Several prediction programs that are accessible through the World Wide Web are useful tools for predicting whether a given protein contains some type of sorting signal or SPase cleavage site. The programs that we used in this and other studies were SignalP-NN and SignalP-HMM, version 2.0 (136), LipoP, version 1.0 (94), PrediSi (76), and Phobius (95). These

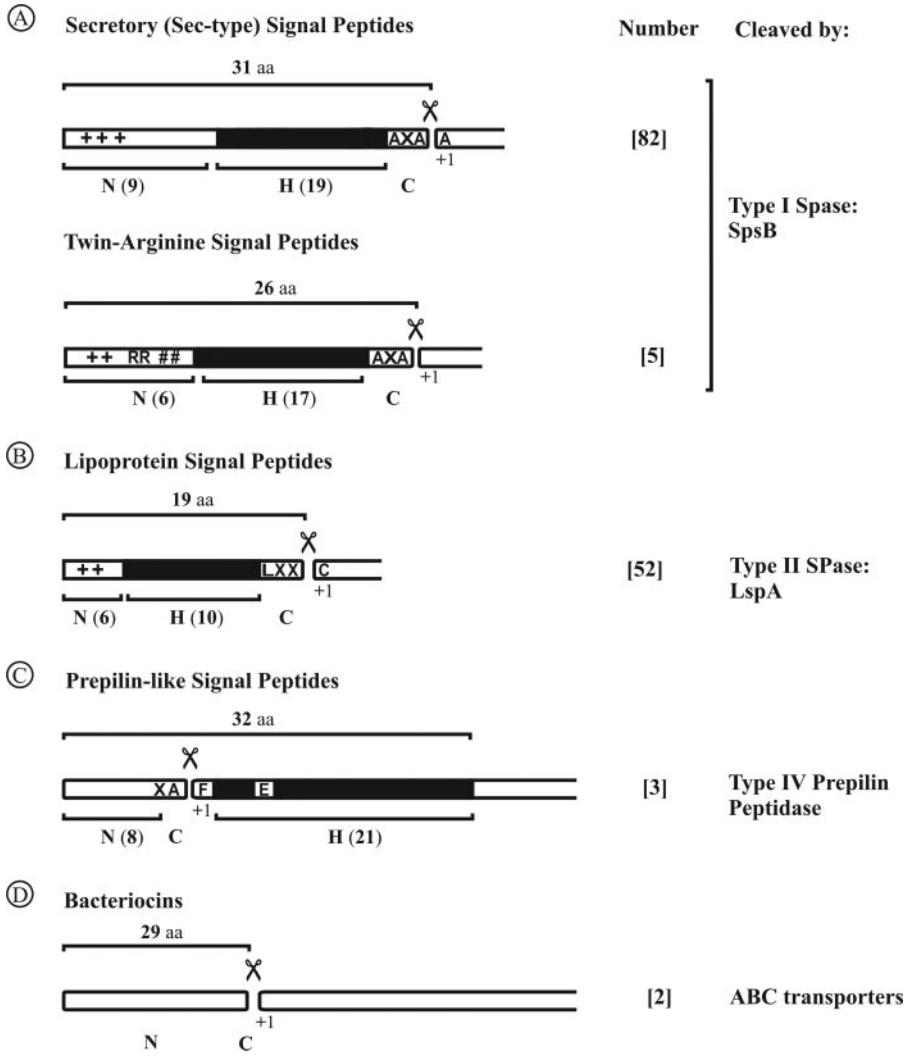

FIG. 5. General properties and classification of *S. aureus* signal peptides. Signal peptide properties are based on SPase cleavage sites and the export pathways by which the preproteins are exported. Predicted signal peptides (144) were divided into the following five distinct classes: secretory (Sec-type) signal peptides, twin-arginine (RR/KR) signal peptides, lipoprotein signal peptides, pseudopilin-like signal peptides, and bacteriocin leader peptides. Most of these signal peptides have a tripartite structure, with a positively charged N domain (N) containing lysine and/or arginine residues (indicated by plus signs), a hydrophobic H domain (H, indicated by a black box), and a C domain (C) that specifies the cleavage site for a specific SPase. Where appropriate, the most frequently occurring amino acid residues at particular positions in the signal peptide or mature protein are indicated. Also, the numbers of signal peptides identified for each class and the respective SPase are indicated.

programs were designed to identify Sec-type signal peptides, N-terminal membrane anchors (Phobius), or lipoprotein signal peptides in gram-negative bacteria (LipoP). The TMHMM program, version 2.0 (41), was used to exclude proteins with (predicted) multiple membrane-spanning domains. Predictions for proteins containing a signal peptide were performed with the SignalP program, using the neural network and hidden Markov model algorithms. Version 2.0 of the SignalP program was preferred above version 3.0 (12) for our signal peptide predictions for *S. aureus* and *S. epidermidis* because the best overall prediction accuracy was obtained with version 2.0 in a recent proteomics-based verification of predicted export and retention signals in *B. subtilis* (179). Specifically, the hidden Markov model in SignalP 2.0 assigns a probability score to each amino acid of a potential signal peptide and indicates whether it is likely to belong to the N, H, or C domain. Proteins with no detectable N, H, or C domain were excluded from the set. Searching for transmembrane domains was performed with the TMHMM program, and proteins with more than one (predicted) transmembrane domain were excluded from the set because they most likely are integral membrane proteins. All proteins with a predicted C-terminal transmembrane segment in addition to a signal peptide were screened for the presence of a conserved motif for covalent cell wall binding. It should be noted that this approach does not automatically result in the exclusion of potential membrane proteins with one N-terminal transmembrane domain. The LipoP program was used to predict lipoproteins. The combined results of all these programs resulted in a list of proteins which have (i) signal peptides with distinctive N, H, and C domains, (ii) no additional transmem-

brane domains, and (iii) predicted extracytoplasmic localizations. These proteins were scanned for the presence of proteomics-based consensus motifs for type I, type II, or pseudopilin-specific SPase recognition and cleavage sites, twin-arginine motifs, and known leader peptides of bacteriocins by BLAST searches and by use of the PATTINPROT program (http://npsa-pbil.ibcp.fr), as previously described (179). To define the core exoproteome and variant exoproteome of the *S. aureus* strains, the sets of proteins with predicted signal peptides were used in multiple BLAST searches with the freeware BLASTall from the NCBI. The output was then filtered using Genome2D (10).

Secretory (Sec-type) signal peptides. Proteomics-based data sets of membrane, cell wall, and extracellular proteins were extremely valuable for a recent verification of signal peptide predictions for *B. subtilis* (179). Such data sets are now becoming available for *S. aureus*, as exemplified by studies on the membrane and cell wall proteomes of *S. aureus* Phillips (127) and the extracellular proteomes of *S. aureus* strains derived from the recently sequenced NCTC8325 and COL strains (208, 209) (Fig. 2). Additionally, the extracellular proteomes of several clinical *S. aureus* isolates have been analyzed (Fig. 2). The membrane, cell wall, and extracellular proteins of *S. aureus* that have been identified by proteomics, involving 2D-PAGE and subsequent mass spectrometry, are listed in Tables 4 and 5. These tables also show the −3-to-+1 amino acid sequences of the respective signal peptidase cleavage sites, if present.

Based on the proteomics data for membrane and extracellular proteins of *B. subtilis*, the optimized −3-to-+1 pattern (AVSTI)-(SEKYHQFLDGPW)-A-(AQVEKDFHLNS) for signal peptide recognition and cleavage by type I SPases of this organism was identified (179). SPase cleavage occurs C-terminal of the invariant Ala residue at the −1 position. The residues in parentheses in the pattern are listed in the order of frequency, with the most frequently identified residue at each position being placed in the first position. By comparing the predicted SPase recognition and cleavage sites for signal peptides of proteomically identified extracellular proteins of *S. aureus* (Table 4), we defined the −3-to-+1 pattern (AVS)-(KHNDQSYEGLR)-A-(AESDIKL) for productive recognition and cleavage by the type I SPase SpsB. Compared to the equivalent pattern in *B. subtilis*, it is interesting that the frequencies of certain residues at the −3, −2, and +1 positions differ, as reflected by the order of frequency with which they are listed in the pattern. Moreover, Asn can be present at the −2 position, while Ile is accepted at the +1 position. These residues are found at the −2 and +1 positions of certain serine proteases, hemolysins, immunoglobulin G (IgG) binding protein A, and aureolysin (Table 4). It should also be noted that compared to the optimized SPase recognition pattern in *B. subtilis*, several residues are not found at the −3, −2, and +1 positions of potential SpsB recognition and cleavage sites in identified extracellular proteins of *S. aureus*. Since such residues may be present in SPase recognition and cleavage sites of proteins that have escaped identification through proteomics, we included them in the −3-to-+1 search pattern (shown in lowercase) for the identification of potential secretory proteins of staphylococci, as follows: (AVSit)-(KHNDQSYEGLRfpw)-A-(AESDIKLfhnqv). This optimized *S. aureus* search pattern was used as an indicator of the quality of signal peptide predictions based on the SignalP-NN, SignalP-HMM, LipoP, PrediSi, Phobius, and TMHMM programs. Proteins with potential signal peptides containing this pattern were assigned to have a high probability of extracytoplasmic localization and a low probability of membrane retention (Tables 6 and 7). Proteins with potential signal peptides that do not contain this pattern were assigned to have a high probability of being retained in the membrane (data not shown). In this case, the uncleaved signal peptide could serve as an N-terminal membrane anchor. Following this approach, sets of 186 to 211 proteins (depending on the *S. aureus* strain) were identified that contain a potential signal peptide or N-terminal transmembrane segment. Scanning for the presence of the SpsB recognition and cleavage motif [(AVSit)-(KHNDQSYEGLRfpw)-A-(AESDIKLfhnqv)] revealed that, depending on the *S. aureus* strain investigated, 78 to 93 proteins carry this motif. These proteins are most likely processed by SPase, liberated from the membrane, and secreted into the extracellular milieu, unless they contain a cell wall retention signal (see below). Most of the other proteins with signal peptides that do not conform to the SpsB recognition and cleavage motif lack the invariant Ala at the −1 position. Also, some of these preproteins contain different residues at the −3, −2, or +1 position. For example, Asp, Glu, Phe, and Lys are highly unlikely residues at the −3 position (187). On the other hand, some preproteins have a Gly (e.g., exotoxins 4 and 5 from *S. aureus* COL) or a Leu at the −3 position (Tables 6 and 7). Since Gly and Leu residues at the −3 position of signal peptides are accepted by the *E. coli* SPase, it seems likely that they are also accepted at this position by SpsB. However, we did not include these residues in the current SpsB recognition and cleavage motif, since we could neither identify these proteins among the secreted proteins of *S. aureus* COL (Fig. 2) nor find published evidence that these proteins are indeed secreted. Among the proteins with predicted cleavable Sec-type signal peptides, there are many known extracellular staphylococcal virulence factors, such as exotoxins, enterotoxins (SEM, SEN, and SEO), hemolysins, TSST-1, leukotoxins (LukD and LukE), a secretory antigen SsaA homologue, and immunodominant antigen A (IsaA). Remarkably, the lists of identified extracellular proteins of *S. aureus* COL and RN6390 (208) (Tables 4 and 5) reveal that about 48% of these proteins lack known signal peptides. This percentage is substantially higher than the initial estimate of 10%, which was based on a limited proteomics-derived data set (174). It is also interesting that the list of identified extracellular proteins without a signal peptide includes enolase, which may be actively exported by an unknown mechanism (19), but lacks EsxA and EsxB, which are exported by the ESAT-6 system (26).

