JB
Journals.ASM.org

Type I Signal Peptidase and Protein Secretion in *Staphylococcus aureus*

Mark A. Schallenberger,[a] Sherry Niessen,[b] Changxia Shao,[a] Bruce J. Fowler,[a] and Floyd E. Romesberg[a]

Department of Chemistry[a] and The Center for Physiological Proteomics[b] at The Scripps Research Institute, La Jolla, California, USA

Staphylococcus aureus **is an important human pathogen whose virulence relies on the secretion of many different proteins. In general, the secretion of most proteins in *S. aureus*, as well as other bacteria, is dependent on the type I signal peptidase (SPase)-mediated cleavage of the N-terminal signal peptide that targets a protein to the general secretory pathway. The arylomycins are a class of natural product antibiotics that inhibit SPase, suggesting that they may be useful chemical biology tools for characterizing the secretome. While wild-type *S. aureus* (NCTC 8325) is naturally resistant to the arylomycins, sensitivity is conferred via a point mutation in its SPase. Here, we use a synthetic arylomycin along with a sensitized strain of *S. aureus* and multidimensional protein identification technology (MudPIT) mass spectrometry to identify 46 proteins whose extracellular accumulation requires SPase activity. Forty-four possess identifiable Sec-type signal peptides and thus are likely canonically secreted proteins, while four also appear to possess cell wall retention signals. We also identified the soluble C-terminal domains of two transmembrane proteins, lipoteichoic acid synthase, LtaS, and *O*-acyteltransferase, OatA, both of which appear to have noncanonical, internal SPase cleavage sites. Lastly, we identified three proteins, HtrA, PrsA, and SAOUHSC_01761, whose secretion is induced by arylomycin treatment. In addition to elucidating fundamental aspects of the physiology and pathology of *S. aureus*, the data suggest that an arylomycin-based therapeutic would reduce virulence while simultaneously eradicating an infection.**

The Gram-positive bacterium *Staphylococcus aureus* is a growing threat as a community-acquired pathogen and a leading cause of nosocomial infections worldwide, creating a large economic and public health burden. *S. aureus* can infect diverse tissues due to a variety of secreted protein virulence factors that are thought to facilitate adhesion and colonization, promote dissemination to other tissues, evade the host immune system, and scavenge nutrients and minerals from the host environment (24, 73, 99, 114). The majority of proteins destined for export out of the cytoplasm are synthesized as preproteins with N-terminal signal sequences that target them to the general secretory (Sec) pathway (25, 27, 93) but ultimately must be proteolytically removed by type I signal peptidase (SPase) (76). The proteins processed by SPase likely constitute the majority of the *S. aureus* secretome, and their identification would further our understanding of SPase, as well as our understanding of this pathogen, how it causes disease, and how such diseases may be most effectively treated.

There has been considerable effort directed toward defining the *S. aureus* secretome (9, 14, 17, 36, 37, 54, 55, 71, 79, 99, 117–119). Some of the earliest efforts were computational approaches that relied on identifying conserved features of Sec-type N-terminal signal sequences. Detailed analysis suggests that canonical signal sequences typically comprise the first 25 to 32 amino acids of the preprotein and that they have a tripartite organization, with a positively charged N terminus, followed by a hydrophobic portion that commonly terminates with a Gly or Pro, and finally a C terminus that includes the SPase recognition site (76). The recognition site itself consists of small, aliphatic residues at positions −1 and −3 relative to the scissile bond, which are both most commonly Ala. Nonetheless, the general prediction of signal peptides remains challenging, in part due to idiosyncratic sequences (3, 4, 40, 61, 83, 103). Moreover, this approach can identify only proteins that have the potential to be secreted, and extrapolation to the actual secretome is complicated by variable levels of transcription and/or translation, as well as the presence of poorly understood and difficult-to-predict cell wall retention signals (11, 40, 105).