Twin-arginine (RR) signal peptides. The consensus RR motif that directs proteins into the Tat pathway has previously been defined as (KR)-R-X-#-#, where # is a hydrophobic residue (39, 89). Dilks et al. (47) used a genomic approach to identify possible Tat substrates for 84 diverse prokaryotes, using the TATFIND 1.2 program. Their study included *S. aureus* Mu50, MW2, and N315. Two potential Tat substrates of unknown function were predicted for *S. aureus* Mu50 and MW2, and one of these was also predicted for *S. aureus* N315 (47). However, both proteins are conserved in all sequenced *S. aureus* strains, including the N315 strain. One of the two pre-

TABLE 4. Identified proteins in extracellular proteomes of various *S. aureus* strains with known signal peptides[a]

Identification no.	Protein	Function	Amino acid at position:				Localization	Motif[f]
			−3	−2	−1	+1		
15925728	SA0022	Hypothetical protein	S	N	A	A	Extracellular	LPKTG
15925799	Plc	1-Phosphatidylinositol phosphodiesterase precursor	A	H	A	S	Extracellular	
15925800	SA0092	Hypothetical protein	T	A	G	C	Extracellular	Lipo
15925815[b]	Spa	Immunoglobulin G binding protein A precursor	A	N	A	A	Membrane/cell wall/ extracellular	LPETG
15925838[c]	SasD	Hypothetical protein	A	H	A	D	Extracellular	LPAAG
15925933	Coa	Staphylocoagulase precursor	A	D	A	I	Extracellular	
15925978	LytM	Peptidoglycan hydrolase	A	D	A	A	Extracellular	
15925983[d]	SA0270	Hypothetical protein	A	Q	A	Y	Extracellular	
15926008	SA0295	Hypothetical protein	A	F	A	K	Extracellular	
15926022	Geh	Glycerol ester hydrolase	A	Q	A	S	Extracellular	
15926073	SA0359	Hypothetical protein	L	T	A	C	Extracellular	Lipo
15926099	Set6	Exotoxin 6	V	Q	A	K	Extracellular	
15926111	Set15	Exotoxin 15	V	K	A	S	Extracellular	
15926112	SA0394	Hypothetical protein	A	E	A	S	Extracellular	
15926142	SA0423	Hypothetical protein	A	N	A	A	Extracellular	LysM
15926239[e]	SdrC	Ser-Asp-rich, fibrinogen-binding bone sialoprotein-binding protein	A	K	A	A	Membrane/cell wall	LPETG
15926241	SdrE	Ser-Asp-rich, fibrinogen-binding bone sialoprotein-binding protein	A	K	A	A	Extracellular	LPETG
15926291	SA0570	Hypothetical protein	A	E	A	A	Extracellular	
15926342	SA0620	Hypothetical protein	A	Q	A	S	Extracellular	
15926373	SA0651	Hypothetical protein	A	L	A	K	Extracellular	
15926417	SA0695	Hypothetical protein	I	S	A	C	Extracellular	Lipo
15926548	GlpQ	Glycerophosphoryl diester phosphodiesterase	A	G	A	E	Extracellular	
15926570	SA0841	Hypothetical protein	V	S	A	A	Extracellular	
15926634	SspB	Cysteine protease precursor	A	K	A	D	Extracellular	
15926635	SspA	Serine protease, V8 protease, glutamyl endopeptidase	A	N	A	L	Extracellular	
15926639	Atl	Autolysin, *N*-acetylmuramyl-L-alanine amidase and endo-β-*N*-acetylglucosaminidase	V	Q	A	A	Extracellular	GW
15926739	SA1001	Hypothetical protein	A	K	A	F	Extracellular	
15926746	SA1007	α-Hemolysin precursor	A	N	A	A	Extracellular	
15926969[c]	SA1221	Thioredoxin reductase	L	G	A	C	Extracellular	Lipo
15927068	SA1318	Hypothetical protein	L	S	G	C	Extracellular	Lipo
15927383	SplF	Serine protease SplF	A	K	A	E	Extracellular	2 TMD
15927384[e]	SplD	Serine protease SplD	A	K	A	E	Membrane/cell wall	2 TMD
15927385	SplC	Serine protease SplC	A	N	A	E	Extracellular	
15927386	SplB	Serine protease SplB	A	K	A	E	Extracellular	
15927387[b]	SplA	Serine protease SplA	A	K	A	E	Membrane/cell wall	
15927389	SA1633	Probable β-lactamase	A	K	A	E	Extracellular	
15927393	LukD	Leukotoxin, LukD	V	D	A	A	Extracellular	
15927394[f]	LukE	Leukotoxin, LukE	S	R	A	N	Extracellular	
15927483	SA1725	Staphopain, cysteine proteinase	A	N	A	E	Extracellular	
15927517	SA1755	Hypothetical protein	A	K	A	F	Extracellular	
15927520[b]	Sak	Staphylokinase precursor	V	S	A	S	Membrane/cell wall	
15927580	SA1812	Hypothetical protein	S	Y	A	K	Extracellular	
15927581	SA1813	Hypothetical protein	A	N	S	A	Extracellular	
15927670	SA1898	Hypothetical protein	A	H	A	S	Extracellular	
15927785	SA2006	Hypothetical protein	A	S	A	D	Extracellular	
15927879	SsaA	Hypothetical protein	A	H	A	S	Extracellular	
15927884	SA2097	Hypothetical protein	A	D	A	A	Extracellular	
15927988[e]	SA2198	Hypothetical protein	L	T	A	C	Membrane/cell wall	Lipo
15927996	Sbi	IgG-binding protein SBI	A	K	A	S	Extracellular	
15927998	HlgC	γ-Hemolysin component C	A	K	A	A	Extracellular	
15927999	HlgB	γ-Hemolysin component B	A	N	A	E	Extracellular	
15928148	IsaA	Immunodominant antigen A	A	H	A	A	Extracellular	
15928216	ClfB	Clumping factor B	A	Q	A	S	Extracellular	LPETG
15928223[b]	Aur	Zinc metalloproteinase aureolysin	A	L	A	I	Membrane/cell wall	
15928230	SA2437	Hypothetical protein	A	Y	A	D	Extracellular	
15928254[e]	IcaB	Intercellular adhesion protein B	A	N	A	D	Membrane/cell wall	
15928257	Lip	Triacylglycerol lipase precursor	A	Q	A	A	Extracellular	
57652419[g]	Pls	Methicillin-resistant surface protein	A	E	A	A	Extracellular	LPDTG
57651319[g]	SACOL0478	Exotoxin 3, putative	V	K	A	S	Extracellular	

Continued on following page

TABLE 4—*Continued*

Identification no.	Protein	Function	Amino acid at position:				Localization	Motif[i]
			−3	−2	−1	+1		
57651320[g]	SACOL0479	Surface protein, putative	A	E	A	S	Extracellular	
57650159[g]	Sek	Staphylococcal enterotoxin	A	S	A	Q	Extracellular	
57650160[g]	Sei	Staphylococcal enterotoxin type I	A	Y	A	D	Extracellular	
57651597[g]	Seb	Staphylococcal enterotoxin B	V	L	A	E	Extracellular	
57651598[g]	SACOL0908	Hypothetical protein	A	K	A	S	Extracellular	
57650600[g]	SACOL1865	Serine protease SplE, putative	A	K	A	E	Extracellular	
57650605[g]	SACOL1870	Hypothetical protein	A	K	A	E	Extracellular	
57650692[g]	Hlb	β-Hemolysin/phospholipase C	A	K	A	E	Extracellular	
57651004[g]	SACOL2505	Cell wall surface anchor family protein	A	E	A	A	Extracellular	LPKTG
24636603[h]	Etd	Exfoliative toxin D	S	H	A	E	Extracellular	
24636604[h]		Probable glutamyl-endopeptidase	V	S	A	S	Extracellular	

[a] The annotation of proteins is based on that of *S. aureus* N315, except for those proteins that are not encoded by the N315 genome.
[b] Also found in membrane/cell wall fraction by Nandakumar et al. (127).
[c] Found only in extracellular proteome described by Ziebandt et al. (208). Note that SA1221 is probably not a thioredoxin reductase, but a phosphate binding protein.
[d] Tyr is not present at the +1 position of the optimized *S. aureus* SpsB search pattern.
[e] Found only in membrane/cell wall fraction by Nandakumar et al. (127).
[f] Arg is not present at the −2 position of the optimized *S. aureus* SpsB search pattern.
[g] Encoded by *S. aureus* COL and absent from N315.
[h] Note that the corresponding gene is not present in the sequenced *S. aureus* genomes.
[i] TMD, transmembrane domain.

dicted Tat substrates has no known function, whereas the other was annotated as a hypothetical protein similar to a ferrichrome ABC transporter (permease). These proteins, however, are not in our list of proteins that have a predicted RR signal peptide. Although they have signal peptides according to the SignalP program, these proteins are localized in the cytoplasm or membrane according to the LipoP, PrediSi, and Phobius programs. It is therefore unlikely that these proteins are destined for secretion. Specifically, the hypothetical permease has eight predicted transmembrane helices.

Our own pattern searches for proteins with a possible RR motif resulted in 24 to 32 positive hits, depending on the *S. aureus* strain investigated. However, most of these proteins have no detectable N, H, or C domain and were therefore discarded from our data set. Also, some other proteins with a possible RR motif are predicted to contain a lipoprotein signal peptide. These predicted lipoproteins were also discarded from the list of potential *S. aureus* Tat substrates, firstly because none of the identified lipoproteins of *B. subtilis* that have an RR motif were shown to be secreted via the Tat pathway (87, 89) and secondly because there is no published evidence for any other bacterium that lipoproteins can be exported Tat dependently. Thus, it appears that only four or five proteins, depending on the *S. aureus* strain investigated, are potentially exported by the Tat pathway and cleaved by SpsB. However, it is noteworthy that none of the *B. subtilis* proteins with a KR motif were secreted Tat dependently, even though KR motifs are capable of directing proteins into the Tat pathways of chloroplasts and gram-negative bacteria, such as *E. coli* and *Salmonella enterica* (77, 82, 123, 167). If KR motifs are also rejected by the *S. aureus* Tat pathway, there would not be a single protein in any sequenced *S. aureus* strain that is secreted Tat dependently. This would be highly remarkable in view of the presence of *tatA* and *tatC* genes in all of these strains. Notably, the only known strictly Tat-dependent extracellular proteins of *B. subtilis* are the phosphodiesterase PhoD (175) and a protein of unknown function, YwbN (88). While a homologue of PhoD is not present in any of the six sequenced *S. aureus* strains, homologues of YwbN are present in all of these strains. Close inspection of the YwbN homologues of *S. aureus* COL, MRSA252, and MSSA476 revealed the presence of an N-terminal RR motif, but a potential signal peptide was not identified as such by the SignalP program. In contrast, the YwbN homologues of *S. aureus* Mu50, MW2, and N315 appear to lack this RR motif. According to comparisons of the deduced amino acid sequences, these three YwbN homologues should be missing the first 40 residues of *B. subtilis* YwbN. This is most likely not the case, since the sequences upstream of the annotated *S. aureus* Mu50, MW2, and N315 *ywbN* genes encode a peptide with an RR motif in the same open reading frame as the *ywbN* structural gene (Table 6). Thus, the RR motifs of the *S. aureus* Mu30, MW2, and N315 YwbN proteins have so far escaped identification due to a systematic difference in sequence annotation. It remains to be investigated whether these sequences with RR motifs serve as signal peptides in the Tat-dependent export of *S. aureus* YwbN homologues.