In principle, the secretome could be defined experimentally by identifying proteins found in the media. However, many proteins isolated from the media are cytoplasmic and/or do not possess signal peptide sequences, suggesting that their presence may result from the cell lysis that unavoidably accompanies bacterial growth (3, 17, 49). To circumvent this challenge, efforts have focused on searching for more direct evidence of secretion. For example, Sibbald et al. demonstrated a role for SecG, a nonessential component of the secretory channel, in the secretion of 11 proteins (98). Alternatively, efforts have focused on comparing the N-terminal sequences of proteins found in the media to their corresponding gene sequences to identify those that appear to have been processed by SPase (20, 36, 84, 99, 104, 110). However, this approach relies on the accurate prediction of signal peptides, does not directly demonstrate the participation of SPase, and is complicated by additional proteolysis, which is common with secreted proteins and which can remove the evidence of SPase activity.

The arylomycins are a class of natural product antibiotics isolated in 2002 and subsequently shown to inhibit *Escherichia coli* SPase *in vitro* (41, 95). We have synthesized a variety of arylomycins, including the derivative arylomycin A-C_{16} (Fig. 1) (formerly referred to as arylomycin C_{16}) (88), and found that they are potent and selective inhibitors of SPase *in vivo*. The antibacterial mechanism of action of the arylomycins suggests that in addition to

Received 14 January 2012 **Accepted** 12 March 2012
Published ahead of print 23 March 2012
Address correspondence to Floyd E. Romesberg, floyd@scripps.edu.
Supplemental material for this article may be found at http://jb.asm.org/.

doi:10.1128/JB.00064-12

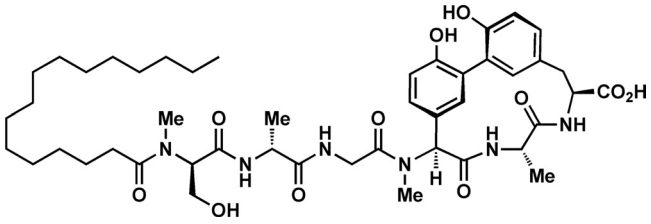

FIG 1 Structure of arylomycin A-C_{16}.

being promising antibiotics, they may also reduce virulence during therapy by inhibiting the secretion of virulence factors. This is in stark contrast to antibiotics that can actually exacerbate virulence and pathogenicity by inducing the production and secretion of virulence factors (15, 35, 58, 75, 78, 97). Moreover, while the arylomycins are active against many bacteria, the activity is bacteriostatic for stationary-phase cells exposed over short periods of time. Thus, the arylomycins should also be valuable chemical biology probes of protein secretion. Indeed, we previously used arylomycin A-C_{16} and two-dimensional (2D) gel electrophoresis coupled with liquid chromatography-tandem mass spectrometry (LC-MS/MS) to profile the secretome of *Staphylococcus epidermidis* (82). Although some human pathogens, including *S. aureus*, are not naturally sensitive to the arylomycins, they may be rendered sensitive by a single mutation in SPase (101), and the availability of the corresponding strains should now make possible the characterization of their secretomes as well.

Here, we use the arylomycin-sensitized *S. aureus* strain PAS8001 [NCTC 8325 SpsB(P29S)] (101), arylomycin A-C_{16}, and multidimensional protein identification technology (MudPIT) mass spectrometry to identify the *S. aureus* proteins whose secretion into the media during stationary-phase growth relies upon SPase activity. Our data suggest that arylomycin A-C_{16} directly inhibits the secretion of 40 proteins, as well as the release from the cell surface of an additional four. Interestingly, we also identified two transmembrane proteins that appear to be processed by SPase at internal sites. Finally, in addition to these 46 proteins whose presence in the media depends on SPase activity, we identified three proteins whose expressions appear to be induced by arylomycin treatment, suggesting that they might be part of a secretion stress response.

MATERIALS AND METHODS

Materials. Components for growth media were manufactured by Difco Laboratories (Detroit, MI). Arylomycin A-C_{16} was synthesized as described previously (88).

Bacterial strains. The construction of the SPase mutant strain PAS8001, *S. aureus* NCTC 8325 SpsB(P29S), was reported previously (101). Strains SCXA108, lacking SAOUHSC_01761, and SCXA81, lacking SAOUHSC_01838 (*htrA*), were constructed from strain PAS8001 using the primers listed in Table S1 in the supplemental material and the allelic exchange vector pMAD as described previously (5). Briefly, two fragments of approximately 1-kb regions upstream and downstream of each target gene were amplified from *S. aureus* NCTC 8325 chromosomal DNA using Phusion Hot Start High-Fidelity DNA polymerase (New England BioLabs) and the appropriate gene-specific primer pair (see Table S1). After purification (PureLink PCR purification kit; Invitrogen), the ~2-kb DNA fragments were amplified using Platinum Pfx DNA polymerase (Invitrogen) and the primers usNF and dsCR (see Table S1). The purified PCR DNA fragments and pMAD were treated with BamHI and SalI (New England BioLabs), mixed in a 3:1 molar ratio, and ligated with T4 DNA ligase (New England BioLabs) to form the deletion cassettes pMADΔ1838 and pMADΔ1761. Each deletion cassette was electroporated into *E. coli* XL10-Gold, and transformants were selected on LB agar containing ampicillin (100 μg/ml). Recovered plasmids were then electroporated into *S. aureus* RN4220 and recovered by selection on tryptic soy agar (TSA) containing erythromycin (5 μg/ml) at 30°C. Plasmids were recovered again and then electroporated into *S. aureus* PAS8001. Colonies of cells harboring the desired integration of the construct were identified by blue/white screening on TSA containing 5 μg/ml erythromycin and 250 μg/ml X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) at 30°C and then cultured in antibiotic-free tryptic soy broth (TSB) for 2 h at 30°C followed by 6 h at 42°C. Serial dilutions were plated on TSA containing erythromycin and X-Gal and incubated at 42°C overnight. The resulting blue colonies were grown in TSB at 30°C overnight, followed by plating serial dilutions on TSA with X-Gal. The candidate white colonies resulting from the desired double-crossover event were assayed for erythromycin sensitivity. Lastly, the identified strains were confirmed for correct gene deletion by PCR with oligonucleotide primers located ~50 bp upstream or downstream of the deleted region (see Table S1).

Protein isolation. *S. aureus* PAS8001 was grown on TSA overnight at 37°C and used to inoculate TSB (40 ml) to an optical density at 590 nm (OD_{590}) of 0.1; the resulting culture was then grown with vigorous shaking at 37°C for 5 h to early stationary phase. Saturated cultures were centrifuged (3,000 × *g*, 15 min) and washed with phosphate-buffered saline (PBS) (40 ml) to remove any residual secreted proteins and then resuspended in 40 ml TSB to a density of ~1.5×10^9 CFU/ml. The resulting saturated 40-ml culture was then divided into 10-ml aliquots, and the following concentrations of arylomycin A-C_{16} were added in a fixed volume of dimethyl sulfoxide (DMSO): 0×, 0.5×, 2.0×, and 8.0× MIC (corresponding to 0, 1.0, 4.0, and 16.0 μg/ml, respectively). After incubation with shaking for 1.5 h at 37°C, the optical density was recorded and appropriate dilutions were plated to determine the number of viable cells in each culture. Cells were then collected by centrifugation (3,000 × *g*, 15 min) and discarded. Supernatants containing secreted proteins were clarified by filtration using a 0.22-μm low-protein-binding membrane. Proteins were then precipitated by the addition of 10% (wt/vol) trichloroacetic acid (TCA) followed by an overnight incubation on ice and then harvested by centrifugation (3,000 × *g*, 1 h). The resulting pellets were washed two times with 90% acetone in water and air dried. Three independently prepared samples were characterized. Protein samples were not normalized before further analysis, as the numbers of viable colonies in each sample were identical within error.