Pseudopilin signal peptides. The signal peptides of pseudopilins differ from the Sec-type signal peptides in the location of SPase cleavage sites. In pseudopilin signal peptides, the cleavage site is located between the N and H domains (105). The consensus recognition and cleavage motif for pseudopilin SPases, such as ComC, is K-G-F-X-X-X-E. Cleavage by pseudopilin SPases occurs within this motif, between the Gly and Phe residues. Upon cleavage, the Phe residue is methylated. For all sequenced *S. aureus* strains, three proteins were found to have the canonical pseudopilin SPase recognition and cleavage motif. These proteins are homologues of the cold shock proteins CspB, CspC, and CspD of *B. subtilis*. However, even though these proteins do contain the pseudopilin SPase recognition and cleavage pattern, they lack the H domain. Since the active sites of pseudopilin SPases are located in the cytoplasm, cleavage of the CspBCD homologues of *S. aureus* would be possible, but their export via the Com pathway is unlikely. Nevertheless,

TABLE 5. Identified proteins in extracellular proteomes of various *S. aureus* strains without known signal peptides

Identification no.	Protein	Function	Identification no.	Protein	Function
15925748[a]	XylR	Hypothetical protein	15926930	CitB	Aconitate hydratase
15926081[a]	AhpF	Alkyl hydroperoxide reductase subunit F	15926982[c]	CspA	Major cold shock protein CspA
15926082[b]	AhpC	Alkyl hydroperoxide reductase subunit C	15926993	OdhA	2-Oxoglutarate dehydrogenase E1
15926091[b]	GuaB	Inositol-monophosphate dehydrogenase	15927003	SA1255	PTS system, glucose-specific enzyme II, A component
15926092	GuaA	GMP synthase	15927005	SA1257	Peptide methionine sulfoxide reductase
15926202	ClpC	HSP100/Clp ATPase	15927057	SA1308	30S ribosomal protein S1
15926205	GltX	Glutamyl-tRNA synthetase	15927062	EbpS	Elastin binding protein
15926225[b]	Fus	Translational elongation factor G	15927092	Gnd	Phosphogluconate dehydrogenase
15926226[b]	TufA	Translational elongation factor TU	15927109	SA1359	Translation elongation factor EF-P
15926266	Pta	Phosphotransacetylase	15927132	Pbp3	Penicillin binding protein 3
15926385	SA0663	Hypothetical protein	15927133	SodA	Superoxide dismutase SodA
15926396[b]	YfnI	Hypothetical protein with five transmembrane segments and a potential SPase I cleavage site	15927160[b]	DnaK	DnaK protein
15926429	SA0707	Hypothetical protein	15927161	GrpE	GrpE protein
15926441	TrxB	Thioredoxin reductase	15927190	GreA	Transcription elongation factor
15926445	ClpP	Peptidase	15927229	SA1475	Hypothetical protein
15926449[b]	Gap	Glyceraldehyde-3-phosphate dehydrogenase	15927254	Tig	Trigger factor (prolyl isomerase)
15926450	Pgk	Phosphoglycerate kinase	15927273	CitZ	Citrate synthase II
15926451	Tpi	Triosephosphate isomerase	15927286	Ald	Alanine dehydrogenase
15926452	Pgm	2,3-Diphosphoglycerate-independent phosphoglycerate mutase	15927287	SA1532	Hypothetical protein
15926453[b]	Eno	Enolase	15927309[b]	Fhs	Formyltetrahydrofolate synthetase
15926468	SA0746	Staphylococcal nuclease with two transmembrane segments and a potential SPase I cleavage site	15927328	SA1572	Hypothetical protein
15926503	SA0775	Hypothetical protein	15927409	TRAP	Signal transduction protein TRAP
15926543	SA0806	Hypothetical protein	15927495	SA1737	Hypothetical protein
15926551	Pgi	Glucose-6-phosphate isomerase A	15927539	SA1774	Hypothetical protein
15926589	SA0859	Hypothetical protein	15927604[b]	GroEL	GroEL protein
15926642	SA0908	Hypothetical protein predicted to have an uncleaved signal peptide	15927605	GroES	GroES protein
15926669	PtsH	Phosphocarrier protein Hpr, phosphohistidine-containing protein	15927640	RsbW	Anti-σ^B factor
15926670	PtsI	Phosphoenolpyruvate-protein phosphatase	15927687	GlyA	Serine hydroxymethyl transferase
15926679	PdhB	Pyruvate dehydrogenase E1 component beta subunit	15927699	FbaA	Fructose-bisphosphate aldolase
15926680	PdhC	Dihydrolipoamide *S*-acetyltransferase component of pyruvate dehydrogenase complex E2	15927712	DeoD	Purine nucleoside phosphorylase
15926681[b]	PdhD	Dihydrolipoamide dehydrogenase component of pyruvate dehydrogenase E3	15927753	SAS074	Hypothetical protein
15926710	SA0974	Hypothetical protein	15927762	Asp23	Alkaline shock protein 23, Asp23
15926723	PheT	$tRNA^{Phe}$ synthetase β chain	15927994	SA2204	Hypothetical protein
15926735	SA0998	Hypothetical protein	15927798	RplM	50S ribosomal protein L13
15926776	IleS	$tRNA^{Ile}$ synthetase	15928070	SA2279	Hypothetical protein
15926829	SucD	Succinyl-coenzyme A synthetase, α subunit	15928081	FnbB	Fibronectin binding protein B
15926840	Tsf	Elongation factor TS	15928082	FnbA	Fibronectin binding protein A
15926857	PnpA	Polyribonucleotide nucleotidyltransferase	15928133	RocA	1-Pyrroline-5-carboxylate dehydrogenase
15926892	GlnA	Glutamine-ammonia ligase	15928185	PanB	3-Methyl-2-oxobutanoate hydroxymethyltransferase
15926915	KatA	Catalase	15928192	SA2399	Fructose-bisphosphate aldolase
15926923[b]	Tkt	Transketolase	15928284	SA2490	Hypothetical protein
			57651372	MetS	Methionyl-tRNA synthetase
			57651442	SACOL0613	Hypothetical protein
			57651702	PdhA	Pyruvate dehydrogenase complex E1 component, α subunit
			24636605[d]	Edin-B	Epidermal cell differentiation inhibitor B

[a] Found only in the extracellular proteome described by Ziebandt et al. (208).
[b] Also found in the extracellular proteome described by Ziebandt et al. (208).
[c] Contains a pseudopilin SPase recognition and cleavage site.
[d] Note that the corresponding gene is not present in the sequenced *S. aureus* genomes.

it should be noted that one of the CspBCD homologues of *S. aureus*, known as CspA, was found in the extracellular proteome of a clinical isolate (Fig. 2 and Table 5). To verify the absence or presence of pseudopilins in *S. aureus*, BLAST searches with the known ComGC, ComGD, ComGE, and ComGG proteins of *B. subtilis* were performed. This revealed the presence of only one potential pseudopilin, which is a homologue of *B. subtilis* ComGC. Although the consensus pseudopilin SPase recognition and cleavage site is absent from *S. aureus* ComGC, a putative cleavage pattern (Q-A-F-T-L-I-E) is present at the position in the ComGC signal peptide where a pseudopilin SPase recognition and cleavage site would be expected. Further analyses revealed that similar observations can be made for ComGC homologues in other gram-positive bacteria, such as *Bacillus cereus*, *B. anthracis*, *L. monocytogenes*, *S. haemolyticus*, and *Oceanobacillus iheyensis*. By comparing the ComGC homologues of these organisms, an expanded search pattern for gram-positive bacterial pseudopilin SPase recognition and cleavage sites was defined as follows: (KEQRS)-(GA)-F-X-X-X-E. Interestingly, using this expanded search pattern, two additional potential pseudopilins of *S. aureus* were identified. These potential pseudopilins show similarity to the ComGD proteins of *B. cereus* and *B. anthracis* and the ComGF proteins of *Bacillus halodurans* and *L. lactis*. It remains to be shown whether the three identified potential pseudopilins of *S. aureus* are indeed able to assemble into pilin-like structures after being processed by the ComC homologue. If so, it will be even more interesting to identify their biological function, for example, in adhesion to surfaces, motility, or export of proteins. Such functions could play a role in virulence and have been attributed to type IV pili and pseudopilins of gram-negative bacteria (106).

TABLE 6. The core exoproteome of *S. aureus*, defined as proteins with predicted Sec-type signal peptides present in all sequenced strains[l]

Identification no.	Protein	Amino acid at position:				Function
		−3	−2	−1	+1	
15925728[a,b]	SA0022	S	N	A	A	Hypothetical protein
15925815[a]	Spa	A	N	A	A	Immunoglobulin G binding protein A precursor
15925838[c]	SA0129	A	H	A	D	Hypothetical protein
15925848[d]	SA0139	S	L	A	I	Hypothetical protein
15925933	Coa	A	D	A	I	Staphylocoagulase precursor
15925978	LytM	A	D	A	A	Peptidoglycan hydrolase
15926008	SA0295	A	F	A	K	Hypothetical protein
15926022[d]	Geh	A	Q	A	S	Glycerol ester hydrolase
15926106[e]	Set13	V	H	A	K	Exotoxin 13
15926112	SA0394	A	E	A	S	Hypothetical protein
15926142[d,f]	SA0423	A	N	A	A	Hypothetical protein
15926239[a,d]	SdrC	A	K	A	A	Ser-Asp-rich fibrinogen-binding protein
15926291[d]	SA0570	A	E	A	A	Hypothetical protein
15926319[d,g,h]	Pbp4	A	Q	A	T	Penicillin binding protein 4
15926342[f,d]	SA0620	A	Q	A	S	Secretory antigen SsaA homologue
15926373[d]	SA0651	A	L	A	K	Hypothetical protein
15926432[f,d]	SA0710	A	H	A	Q	Hypothetical protein
15926464[a]	ClfA	A	D	A	S	Fibrinogen-binding protein A
15926466	Ssp	A	K	A	A	Extracellular matrix and plasma binding protein
15926467	SA0745	A	N	A	L	Hypothetical protein
15926548[h]	GlpQ	A	G	A	E	Glycerophosphoryl diester phosphodiesterase
15926570	SA0841	V	S	A	A	Hypothetical protein
15926634	SspB	A	K	A	D	Cysteine protease precursor
15926635[d]	SspA	A	N	A	L	Serine protease
15926639[f,d]	Atl	V	Q	A	A	Autolysin
15926713[a]	IsdB	A	Q	A	A	Hypothetical protein
15926714[a]	IsdA	V	N	A	A	Cell surface protein
15926715[i]	IsdC	A	N	A	A	Hypothetical protein
15926738	SA1000	S	H	A	Q	Hypothetical protein
15926741	Efb	A	D	A	S	Hypothetical protein
15926742	SA1004	A	D	A	S	Hypothetical protein
15926750	SA1010	S	E	A	K	Hypothetical protein
15926830[f]	LytN	A	Y	A	D	LytN protein
15927055[d]	GpsA	V	L	A	E	Glycerol-3-phosphate dehydrogenase
15927385	SplC	A	N	A	E	Serine protease SplC
15927456[d]	SA1698	S	L	A	D	Hypothetical protein
15927483[d]	SspB2	A	N	A	E	Staphopain, cysteine proteinase
15927580	SA1812	S	Y	A	K	Hypothetical protein
15927607[d]	SA1839†	V	E	A	K	Hypothetical protein
15927670[d]	SA1898	A	H	A	S	Hypothetical protein
15927785	SA2006	A	S	A	D	Hypothetical protein
15927879[d]	SsaA	A	H	A	S	Hypothetical protein
15927884[d]	SA2097	A	D	A	A	Hypothetical protein
15927996	Sbi	A	K	A	S	IgG-binding protein Sbi
15927997	HlgA	S	K	A	E	γ-Hemolysin chain II precursor
15927998	HlgC	A	K	A	A	γ-Hemolysin component C
15927999	HlgB	A	N	A	E	γ-Hemolysin component B
15928114	SA2323	A	Y	A	H	Hypothetical protein
15928123[d]	SA2332	S	H	A	A	Hypothetical protein
15928145[d]	SA2353	A	Q	A	A	Hypothetical protein
15928148[d]	IsaA	A	H	A	A	Immunodominant antigen A
15928216[a]	ClfB	A	Q	A	S	Clumping factor B
15928223[d,j]	Aur	A	L	A	I	Zinc metalloproteinase aureolysin
15928224[d,k]	IsaB	A	Q	A	A	Immunodominant antigen B
15928230[d]	SA2437	A	Y	A	D	Hypothetical protein
15928232[c,d]	SasF	A	Q	A	A	Conserved hypothetical protein
15928254	IcaB	A	N	A	D	Intercellular adhesion protein B
15928257[d]	Lip	A	Q	A	A	Triacylglycerol lipase precursor

[a] Contains an LPXTG motif.
[b] Protein homologue of *B. subtilis* is found in extracellular proteome (174).
[c] Contains an LPXAG motif.
[d] Also present in *S. epidermidis*.
[e] Only the *S. aureus* COL protein has a Thr at the +1 position, which is not included in the recognition and cleavage pattern. All other *S. aureus* strains conform to the proposed pattern.
[f] Contains a LysM or GW domain motif.
[g] Only the *S. aureus* N315 protein has a Thr at the +1 position, which is not included in the recognition and cleavage pattern. All other *S. aureus* strains conform to the proposed pattern.
[h] Protein homologues of *B. subtilis* are classified as Sec-attached membrane proteins (179).
[i] Contains an NPQTN motif.
[j] Protein homologue of *B. subtilis* classified as a secretory protein (179).
[k] Only for the *S. aureus* MRSA252 protein; protein homologues in other strains are predicted to have two transmembrane domains and were excluded from the list.
[l] Signal peptide predictions were performed with SignalP-NN and SignalP-HMM, version 2.0 (136; http://www.cbs.dtu.dk/services/SignalP-2.0/), PrediSi (76; http://www.predisi.de/), Phobius (95; http://phobius.cgb.ki.se/), and LipoP, version 1.0 (94; http://www.cbs.dtu.dk/services/LipoP/). These programs are designed to identify Sec-type signal peptides, amino-terminal membrane anchors (Phobius), or lipoprotein signal peptides in gram-negative bacteria (LipoP). The TMHMM program, version 2.0 (41; http://www.cbs.dtu.dk/services/TMHMM/), was used to identify transmembrane segments in proteins.

TABLE 7. Variant exoproteome of *S. aureus*, defined as proteins with predicted Sec-type signal peptides present in at least one sequenced strain

Identification no.	Protein	Amino acid at position:				Function
		−3	−2	−1	+1	
15925799	Plc	A	H	A	S	1-Phosphatidylinositol phosphodiesterase precursor
15926099[a]	Set6	V	Q	A	K	Exotoxin 6
15926100[a]	Set7	V	H	A	E	Exotoxin 7
15926101[a]	Set8	V	K	A	E	Exotoxin 8
15926103[a]	Set10	V	N	A	S	Exotoxin 10
15926104[a]	Set11	V	H	A	K	Exotoxin 11
15926105[a]	Set12	V	N	A	K	Exotoxin 12
15926106[a]	Set13	V	H	A	K	Exotoxin 13
15926111[a]	Set15	V	K	A	S	Exotoxin 15
15926113	SA0395	A	D	A	K	Hypothetical protein
15926465	SA0743	A	S	A	V	Hypothetical protein
15926739	SA1001	A	K	A	F	Hypothetical protein
15926746	HlY	A	N	A	A	α-Hemolysin precursor
15927016[b]	EbhB	A	H	A	A	Hypothetical protein
15927308[c]	Fhs	A	Q	A	A	Hypothetical protein
15927386	SplB	A	K	A	E	Serine protease SplB
15927387	SplA	A	K	A	E	Serine protease SplA
15927389	SA1633	A	K	A	E	Probable β-lactamase
15927393	LukD	V	D	A	A	Leukotoxin, LukD
15927398	SEG	V	N	A	Q	Extracellular enterotoxin type G precursor
15927399	SEN	V	N	A	E	Enterotoxin SeN
15927402	SEI	T	Y	A	Q	Extracellular enterotoxin type I precursor
15927404	SEO	A	Y	A	N	Enterotoxin SeO
15927512	Map	A	S	A	A	Truncated MapW protein
15927513	Hlb	A	K	A	E	Truncated β-hemolysin
15927516	SA1754	A	Q	A	S	Hypothetical protein
15927517	SA1755	A	K	A	F	Hypothetical protein
15927520	Sak	V	S	A	S	Staphylokinase precursor
15927522	SA1760	A	K	A	I	Hypothetical protein
15927585	SEC3	V	L	A	E	Enterotoxin type C3
15927586	SA1818	A	K	A	E	Hypothetical protein
15927587	TSST-1	A	K	A	S	Toxic shock syndrome toxin 1
15927741[c]	FmtB	A	S	A	A	FmtB protein
15928076[b,c]	SA2285	A	E	A	A	Hypothetical protein
15928174[c]	SA2381	A	N	A	E	Hypothetical protein
15928182	SA2389	V	L	A	D	Hypothetical protein
16119203	SAP003	A	Y	A	N	Hypothetical protein
16119219	SAP019	A	E	A	A	Hypothetical protein
14141830	SAVP008	A	N	A	E	Hypothetical protein
21282111	Set16	V	Q	A	K	Hypothetical protein
21282116	Set21	V	K	A	A	Hypothetical protein
21282123	Set26	V	K	A	I	Hypothetical protein
21283107	LukF	V	D	A	A	Panton-Valentine leukocidin chain F precursor
21283108	LukS	S	K	A	D	Panton-Valentine leukocidin chain S precursor
21284341[c]	Cna	A	L	A	A	Collagen adhesin precursor
49482651	SAR0423	V	H	A	E	Exotoxin
49482652	SAR0424	A	N	A	E	Exotoxin
49482653	SAR0425	A	N	A	E	Exotoxin
49482925[b]	SAR0721	T	F	A	E	Multicopper oxidase protein
49484047	SAR1886	A	Y	A	F	Putative exported protein
49484059[b]	SAR1905	A	K	A	E	Serine protease
49484898	SAR2788	A	E	A	S	Putative exported protein
49484957	SEH	A	K	A	E	Enterotoxin H
49485293	SAS0389	V	K	A	A	Exotoxin
57650600	SACOL1865	A	K	A	E	Serine protease SplE, putative
57650605	SACOL1870	A	K	A	E	Hypothetical protein
57650609	EpiP	A	S	A	S	Epidermin leader peptide processing serine protease EpiP
57651309	SACOL0468	V	Q	A	K	Exotoxin 3, putative
57651311	SACOL0470	V	K	A	E	Exotoxin, putative
57651319	SACOL0478	V	K	A	S	Exotoxin 3, putative
57652419[c]	Pls	A	E	A	A	Methicillin-resistant surface protein

[a] Although these proteins share homology with exotoxins from other *S. aureus* strains, they are highly variable (80).
[b] Also present in *S. epidermidis*.
[c] Contains an LPXTG motif.

Bacteriocin leader peptides. Bacteriocins form a distinct group of proteins with cleavable N-terminal signal peptides, which are often called leader peptides. These leader peptides only have N and C domains and completely lack the hydrophobic H domain. The bacteriocin leader peptides are invoked in posttranslational modification and the prevention of premature antimicrobial activity, which would be deleterious to the producing organism. Of the sequenced *S. aureus* bacteriocins, C55α and C55β contain a leader peptide (130), whereas leader peptides are absent from aureocin A53 (134) and aureocin A70 (134). Two potential lantibiotics with leader peptides were identified by sequencing the genomes of *S. aureus* MW2 (7) (GI numbers 49486642 and 49486641) and MSSA476. In both strains, the corresponding genes are located on the genomic island *v*SAβ. Both *S. aureus* proteins show similarity to the lantibiotic gallidermin precursor GdmA of *Staphylococcus gallinarum* and to the lantibiotic epidermin precursor EpiA of *S. epidermidis*. Notably, the *S. aureus* COL strain contains only one of these two potential lantibiotics, which is most similar to the potential MW2 lantibiotic under GI number 49486641. Two additional putative bacteriocins that were identified by genome sequencing seem to be homologous to *L. lactis* lactococcin 972. The hypothetical protein SAP019 (N315 annotation) is plasmid encoded in *S. aureus* N315 and MSSA476 and chromosomally encoded in *S. aureus* MRSA252. The other hypothetical bacteriocin, SAS029, is chromosomally encoded in all sequenced *S. aureus* strains. No published data are presently available on the characteristics of these proteins, so it remains to be seen whether they are genuine bacteriocins.

Potential ESAT-6 export signal. As described above, the EsxA and EsxB proteins are secreted by *S. aureus* via the ESAT-6 route (26). Both proteins lack a known signal peptide but are specifically transported across the membrane nonetheless. This implies that these two proteins must contain an export signal that is recognized by one or more ESAT-6 pathway components. The nature of this signal is presently unknown. The only common feature of proteins that are known (or predicted) to be translocated across the membrane via the ESAT-6 pathway is a WXG motif, which is located ~100 amino acids from the N terminus of the protein (140). The involvement of the WXG motif in ESAT-6 targeting remains to be demonstrated.

Retention Signals

Lipoproteins. Lipoproteins appear to be exported via the Sec pathway. During or shortly after translocation, the invariant Cys in the lipobox is diacylglyceryl modified by Lgt. This results in signal peptide cleavage by SPase II and retention of the mature lipoprotein in the membrane. Based on the cleavage sites of lipoproteins that have been identified in various gram-positive bacteria, Sutcliffe and Harrington (171) reported the −4-to-+2 lipobox pattern (LIVMFESTAG)-(LVIAMGT)-(IVMSTAFG)-(AG)-C-(SGANERQTL). Furthermore, they reported that neither the charged residues Asp, Glu, Arg, and Lys nor Gln is present in the region between 6 and 20 residues N-terminal of the lipobox. A search of the translated proteins encoded by the six *S. aureus* genomes with the pattern shown above, using the PATTINPROT program, revealed about 50 proteins with this motif (Tables 8 and 9). A comparison of the PATTINPROT results to the results obtained with the LipoP program showed that 10 to 16 more potential lipoproteins may be present in *S. aureus*. Most of these extra predicted lipoproteins contain an amino acid at the −1 position (mostly Ser) or the +2 position (mostly Asp) that differs from the lipobox pattern reported by Sutcliffe and Harrington (171). Recently, Tjalsma and van Dijl (179) proposed the lipobox search pattern (LITAGMV)-(ASGTIMVF)-(AG)-C-(SGENTAQR) for potential lipoproteins of *B. subtilis* on the basis of published proteomics data. The only difference compared to the pattern by Sutcliffe and Harrington (171) is that Leu is absent from the +2 position, which is due to the fact that no potential *B. subtilis* lipoprotein with Leu at this position was identified by proteomics. Consistently, none of the predicted *S. aureus* lipoproteins has a Leu at the +2 position (Tables 8 and 9). It is also noteworthy that some lipoproteins contain a (KR)-R-X-#-# motif in their signal peptides, although it has not been shown yet that lipoproteins can be transported via the Tat pathway. Finally, the hypothetical protein Lpl2 of *S. aureus* N315 and Mu50 was excluded from our lipoprotein predictions because Asp does not seem to occur at the +2 position of lipoproteins from gram-positive bacteria (94, 179). Nevertheless, the homologues of Lpl2 of the other sequenced *S. aureus* strains are classified as lipoproteins because they have residues at the +2 position that conform to the lipobox consensus.