Liquid chromatography-MudPIT mass spectrometry. Precipitated proteins were resuspended in an equal volume (50 μl) of 8 M urea 50 mM Tris (pH 8.0). An aliquot (7 μl) of each sample (corresponding to 10 to 20 μg protein) was reduced with 10 mM Tris(2-carboxyethyl) phosphine hydrochloride (TCEP; Sigma) for 30 min at room temperature and then alkylated with 12.5 mM fresh iodoacetamide (IAA; Sigma). The concentration of urea was reduced to 2 M with the addition of 50 mM Tris (pH 8.0). Proteins were digested overnight at 37°C in the presence of 1 mM $CaCl_2$ and 0.2 μg trypsin (1:50 enzyme to substrate). Peptides were acidified to a final concentration of 5% formic acid and centrifuged at 17,000 × *g* for 15 min, and half of the digestion (5 to 10 μg protein) was pressure loaded onto a biphasic (strong cation exchange/reverse phase) capillary column for MudPIT analysis. Peptides were separated and analyzed by 2D LC in combination with MS/MS as previously described using an 11-step gradient (111). Data-dependent MS/MS analysis was performed using an LTQ-Orbitrap XL hybrid mass spectrometer (Thermo Scientific). Full MS spectra were acquired in centroid mode, with a mass range of 400 to 1,800 in the Orbitrap analyzer with resolution set at 30,000 followed by 7 MS/MS scans in the ion trap. Dynamic exclusion was enabled with a repeat count of 1, a repeat duration of 20 s, exclusion duration of 90 s, and an exclusion list size of 300. All tandem mass spectra were collected using a normalized collision energy of 35% and an isolation window of 2 Da. One microscan was applied for all experiments in the

Orbitrap or LTQ. The spray voltage was set to 2.50 kV, and the flow rate through the column was 0.20 μl/min.

Analysis of MS data. RAW files were generated from mass spectra using XCalibur and MS/MS spectra data extracted using RAW Xtractor (version 1.9.1), which is publicly available (http://fields.scripps.edu/downloads.php). MS/MS spectral data were searched using the SEQUEST algorithm (version 3.0) against a custom database containing 25,827 sequences (2,892 *S. aureus* [31] and 22,935 human International Protein Index [IPI] sequences) that were concatenated to a decoy database in which the sequence for each entry in the original database was reversed (28). In total, the search database contained 51,654 protein sequence entries (25,827 real sequences and 25,827 decoy sequences). SEQUEST searches allowed for oxidation of methionine residues (15.99491 Da), static modification of cysteine residues (57.02146 Da; due to alkylation), and no enzyme specificity. As the peak selected for MS/MS analysis by the instrument control software is often not the monoisotopic ion, multiple isotopes, with a 50-ppm mass tolerance for each possible theoretical isotope peak, were also considered part of the search algorithm. The validity of peptide/spectrum matches was assessed using DTASelect2 (version 2.0.27) and three SEQUEST-defined parameters, the cross-correlation score (XCorr), normalized difference in cross-correlation scores (DeltaCN), and DeltaMass, where DeltaMass is the absolute difference between the experimental precursor ion mass and the nearest theoretical isotope peak. The search results were grouped by charge state (+1, +2, +3, +4), tryptic status, and modification status (modified and unmodified peptides), resulting in 24 distinct subgroups. In each of these subgroups, the distribution of Xcorr, DeltaCN, and DeltaMass values for the direct and decoy database hits was obtained, and then the direct and decoy subsets were separated by discriminant analysis. Outlier points in the two distributions were discarded. Full separation of the direct and decoy subsets is not generally possible, so the discriminant score was set such that a false discovery rate of less than 1% was determined based on the number of accepted decoy database peptides (number of decoy database hits/number of filtered peptides identified × 100). This procedure was independently performed on each data subset, resulting in a false discovery rate independent of tryptic status, modification status, or charge state. In addition, a minimum peptide length of seven residues was imposed, and protein identification required the matching of at least two peptides per protein. Such criteria resulted in the elimination of most decoy database hits. In our data set, the identification of nontryptic peptides included half-tryptic peptides from the N and C termini of the identified proteins. Other nontryptic peptides that were identified may represent endogenous activities of cellular proteases or peptides generated by in-source fragmentation. The resulting data files were then imported for statistical analysis using the PatternLab software (19).