Lipoprotein release determinant. Although lipoproteins were generally believed to be retained at the membrane-cell wall interface, the presence of lipoproteins in the growth medium of *B. subtilis* was documented by Antelmann et al. (3). This unexpected finding was correlated with the proteolytic removal of the amino-terminal, lipid-modified Cys, which suggests that the observed lipoprotein release into the growth medium was caused by proteolytic "shaving" after processing by LspA. In most of these lipoproteins, Tjalsma and van Dijl (179) identified the +1-to-+10 consensus sequence C-G-(NSTF)-X-(SGN)-X-(SGKAE)-X-X-(SGA), which might represent the recognition site for an as yet unidentified shaving protease. Most probably, a Gly at the +2 position is of major importance for lipoprotein release into the growth medium, while a Ser at this position seems to inhibit the process. In the sequenced genomes of *S. aureus*, a gene for only one lipoprotein with the exact motif described above could be found. By searching for patterns with 80% similarity to the consensus sequence (i.e., one different residue), five to seven additional lipoproteins could be found, depending on the *S. aureus* strain. Thr was not found at the +3 position in any of these proteins. Instead, a Lys was identified at this position in one or two predicted lipoproteins with a potential release motif, depending on the *S. aureus* strain. In other predicted lipoproteins with a potential release motif, no Gly or Ala residue was found at the +7 position. However, one of these lipoproteins contains a predicted release motif with a Gln at the +7 position. To date, a total of four potential lipoproteins have been identified in the extracellular milieu of *S. aureus*. The first one was identified by Ziebandt et al. (208). This protein was annotated as a thioredoxin reductase (Table 4), but it shows no similarity to known thioredoxin reductases. Instead, it is highly similar to phosphate-binding lipoproteins, such as PstS of *B. subtilis*. It should be noted that this protein was not predicted to be a lipoprotein because the signal peptide contains a Gln residue in the

TABLE 8. Core lipoproteome of *S. aureus*, defined as proteins with predicted lipoprotein signal peptides present in all sequenced strains

Identification no.	Protein	Amino acid at position:					Function
		−3	−2	−1	+1	+2	
15925800	SA0092	T	A	G	C	G	Hypothetical protein
15925819	SirA	L	A	G	C	S	Lipoprotein
15925847[a]	SA0138	A	A	A	C	G	Hypothetical protein, similar to alkylphosphonate ABC transporter
15925912	Slp	L	S	G	C	G	RGD-containing lipoprotein
15925918	SA0207	V	T	A	C	G	Hypothetical protein, similar to maltose/maltodextrin-binding protein
15925928[a,b]	SA0217	L	S	S	C	A	Hypothetical protein, similar to periplasmic-iron-binding protein BitC
15925940[c]	SA0229	L	S	G	C	G	Hypothetical protein, similar to nickel ABC transporter nickel-binding protein
15926044	SA0331	I	A	A	C	G	Conserved hypothetical protein
15926073	SA0359	L	T	A	C	G	Conserved hypothetical protein
15926079[a]	SA0363	L	T	G	C	A	Hypothetical protein
15926141[a,d]	SA0422	L	A	A	C	G	Hypothetical protein, similar to lactococcal lipoprotein
15926287[a]	SA0566	L	S	G	C	G	Hypothetical protein, similar to iron-binding protein
15926308[a]	SA0587	V	A	A	C	G	Lipoprotein, streptococcal adhesin PsaA homologue
15926354[a]	SA0632	L	T	G	C	G	Conserved hypothetical protein
15926385[a,e]	SA0663	L	G	A	C	G	Hypothetical protein
15926413[a,f]	SA0691	L	A	A	C	G	Lipoprotein, similar to ferrichrome ABC transporter
15926417[a]	SA0695	I	S	A	C	G	Hypothetical protein
15926461	SA0739	L	G	A	C	G	Conserved hypothetical protein
15926499[a,g]	SA0771	L	A	A	C	G	Conserved hypothetical protein
15926579[a]	SA0849	L	S	G	C	A	Hypothetical protein, similar to peptide binding protein OppA
15926625	SA0891	V	A	G	C	G	Hypothetical protein, similar to ferrichrome ABC transporter
15926678[a]	SA0943	L	A	G	C	T	Conserved hypothetical protein
15926717[h]	IsdE	L	T	S	C	Q	Hypothetical protein
15926796[a]	SA1056	V	A	G	C	S	Hypothetical protein
15926969[h]	SA1221	L	G	A	C	G	Thioredoxin reductase
15927111[a,e]	SA1361	L	A	G	C	G	Hypothetical protein
15927372[h]	SA1616	L	S	S	C	G	Hypothetical protein
15927375[a]	SA1619	L	T	A	C	G	Hypothetical protein
15927415[a]	PrsA	L	G	A	C	G	Peptidyl-prolyl *cis*/*trans* isomerase homolog
15927477[a]	SA1719	L	A	A	C	G	Conserved hypothetical protein
15927757[a,i]	SA1979	V	A	A	C	G	Hypothetical protein, similar to ferrichrome ABC transporter (binding protein)
15927864[g]	SA2079	L	A	A	C	G	Hypothetical protein, similar to ferrichrome ABC transporter FhuD precursor
15927948[a]	SA2158	L	A	A	C	G	Hypothetical protein, similar to TpgX protein
15927961[a,h]	SA2171	L	I	V	C	I	Hypothetical protein
15927984[a]	DsbA	L	A	A	C	G	DsbA; hypothetical protein similar to Zn-binding lipoprotein AdcA
15927987[c]	SA2197	L	T	A	C	G	Conserved hypothetical protein
15927988[c]	SA2198	I	S	G	C	G	Hypothetical protein
15927992[a,g]	SA2202	L	A	A	C	G	Hypothetical protein, similar to ABC transporter, periplasmic amino acid-binding protein
15928025[a]	OpuCC	L	S	G	C	S	Glycine betaine/carnitine/choline ABC transporter OpuC
15928037[a]	SA2247	L	S	A	C	G	Conserved hypothetical protein
15928046[a]	Opp-1A	L	T	G	C	G	Oligopeptide transporter putative substrate binding domain
15928066[a,i]	SA2275	I	G	A	C	G	Hypothetical protein
15928267[h]	SA2473	L	Y	S	C	S	Hypothetical protein

[a] Also present in *S. epidermidis*.
[b] Excluded for *S. aureus* Mu50 and N315 because of the motif proposed by Sutcliffe and Harrington (171).
[c] Excluded for *S. epidermidis* because of the motif proposed by Sutcliffe and Harrington (171).
[d] Contains the lipoprotein release motif (179) with one amino acid change, except for *S. aureus* COL and *S. epidermidis*.
[e] Contains the lipoprotein release motif (179) with one amino acid change for all staphylococcal strains.
[f] Contains the exact lipoprotein release motif (179), except in *S. epidermidis*.
[g] Contains the lipoprotein release motif (179) with one amino acid change, except in *S. epidermidis*.
[h] Excluded for all *S. aureus* strains because of the motif proposed by Sutcliffe and Harrington (171).
[i] Contains the lipoprotein release motif (179) with one amino acid change, only for *S. epidermidis*.

N domain. According to the search profile of Sutcliffe and Harrington (171), lipoproteins do not contain a Gln residue at this position. On the other hand, PstS of *B. subtilis* is a lipoprotein, and it would seem quite likely that this is also true for its *S. aureus* homologue. The other three potential lipoproteins were identified in the growth media of clinical isolates (Table 4). Remarkably, none of the four lipoproteins with extracellular localization contain the complete lipoprotein release motif that was identified in extracellular lipoproteins of *B. subtilis*. However, they do contain a Gly residue at the +2 position,

TABLE 9. Variant lipoproteome of *S. aureus*, defined as proteins with predicted lipoprotein signal peptides present in at least one sequenced strain

Identification no.	Protein	Amino acid at position:					Function
		−3	−2	−1	+1	+2	
15925801[a]	SA0093	F	A	G	C	G	Hypothetical protein
15925803	SA0095	T	A	G	C	G	Hypothetical protein
15925804	SA0096	T	A	G	C	G	Hypothetical protein
15925877[a]	SA0167	I	T	G	C	D	Hypothetical protein, similar to membrane lipoprotein SrpL
15926004	SA0291	L	A	G	C	S	Hypothetical protein
15926114[b]	Lpl1	I	A	G	C	G	Hypothetical protein
15926115[a]	Lpl2	I	I	G	C	D	Hypothetical protein
15926116	Lpl3	I	A	G	C	G	Hypothetical protein
15926118	Lpl4	I	I	G	C	G	Hypothetical protein
15926119	Lpl5	V	A	G	C	G	Hypothetical protein
15926120[a]	Lpl6	I	I	G	C	D	Hypothetical protein
15926122[a]	Lpl8	A	T	S	C	G	Hypothetical protein
15926123	Lpl9	I	G	G	C	G	Hypothetical protein
15926465[a]	SA0743	G	A	L	C	V	Hypothetical protein
15926580[c]	SA0850	L	S	A	C	G	Hypothetical protein, similar to oligopeptide ABC transporter oligopeptide-binding protein
15927067	SA1317	L	S	G	C	S	Hypothetical protein
15927068[d]	SA1318	L	S	G	C	S	Hypothetical protein
15927069	SA1319	L	S	G	C	S	Hypothetical protein
15927373[b]	SA1617	L	S	A	C	S	Hypothetical protein
15927396[c]	SA1640	L	V	A	C	G	Conserved hypothetical protein
15927859[d]	ModA	L	A	G	C	S	Probable molybdate-binding protein
19528064[a]	SA2273	I	G	G	C	I	Hypothetical protein
21281801	Mw0072	T	A	G	C	G	*Staphylococcus* tandem lipoprotein
21282126	Lpl10	I	A	G	C	G	Hypothetical protein
21282127[a]	Lpl11	V	T	S	C	G	Hypothetical protein
21282129[a]	Lpl13	I	I	G	C	D	Hypothetical protein
21283103	MW1374	L	S	G	C	S	Conserved hypothetical protein
21283167	MW1438	L	T	A	C	G	Hypothetical protein
21284135	MW2406	I	G	A	C	G	Hypothetical protein
21284306	MW2577	V	S	G	C	S	Hypothetical protein
49482670	SAR0445	I	G	G	C	G	Putative lipoprotein
49483993[d]	BlaZ	L	S	A	C	N	β-Lactamase precursor
49484246[d]	SAR2104	L	S	A	C	G	Putative lipoprotein
49484287[a]	SAR2149	L	I	V	C	G	Hypothetical protein
49484977[d,e]	SAS0074	I	G	G	C	G	Putative lipoprotein
57650161	SACOL0888	L	G	A	C	G	Pathogenicity island, putative lipoprotein
57650441	SACOL1528	L	S	G	C	S	Hypothetical protein
57650444	SACOL1531	L	S	G	C	S	Hypothetical protein
57650485	SACOL1574	L	S	A	C	G	Hypothetical protein
57650696	SACOL2010	L	T	A	C	S	Iron compound ABC transporter, iron compound-binding protein
57651323[a]	SACOL0482	I	M	G	C	D	*Staphylococcus* tandem lipoprotein
57652445	SACOL0081	T	A	G	C	G	Hypothetical protein
14141829[a]	SAVP006	L	V	S	C	N	Hypothetical protein

[a] Excluded for all *S. aureus* strains because of the motif proposed by Sutcliffe and Harrington (171).
[b] Excluded for *S. aureus* COL, MSSA476, Mu50, MW2, and N315 because of the motif proposed by Sutcliffe and Harrington (171).
[c] Contains the lipoprotein release motif (179) with one amino acid change.
[d] Also present in *S. epidermidis*.
[e] Contains the lipoprotein release motif for *S. epidermidis* (179), with one amino acid change.

which strengthens the idea that this amino acid residue is probably important for lipoprotein release. It is interesting that in lipoproteins of gram-negative bacteria, an Asp, Gly, Phe, or Trp residue at the +2 position prevents transport of the mature lipoprotein to the outer membrane (128, 181). In gram-positive bacteria, no outer membrane is present, and it is currently not known whether the residue at the +2 position has a role in subcellular protein sorting. However, a Gly at this position does seem to promote lipoprotein release into the extracellular milieu, not only in *B. subtilis* but also in *S. aureus*.

Cell wall binding domains. Proteins that have to be displayed on the bacterial surface must be retained by noncovalent or covalent binding to the peptidoglycan moiety of the cell wall. In *B. subtilis*, several proteins involved in cell wall turnover contain repeated domains in the C-terminal part of the protein which have affinity for cell wall components (62, 110, 150). Specifically, the *B. subtilis* proteins LytD, WapA, YocH, YvcE, and YwtD have been reported to bind to the cell wall (62, 110, 150). While WapA is not conserved in staphylococci, various *S. aureus* proteins with regions that show amino acid sequence similarity to LytD, YocH, YvcE, and YwtD of *B.*

subtilis can be found by BLAST searches. Accordingly, these *S. aureus* proteins may be cell wall bound, but this remains to be shown.

One of the domains that have affinity for cell wall components is the "lysin motif," or LysM domain, which was first described for bacterial lysins (146). The number of LysM domains can differ for wall-bound proteins from different gram-positive bacterial species (168). For example, XlyA of *B. subtilis* contains only one LysM domain, whereas three domains can be detected in AcmA of *L. lactis* and five or six domains can be detected in muramidases from *Enterococcus* species (92). Using the LysM domain of AcmA from *L. lactis* in BLAST searches against the six sequenced and annotated *S. aureus* genomes, four proteins with one or more LysM domains were detected. These proteins include a hypothetical protein similar to autolysins (SA0423), a secretory antigen SsaA homologue (SA0620), a conserved hypothetical protein (SA0710), and the LytN protein.

A different domain that can facilitate protein binding to the cell wall is the GW domain. In *L. monocytogenes*, the surface-exposed InlB protein contains three C-terminal GW domains. Each domain consists of ~80 amino acids and starts with a Gly and a Trp residue (22). This domain specifically binds to lipoteichoic acids in the cell wall (91), thereby facilitating the interaction of *L. monocytogenes* with components of human host cells. The only protein with GW domains found in the sequenced *S. aureus* strains is the autolysin protein Atl (5, 6). This bifunctional autolysin contains three GW repeats of ~97 amino acids. The protein is exported as a prepro-Atl precursor of 1,256 amino acids. Subsequent processing steps result in the removal of the signal peptide and the propeptide and the separation of the mature region into an amidase and a glucosaminidase (139). A similar separation of the mature region into an amidase and glucosaminidase has been reported for the AtlE protein of *S. epidermidis* (74). The GW repeats are both necessary and sufficient to direct reporter proteins to the equatorial surface rings of *S. aureus* cells, where cell division starts.

Other *S. aureus* wall proteins that contain repeated domains with potential wall binding properties have been described. These include the clumping factors A and B (ClfAB) (70, 135), several serine-aspartate repeat proteins (SdrCDE) (93), the homologue of *S. gordonii* GspB (SasA) (165) (see "Covalent attachment to the cell wall" below), and an extracellular matrix-binding protein homologue (34). Although not documented in the literature, additional proteins with Sec-type signal peptides and potential cell wall binding repeats can be recognized readily. These are the cell wall surface anchor family protein SACOL2505 and the methicillin-resistant surface protein SACOL0050, which both contain C-terminal repeat regions of ~130 amino acids. The latter protein, which shows a high degree of sequence similarity to the SACOL2505 protein, is found only in *S. aureus* COL, not in the five other sequenced strains. This is due to the fact that the gene for SACOL0050 is localized on *mec* cassette 1 and therefore not present in the other strains. Notably, the SACOL2505 homologues in *S. aureus* Mu50 and N315 seem to lack the C-terminal part of the protein with the repeats. A close inspection of the sequences of the corresponding genes in these strains revealed that there is a frameshift mutation or sequencing error in these genes, resulting in an apparent or real C-terminal truncation of the corresponding proteins. Thus, the C-terminal cell wall binding repeats are absent or appear to be absent. Interestingly, most of the aforementioned proteins with cell wall binding motifs also contain the motif LPXTG for covalent attachment to the cell wall by sortase A or sortase B (see below).

It should be noted that a variety of known cell wall binding domains, such as the choline binding domain (205), the Cpl-7 cell wall binding domain (59), and the fructosyltransferase cell wall binding domain (81, 122, 151), appear to be absent from staphylococcal proteins.

Covalent attachment to the cell wall. Cell wall sorting proteins, known as sortases, exist in many gram-positive bacteria and serve to anchor proteins that are destined for cell surface display to the cell wall (50, 182). In almost all gram-positive bacteria, there is at least one sortase present, and often genes for more than one sortase-like protein can be detected in a single genome. These transpeptidases catalyze the formation of an amide bond between the carboxyl group of a Thr and the free amino end of pentaglycine cross bridges in peptidoglycan precursors. Subsequently, the peptidoglycan precursors with covalently bound proteins are incorporated into the cell wall. More recently, it was shown that sortases can also be involved in protein polymerization, leading to the assembly of pili on the surfaces of gram-positive bacteria, such as *Corynebacterium diphtheriae* (61, 182). The three-dimensional structure of sortase A (SrtA) of *S. aureus* revealed that this protein has a unique β-barrel structure in which a catalytic Cys residue is positioned close to a His residue. This suggests that sortase A forms a thiolate-imidazolium ion pair for catalysis (83, 183). Furthermore, it has been shown that a conserved Arg residue is needed for efficient catalysis (112). The catalytic cysteine is part of a conserved motif, TLXTC, which can be found in the C-terminal part of the protein (X is usually Val, Thr, or Ile). Recently, a classification of sortases was proposed by Dramsi et al. (50), based on phylogenetic analyses of 61 sortases that are encoded by the genomes of 22 gram-positive bacteria. These analyses showed that sortases can be grouped into four different classes (A to D). Class A consists of sortases from many low-GC% gram-positive bacteria, including *L. monocytogenes*, *Streptococcus pyogenes*, and *S. aureus*. The second class (class B) is present in only a few low-GC% gram-positive bacteria, including *L. monocytogenes*, *B. anthracis*, and *S. aureus*. Sortases of this class contain three amino acid segments that are not present in the sortases of class A. These sortases recognize a different motif (NPQTN in *S. aureus*). The genes for substrates of class B sortases are often found at the same locus as the sortase gene. The largest class (class C) consists of sortases from high-GC% and low-GC% gram-positive bacteria. The genes for class C sortases are often present in multiple copies per genome. The characteristics of this class of sortases include a C-terminal hydrophobic domain that might serve as a membrane anchor and a conserved proline residue behind the catalytic site. Finally, class D sortases are present in high- and low-GC% gram-positive bacteria. This class can be divided into three subclusters, depending on whether the sortases are present in bacilli, clostridia, or actinomycetes. Since class C and D sortases are absent from *S. aureus*, the (potential) substrates of these enzymes are not reviewed here.

(i) Sortase A recognition signal. For interaction with host cells during infection, many proteins are anchored to the cell

walls of staphylococcal cells, thereby enabling the cells to adhere to and invade the host cells or to evade the immune system. Many of these proteins contain an LPXTG motif in their C-terminal part, which is recognized by the cell wall sorting protein sortase A. In each of the six sequenced *S. aureus* strains, there is only one sortase gene present, which encodes a class A sortase. The LPXTG motif of sortase A substrates is followed by a stretch of hydrophobic amino acids and at least one positively charged amino acid (Lys or Arg) at the C terminus. After protein translocation across the membrane, the LPXTG motif is recognized by SrtA and subsequently cleaved between the Thr and Gly residues (115, 131). Transpeptidation is then mediated by SrtA through amide linkage of the C-terminal Thr of the protein to pentaglycine cross bridges. It has been suggested that SrtA actually uses lipid II as a peptidoglycan substrate and that the proteins linked to lipid II are subsequently incorporated into the cell wall. In addition to the canonical LPXTG motif, an LPXAG motif can also be recognized and cleaved by SrtA (156).

It has been reported that *S. aureus* has 19 proteins that carry the LPXTG motif and 2 proteins that carry the LPXAG motif at their C termini (156). Many of these proteins have been shown to be expressed. These include protein A (Spa), two clumping factors (ClfA and ClfB; also contain potential wall binding repeats), a collagen-binding protein (Cna), three serine-aspartate repeat proteins (SdrC, SdrD, and SdrE; also contain potential wall binding repeats), two fibronectin-binding proteins (FnbpA and FnbpB) (reviewed by Foster and Hook [58]), a plasmin-sensitive protein (Pls) (163), FmtB (98), and several *S. aureus* surface (Sas) proteins (156). A recent study on the cell wall and membrane proteome by Nandakumar et al. (127) resulted in the identification of two proteins with an LPXTG cell wall sorting signal. Many of the LPXTG-containing proteins contain a conserved motif, (YF)-SIRK (with some variance), in their N-terminal signal peptides, which has also been observed for other proteins that are substrates for SrtA in several gram-positive bacteria (9). However, this sequence is not found in all SrtA substrates and can also be found in non-cell-wall proteins. This suggests that (YF)-SIRK is not a specific SrtA targeting sequence. One of the *sas* genes, *sasA*, is situated in the *secA2*/*secY2* cluster and has an unusually long signal peptide (90 residues), which might indicate that the accessory SecA2/SecY2 system is needed for the transport of SasA across the membrane. If so, this would be similar to the case reported for the cell wall-bound GspB protein of *S. gordonii* (13).

Depending on the sequenced *S. aureus* strain, 10 to 13 proteins with an LPXTG cell wall sorting signal followed by a hydrophobic stretch of residues and a positively charged C terminus can be found (Table 10). Among these proteins are fibrinogen-binding protein A (ClfA), the immunoglobulin G binding protein A precursor (Spa), and the Ser-Asp-rich, fibrinogen-binding bone sialoprotein-binding protein (SdrC). Five additional proteins (SdrD, SdrE, SasC, FnbA, and FnbB) with an LPXTG motif can be found among the *S. aureus* strains (Table 10). These five proteins were excluded from our initial list because the corresponding SignalP scores were lower than our (high) score criterion. However, since some of the domains present in these proteins (besides the LPXTG motif) are conserved in well-described cell wall proteins, they were included in Table 10. The remaining proteins with a cell wall sorting signal either are missing in one or more *S. aureus* strains or have been annotated wrongly. Interestingly, *S. epidermidis* ATCC 12228 contains a gene for a class C sortase (*srtC*) which seems to be absent from other staphylococci. This SrtC protein is most closely related to sortases of *L. lactis* and *Streptococcus suis*. Two proteins with LPXTG motifs which are encoded by the same genomic island as SrtC also seem to be strain specific (63).

(ii) Sortase B recognition signal. All sequenced *S. aureus* strains contain sortase B (SrtB) in addition to SrtA. The gene for SrtB is situated at a locus which is involved in the uptake of heme iron (116). This locus also contains the gene for the cell wall protein IsdC, which contains the SrtB recognition sequence NPQTN. In addition, this locus contains the genes for the SrtA substrates IsdA and IsdB, which both contain LPXTG motifs. Notably, IsdC is so far the only protein known to be anchored to the cell wall by sortase B. IsdC is cleaved by SrtB between the Thr and Asn residues of the NPQTN motif. The only other *S. aureus* protein with a motif that resembles NPQTN is the DNA-binding protein II, but this protein is probably not cell wall bound because it lacks a signal peptide for export from the cytoplasm.