qRT-PCR. *S. aureus* PAS8001 was grown and treated with arylomycin A-C_{16} (0, 1, 4, or 8 μg/ml) and then lysed using the RNAprotect bacterial reagent (Qiagen). Lysates were subjected to total RNA isolation with the RNeasy minikit (Qiagen). Total RNA was treated with DNase I (RNase-free; New England BioLabs) and converted to cDNA using the Superscript III first-strand synthesis kit (Invitrogen). The cDNA was subjected to quantitative real-time PCR (qRT-PCR) using *Taq* polymerase (New England BioLabs), SYBR green, and gene-specific primer pairs (see Table S1 in the supplemental material) in an MyiQ real-time detection system (Bio-Rad). Primer pairs were evaluated to be free of secondary products and primer dimers via melt-curve analysis. Relative quantification of each gene in the arylomycin-treated samples compared to the untreated control was done using the standard curve method and normalization relative to 16S RNA. The fold changes observed in treated samples relative to the untreated controls are reported as the averages and standard deviations from 2 to 3 replicates of 2 to 3 independent biological samples.

***In silico* analysis of identified proteins.** SPase cleavage sites, membrane anchor sequences, and transmembrane segments were predicted using SMART (62) (http://smart.embl-heidelberg.de/), TMHMM (56) (http://www.cbs.dtu.dk/services/TMHMM/), LocateP (116) (http://www.cmbi.ru.nl/locatep-db/cgi-bin/locatepdb.py), SignalP 3.0 neural networks (SignalP-NN), and hidden Markov model (SignalP-HMM) algorithms (http://www.cbs.dtu.dk/services/SignalP/) (12), PrediSi (Institute for Microbiology, Technical University of Braunschweig; http://www.predisi.de), and LipoP 1.0 (http://www.cbs.dtu.dk/services/LipoP/) (50). Default settings (for Gram-positive bacteria) for these Web-based programs were used in all cases.

Hemolysin activity. The secretion of δ-hemolysin was evaluated essentially as described, relying on the action of β-hemolysin to enhance the lytic property of δ-hemolysin and inhibit that of α-hemolysin (1, 107). Briefly, cation-adjusted Mueller-Hinton agar plates containing 5% sheep blood were spread with a filter-sterilized and 2-fold-concentrated medium fraction of an overnight culture of an *S. aureus* strain (RN4220) that produces only β-hemolysin (not α-, γ-, or δ-hemolysin). Plates were also spread with a solution of arylomycin A-C_{16} in DMSO to produce a final concentration of 8 μg/ml or an equal volume of DMSO alone. Plates were then inoculated with dilutions of freshly grown cultures of *S. aureus* NCTC 8325 or PAS8001 and incubated overnight at 37°C. Under these conditions, colonies secreting δ-hemolysin are distinguished by their surrounding clear zone of hemolysis.

One-dimensional PAGE analysis of protein secretion. *S. aureus* strains PAS8001, SCXA81, and SCXA108 were grown as for MudPIT mass spectrometry to generate stationary-phase cultures (1.5×10^9 CFU/ml). Cultures were treated with DMSO alone or an equal volume of a DMSO solution of arylomycin A-C_{16} to a final concentration of 2× the respective arylomycin A-C_{16} MIC for each strain. Cells were grown for an additional 2 h and then subjected to 0.22-μm-pore-size filtration to remove all cells. The resulting culture supernatants were precipitated as for MudPIT mass spectrometry, normalized by the number of viable cells present at the time the culture was collected, and then separated on 10% SDS-PAGE and visualized with Coomassie brilliant blue.