COMPARATIVE SECRETOME ANALYSIS

Comparison of the predicted secretomes of *S. aureus* and *S. epidermidis* with those of *B. subtilis* and other gram-positive bacteria revealed that most of the known components of the translocation machinery are present in *S. aureus*. The most notable differences are the second set of *secA* and *secY* genes in *S. aureus*, the absence of known signal peptide peptidases from *S. aureus* and *S. epidermidis*, the absence of a BdbC homologue from *S. aureus* and *S. epidermidis*, the presence of a second *lspA* gene in *S. epidermidis*, the absence of a Tat system from *S. epidermidis*, and the absence of two potential components in the ESAT-6 pathway from *S. aureus* MRSA252 (Table 3). So far, no evidence has been published on whether SecA2 and SecY2 are involved in the export of important virulence factors in *S. aureus*. However, it has been shown that the second *secA*/*secY* set is involved in the export of virulence factors in other pathogens (172). Although most known determinants for protein export, processing, and posttranslocational modification in other gram-positive bacteria are also present in *S. aureus*, in many cases it remains to be investigated to what extent they are necessary for protein export in general and the export of virulence factors in particular.

As shown by multiple BLAST comparisons, the core exoproteome of the sequenced *S. aureus* strains consists of 58 proteins (Table 6). All of these proteins have a signal peptide with a potential SpsB recognition and cleavage site. Thirty-three of these core exoproteins have already been identified in the extracellular milieus and/or membrane/cell wall proteomes of different *S. aureus* isolates (127, 208). Interestingly, 26 core exoproteins of *S. aureus* are also conserved in *S. epidermidis*, suggesting that they belong to a core staphylococcal exoproteome, which is presently still poorly defined. Interestingly, the core exoproteome of *S. aureus* seems to be largely composed of enzymes, such as proteases, and other factors, such as fibrinogen- and IgG-binding proteins, that are required for life in

TABLE 10. Staphylococcal proteins with (potential) Sec-type signal peptides and (potential) signals for covalent cell wall binding

Identification no.	Protein	Function	Signal
15925728	SA0022	Hypothetical protein, similar to 5′-nucleotidase	LPKTG
15925815	Spa	Immunoglobulin G binding protein A precursor	LPETG
15925838	SasD	Hypothetical protein	LPAAG
15926239[a]	SdrC	Ser-Asp-rich, fibrinogen-binding bone sialoprotein-binding protein	LPETG
15926240[b,c]	SdrD	Ser-Asp-rich, fibrinogen-binding bone sialoprotein-binding protein	LPETG
15926241[a,c]	SdrE	Ser-Asp-rich, fibrinogen-binding bone sialoprotein-binding protein	LPETG
15926464	ClfA	Fibrinogen-binding protein A, clumping factor	LPDTG
15926713	IsdB	Conserved hypothetical protein	LPKTG
15926714	IsdA	Cell surface protein	LPKTG
15926715	IsdC	Conserved hypothetical protein	NPQTN
15927308[b]	HarA	Hypothetical protein	LPKTG
15927333[c,d]	SasC	Hypothetical protein, similar to FmtB protein	LPNTG
15927741[a,e]	SasB	FmtB protein	LPDTG
15928076[a,b,f]	Aap	Hypothetical protein, similar to accumulation-associated protein	LPKTG
15928081[b,c]	FnbB	Fibronectin-binding protein homolog	LPETG
15928082[c]	FnbA	Fibronectin-binding protein homolog	LPETG
15928174[g]	SasK	Hypothetical protein	LPKTG
15928216	ClfB	Clumping factor B	LPETG
15928232[a]	SasF	Conserved hypothetical protein	LPKAG
15928240[h]	SasA	Hypothetical protein, similar to streptococcal hemagglutinin protein	LPDTG
57652419[i]	Pls	Methicillin-resistant surface protein	LPDTG
21284341[j]	Cna	Collagen adhesin precursor	LPKTG
27467746[k]	SE0828	Lipoprotein VsaC	LPETG
27468418[k]	SE1500	Hypothetical protein	LPKTG
27468419[k]	SE1501	Hypothetical protein	LPNTG
27468546[k]	SE1628	Hypothetical protein	LPETG
27469070[k]	SE2152	Hypothetical protein	LPNTG

[a] Also present in *S. epidermidis*.
[b] The genes encoding SdrC, HarA, and FnbB are not present in *S. aureus* MRSA252.
[c] Has a lower SignalP score than our threshold score.
[d] Truncated in *S. aureus* Mu50, thereby missing the C-terminal part containing the LPXTG motif.
[e] The gene encoding SasB is not present in *S. aureus* MRSA252 and MSSA476 and is truncated in *S. aureus* MW2.
[f] Truncated in *S. aureus* Mu50 and N315, thereby missing the C-terminal part containing the LPXTG motif.
[g] The gene encoding SasK is not present in *S. aureus* COL, MRSA252, and MSSA476 and is truncated in *S. aureus* MW2, thereby missing the N-terminal signal peptide.
[h] Contains an unusually long signal peptide (90 amino acids).
[i] The gene encoding Pls is present only in *S. aureus* COL.
[j] The gene encoding Cna is not present in *S. aureus* COL, Mu50, and N315.
[k] Present only in *S. epidermidis*.

the ecological niches provided by the human host (Table 6). This is particularly true for the proteins that have the potential to be covalently bound to the cell wall (Table 10). In contrast, the variant exoproteome of *S. aureus* contains most of the known staphylococcal toxins and immunomodulating factors (Tables 7 and 11). This suggests that the components of the variant exoproteome should be regarded as specific gadgets of *S. aureus* that help this organism to conquer certain protected niches of the human host, thereby causing disease. If this idea is correct, then proteins of unknown function that belong to the variant exoproteome should be regarded as potentially important virulence factors.

The (predicted) extracellular toxins of *S. aureus* are not present in *S. epidermidis*. This is mainly due to the fact that these toxins are encoded by pathogenicity islands in the genomes of *S. aureus* strains that have thus far not been observed in *S. epidermidis* genomes. Proteins with predicted signal peptides that are specific for *S. epidermidis* are listed in Table 12. Notably, the majority (i.e., 26 of 30) of predicted *S. epidermidis* exoproteins that have homologues in *S. aureus* share this homology with components of the core exoproteome of *S. aureus* (Tables 6 and 7). This suggests that in *S. epidermidis*, the core exoproteome is also involved in housekeeping functions. In contrast to the case for the exoproteome, it is presently difficult to speculate about housekeeping and disease-causing roles of the constant and variant lipoproteomes of *S. aureus*. This is due to the fact that the functions of only a few *S. aureus* lipoproteins are known (Tables 8, 9, and 13). In general terms, it is presently not clear why *S. epidermidis* seems to export a smaller number of different proteins (101 in total) than does *S. aureus* (~135 in total). This difference is all the more remarkable since the total numbers of proteins encoded by the genomes of *S. aureus* (~2,600) and *S. epidermidis* (~2,500) are comparable.

Compared to *B. subtilis* and *B. licheniformis* (193), *S. aureus* is also predicted to export a relatively large number of proteins from the cytoplasm to the membrane-cell wall interface, the cell wall, and the extracellular milieu. The genomes of *B. subtilis* and *B. licheniformis* contain ~4,100 protein-encoding genes, while *S. aureus* genomes contain significantly fewer genes (~2,600). Using the most recent prediction protocols (179), *B. subtilis* is predicted to export 190 proteins to an extracytoplasmic location, whereas, depending on the strain investigated, *S. aureus* is predicted to export 130 to 145 proteins (this review). Accordingly, as judged by the relative numbers of protein-encoding genes, *S. aureus* strains appear to

TABLE 11. Composition of the variant exoproteome of sequenced *S. aureus* strains

Identification no.	Protein	Function	Presence of protein in strain[a]					
			COL	MRSA252	MSSA476	Mu50	MW2	N315
15925799	Plc	1-Phosphatidylinositol phosphodiesterase precursor	Y	Y	N	Y	Y	Y
15926099	Set6	Exotoxin 6	N	N	N	Y	N	Y
15926100	Set7	Exotoxin 7	Y	N	Y	Y	Y	Y
15926101	Set8	Exotoxin 8	N	N	Y	Y	Y	Y
15926103	Set10	Exotoxin 10	N	Y	Y	Y	Y	Y
15926104	Set11	Exotoxin 11	N	Y	Y	Y	Y	Y
15926105	Set12	Exotoxin 12	N	N	Y	Y	Y	Y
15926106	Set13	Exotoxin 13	Y[b]	Y	Y	Y	Y	Y
15926111	Set15	Exotoxin 15	N	N	N	Y	N	Y
15926113	SA0395	Hypothetical protein	Y	Y	Y	N	Y	Y
15926465	SA0743	Hypothetical protein	Y	N	Y	Y	Y	Y
15926739	SA1001	Hypothetical protein	Y	N	Y	Y	Y	Y
15926746	HlY	α-Hemolysin precursor	Y	N	Y	Y	Y	Y
15927016	EbhB	Hypothetical protein	Y	Y	N	Y	Y	Y
15927308	Fhs	Hypothetical protein	Y	N	Y	Y	Y	Y
15927386	SplB	Serine protease SplB	Y	N	Y	Y	Y	Y
15927387	SplA	Serine protease SplA	Y	N	Y	Y	Y	Y
15927389	SA1633	Probable β-lactamase	N	N	N	Y	N	Y
15927393	LukD	Leukotoxin, LukD	Y	N	Y	Y	Y	Y
15927398	SEG	Extracellular enterotoxin type G precursor	N	Y	N	Y	N	Y
15927399	SEN	Enterotoxin SEN	N	Y	N	Y	N	Y
15927402	SEI	Extracellular enterotoxin type I precursor	N	Y	N	Y	N	Y
15927404	SEO	Enterotoxin SEO	N	Y	N	Y	N	Y
15927512	Map	Truncated MapW protein	Y	Y	N	Y	Y	Y
15927513	Hlb	β-Hemolysin/phospholipase C	Y	N	N	Y	Y	Y
15927516	SA1754	Hypothetical protein	N	Y	Y	Y	Y	Y
15927517	SA1755	Hypothetical protein	N	Y	N	N	N	Y
15927520	Sak	Staphylokinase precursor	N	Y	Y	Y	Y	Y
15927522	SA1760	Hypothetical protein	N	Y	Y	Y	Y	Y
15927585	SEC3	Enterotoxin type C3	Y	Y	N	Y	Y	Y
15927586	SA1818	Hypothetical protein	Y	N	N	Y	Y	Y
15927587	TSST-1	Toxic shock syndrome toxin 1	N	N	N	Y	N	Y
15927741	FmtB	FmtB protein	Y	N	N	Y	Y	Y
15928076	SA228	Hypothetical protein	Y	N	Y	Y	Y	Y
15928174	SA2381	Hypothetical protein	N	N	Y	Y	Y	Y
15928182	SA2389	Hypothetical protein	N	N	N	Y	N	Y
16119219	SAP019	Hypothetical protein	N	Y	Y	N	N	Y
14141830	SAVP008	Hypothetical protein	N	N	N	Y	N	N
21282111	Set16	Hypothetical protein	N	Y	Y	N	Y	N
21282116	Set21	Hypothetical protein	N	N	Y	N	Y	N
21282123	Set26	Hypothetical protein	N	Y	Y	N	Y	N
21283107	LukF	Panton-Valentine leukocidin chain F precursor	N	N	N	N	Y	N
21283108	LukS	Panton-Valentine leukocidin chain S precursor	N	N	N	N	Y	N
21284341	Cna	Collagen adhesin precursor	N	Y	Y	N	Y	N
49482651	SAR0423	Exotoxin	N	Y	N	N	N	N
49482652	SAR0424	Exotoxin	N	Y	N	N	N	N
49482653	SAR0425	Exotoxin	N	Y	N	N	N	N
49482925	SAR0721	Multicopper oxidase protein	N	Y	N	N	N	N
49484047	SAR1886	Putative exported protein	N	Y	N	N	N	N
49484059	SAR1905	Serine protease	N	Y	N	N	N	N
49484898	SAR2788	Putative exported protein	N	Y	N	N	N	N
49484957	SEH	Enterotoxin H	N	N	Y	N	Y	N
49485293	SAS0389	Exotoxin	N	N	Y	N	Y	N
57650600	SACOL1865	Serine protease SplE, putative	Y	Y	N	N	N	N
57650605	SACOL1870	Hypothetical protein	Y	N	Y	N	Y	N
57650609	EpiP	Epidermin leader peptide processing serine protease EpiP	Y	N	Y	N	Y	N
57651309	SACOL0468	Exotoxin 3, putative	Y	N	N	N	N	N
57651311	SACOL0470	Exotoxin, putative	Y	N	N	N	N	N
57651319	SACOL0478	Exotoxin 3, putative	Y	N	N	N	N	N
57652419	Pls	Methicillin-resistant surface protein	Y	N	N	N	N	N

[a] Y, yes; N, no.
[b] This protein has a Thr at the +1 position, which is not included in the SpsB recognition and cleavage pattern. All other *S. aureus* strains conform to the proposed pattern.

TABLE 12. Specific *S. epidermidis* proteins with predicted signal peptides

Signal peptide type	Identification no.	Protein	Amino acid at position:					Function
			−3	−2	−1	+1	+2	
Sec	27466926[a]	SE0008	T	Y	A	S		Truncated β-hemolysin
	27467163	SE0245	V	H	A	A		Triacylglycerol lipase precursor
	27467176	SE0258	A	K	A	Q		Immunodominant antigen B
	27467249[b,c]	SE0331	A	K	A	E		Ser-Asp-rich, fibrinogen-binding bone sialoprotein-binding protein
	27467501[a]	SE0583	S	F	A	N		Hypothetical protein
	27467545[a]	SE0627	T	L	A	D		Poly-D-alanine transfer protein
	27467746[b]	SE0828	A	H	A	E		Lipoprotein VsaC
	27467938[a]	SE1020	I	K	A	Q		Hypothetical protein
	27468418[b]	SE1500	S	Y	A	Q		Hypothetical protein
	27468493	SE1575	A	Q	A	H		Immunodominant antigen B
	27468546[b]	SE1628	V	Y	A	D		Hypothetical protein
	27468801[a]	SE1883	V	S	A	K		Lyt divergon expression attenuator LytR
	27468838	SE1920	V	Y	A	Q		Hypothetical protein
	27469060[a]	SE2142	T	L	A	F		2-Dehydropantoate 2-reductase
	27469070[b]	SE2152	T	H	A	A		Hypothetical protein
	27469115[a]	SE2197	S	Y	A	S		Alkaline phosphatase III precursor
	27469119	SE2201	A	D	A	Z		Phage-related protein
	27469291	SE2373	A	Q	A	S		1,4-β-*N*-Acetylmuramidase
	27469316	SE2398	S	S	A	S		Hypothetical protein
	32470521	P601	A	S	A	S		Hypothetical protein
	32470527	P607	I	N	A	D		Hypothetical protein
	32470549[b]	P517	A	K	A	E		Hypothetical protein
	32470583	P202	T	F	A	L		Hypothetical protein
Lipoprotein	27466952	SE0034	L	S	A	C	S	Hypothetical protein
	27467000	SE0082	L	T	A	C	G	Hypothetical protein
	27467062	SE0144	V	S	G	C	G	Hypothetical protein
	27467063	SE0145	V	S	G	C	G	Hypothetical protein
	27467067[d]	SE0149	L	A	G	C	D	Hypothetical protein
	27467309[d]	SE0391	L	T	T	C	S	Hypothetical protein
	27468024	SE1106	V	T	A	C	S	ABC transporter
	27468425[d]	SE1507	L	Y	G	C	G	Hypothetical protein
	27469012[d]	SE2094	L	I	I	C	S	Hypothetical protein
	27469069[d]	SE2151	L	A	G	C	G	Hypothetical protein
	27469130	SE2212	V	S	G	C	S	Hypothetical protein
	27469141[d]	SE2223	L	G	S	C	S	Hypothetical protein
	27469317	SE2399	L	S	A	C	G	Hypothetical protein

[a] All *S. aureus* strains have one or more residues in the cleavage site which are not included in the search pattern.
[b] Contains an LPXTG motif.
[c] Has a lower SignalP score than our threshold score for all *S. aureus* strains.
[d] Excluded for *S. epidermidis* because of the motif proposed by Sutcliffe and Harrington (171).

export 6 to 20% more proteins to an extracytoplasmic location than do the aforementioned bacilli. Most probably, this is related to the fact that *S. aureus* needs an arsenal of virulence factors, such as toxins and surface proteins, for colonization of and survival in its preferred niches in the human host. Such proteins are of less importance for soil bacteria, such as *B. subtilis* and *B. licheniformis*, which thrive predominantly on dead organic matter.

PERSPECTIVES

The present review provides a survey of possible protein transport pathways in staphylococcal pathogenesis. In many cases, the knowledge gathered from protein secretion studies in other organisms has been projected on *S. aureus*, assuming that similar pathways or pathway components have similar functions in different organisms. Clearly, this leaves room for surprises when such pathways are investigated thoroughly for *S. aureus*. The same was true for studies on protein secretion in *B. subtilis*. These studies showed, for example, that the absence of SecDF has barely any consequences for protein secretion by *B. subtilis*, whereas SecD and SecF are of key importance for protein translocation in *E. coli*, the organism in which SecD/F was first discovered (20). Likewise, LspA was shown to be dispensable in *B. subtilis* but not in *E. coli* (147, 177). Thus, the relative importance of different secretion machinery components of *S. aureus* needs to be assessed in a systematic manner, preferably in an isogenic background. Such studies would need to address the importance of secretion machinery components for in vitro growth on different substrates (e.g., broth or blood) and for virulence in vivo in model systems (e.g., *C. elegans*, *Drosophila melanogaster*, mice, or rats) (8, 60, 173). These studies should be complemented with proteomic verification of our present lipoproteome, wall proteome, and exoproteome predictions. Such a verification could involve both gel-based proteomics approaches, as outlined in this review, and more sophisticated gel-free proteomics approaches (194). This would lead to an improved understanding of the contribution

TABLE 13. Composition of the variant lipoproteome of sequenced *S. aureus* strains

Identification no.	Protein	Function	Presence of protein in strain[a]					
			COL	MRSA252	MSSA476	Mu50	MW2	N315
15925801	SA0093	Hypothetical protein	N	N	N	Y	N	Y
15925803	SA0095	Hypothetical protein	N	N	N	Y	N	Y
15925804	SA0096	Hypothetical protein	Y	N	N	Y	N	Y
15925877	SA0167	Hypothetical protein	N	Y	Y	Y	Y	Y
15926004	SA0291	Hypothetical protein	Y	N	Y	Y	Y	Y
15926114	Lpl1	Hypothetical protein	Y	Y	N	Y	N	Y
19526115	Lpl2	Hypothetical protein	N	N	N	Y	N	Y
15926116	Lpl3	Hypothetical protein	Y	N	N	Y	N	Y
15926118	Lpl4	Hypothetical protein	N	N	N	Y	N	Y
15926119	Lpl5	Hypothetical protein	N	N	N	Y	N	Y
15926120	Lpl6	Hypothetical protein	N	N	Y	Y	Y	Y
15926122	Lpl8	Hypothetical protein	Y	Y	N	Y	N	Y
15926123	Lpl9	Hypothetical protein	Y	Y	Y	Y	N	Y
15926465	SA0743	Hypothetical protein, similar to staphylocoagulase precursor	Y	N	Y	Y	Y	Y
15926580	SA0850	Hypothetical protein, similar to oligopeptide ABC transporter oligopeptide-binding protein	Y	N	Y	Y	Y	Y
15927067	SA1317	Hypothetical protein	N	N	N	Y	N	Y
15927068	SA1318	Hypothetical protein	N	N	Y	Y	Y	Y
15927069	SA1319	Hypothetical protein	N	Y	Y	Y	Y	Y
15927373	SA1617	Hypothetical protein, similar to latent nuclear antigen	Y	Y	Y	Y	Y	Y
15927396	SA1640	Conserved hypothetical protein	N	N	N	Y	N	Y
15927859	ModA	Probable molybdate-binding protein	Y	Y	Y	N	Y	Y
15928064	SA2273	Hypothetical protein	Y	N	N	Y	N	Y
21281801	MW0072	Hypothetical protein	Y	N	Y	N	Y	N
21282126	Lpl10	Hypothetical protein	Y	Y	Y	N	Y	N
21282127	Lpl11	Hypothetical protein	N	Y	Y	N	Y	N
21282129	Lpl13	Hypothetical protein	N	N	Y	N	Y	N
21283103	MW1374	Conserved hypothetical protein	N	N	Y	N	Y	N
21283167	MW1438	Hypothetical protein	N	Y	Y	N	Y	N
21284135	MW2406	Hypothetical protein	N	N	Y	N	Y	N
21284306	MW2577	Hypothetical protein	N	N	Y	N	Y	N
49482670	SAR0445	Putative lipoprotein	N	Y	N	N	N	N
49483993	BlaZ	β-Lactamase precursor	N	Y	N	N	N	N
49484246	SAR2104	Putative lipoprotein	N	Y	N	Y	N	N
49484287	SAR2149	Putative exported protein	N	Y	N	N	N	N
49484977	SAS0074	Putative lipoprotein	N	N	Y	N	N	N
57650161	SACOL0888	Pathogenicity island, putative lipoprotein	Y	N	N	N	N	N
57650441	SACOL1528	Hypothetical protein SA1528	Y	Y	N	N	N	N
57650444	SACOL1531	Hypothetical protein SA1531	Y	N	N	N	N	N
57650485	SACOL1574	Hypothetical protein SA1574	Y	Y	N	N	N	N
57650696	SACOL2010	Iron compound ABC transporter, iron compound-binding protein	Y	N	Y	Y	Y	N
57651323	SACOL0482	*Staphylococcus* tandem lipoprotein	Y	N	N	N	N	N
57652445	SACOL0081	Hypothetical protein SA0081	Y	N	N	N	N	N
14141829	SAVP006	Hypothetical protein	N	N	N	Y	N	N

[a] Y, yes; N, no.

of each protein transport pathway and its substrate proteins to staphylococcal cell physiology and virulence. Since the virulence of different *S. aureus* strains will depend not only on the presence (or absence) of particular genes for virulence factors but also on their expression, such proteomic studies should also include experiments on the impacts of major regulatory systems, such as *agr*, *sae*, and *sarA*. On this basis, it should be possible to identify the most promising candidate drug targets in the staphylococcal secretome. Alternatively, secretome components thus identified could represent promising candidates for novel vaccines. For all of these efforts, comparative secretomics approaches will provide essential information on those potential drug targets that are most important for both bacterial housekeeping functions and virulence. Novel drugs and vaccines designed against these targets are likely to have the highest impact (65).

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