RESULTS

To identify *S. aureus* proteins whose secretion relies on SPase, we performed MudPIT analysis of the SpsB(P29S) mutant strain of *S. aureus* NCTC 8325 in the presence of various concentrations of arylomycin A-C_{16}. Briefly, the bacteria were grown to early stationary phase in TSB, washed with PBS to remove any residual extracellular protein, and then resuspended in TSB with 0×, 0.5×, 2.0×, and 8.0× the MIC of the inhibitor. After 1.5 h of incubation, the number of CFU was determined at each concentration of arylomycin A-C_{16}, and the total extracellular protein was isolated. No statistically significant differences in growth or viability were observed, regardless of arylomycin concentration, and thus no attempt was made to normalize the data. In total, 295 extracellular proteins were identified by MudPIT with greater than 10 average spectral counts in the untreated (0× the MIC) samples (see Table S2 in the supplemental material), and 47 were identified whose spectral counts decreased significantly in the presence of arylomycin A-C_{16} at 0.5×, 2.0×, and 8.0× the MIC (Table 1).

Among the 47 proteins, one (SAOUHSC_00472; a putative ribose-phosphate pyrophosphokinase) does not have an identifiable SPase cleavage site and is a known cytoplasmic protein. Moreover, the spectral counts observed in the untreated control samples were low and barely satisfied our cutoff, and thus we assumed that it was a false positive and it was not further considered. Forty of the remaining 46 proteins possess a signal peptide (as predicted by SignalP 3.0) and lack a cell wall retention signal (Table 1). Thus, these proteins are likely to be canonically secreted via the Sec pathway. Interestingly, this group of proteins contains two lipases, Lip1 and Lip2, whose signal peptides contain the YSIRK sequence, which is common among cell wall-retained proteins (7). How-

TABLE 1 *S. aureus* proteins present at decreased levels in the media in response to varying concentrations of arylomycin A-C_{16}

Locus	Description	Predicted signal peptides[b]	Spectral count ± SEM[a]			
			0× MIC	0.5× MIC	2× MIC	8× MIC
Sec-type signal sequence, no cell wall retention signal						
00051	1-Phosphatidylinositol phosphodiesterase (*plc*)	MKKCIKTLFLSIILVVMSGWYHSAHA	592 ± 74	71 ± 16	126 ± 2	77 ± 25
00192	Staphylocoagulase	MKKQIISLGALAVASSLFTWDNKADA	58 ± 39	20 ± 13	33 ± 19	6 ± 1
00256	Conserved hypothetical protein	MKKTILLTMTTLTLFSMSPNSAQA	32 ± 4	15 ± 7	27 ± 12	16 ± 4
00300	Lipase (*lip2*)	MLRGQEERKYSIRKYSIGVVSVLAATMFVVSSHEAQA	642 ± 40	109 ± 22	129 ± 44	51 ± 16
00354	Superantigen-like protein	MFKKYDSKNSIVLKSILSLGIIYGGTFGIYPKADA	21 ± 10	12 ± 9	17 ± 13	ND
00399	Superantigen-like protein	MKLKNIAKASLALGILTTGMITTTAQPVKA	25 ± 1	8 ± 2	11 ± 4	5 ± 2
00427	Autolysin precursor (*sle*)	MQKKVIAAIIGTSAISAVAATQANA	250 ± 56	33 ± 24	61 ± 40	19 ± 11
00617	Conserved hypothetical protein	MKKLLTASIIACSVVMGVGLVNTSAEA	394 ± 35	248 ± 90	293 ± 73	126 ± 17
00818	Thermonuclease precursor (*nuc*)	[aa25]SNVSKGQYAKRFFYFATSCLVLTLVVVSSLSSSANA	59 ± 14	19 ± 5	11 ± 2	13 ± 2
00897	Glycerophosphoryl diester phosphodiesterase (*glpQ*)	MTNSSKSFTKFMAASAVFTMGFLSVPTAGA	180 ± 32	58 ± 6	79 ± 15	63 ± 25
00987	Putative cysteine protease precursor (*sspB*)	MNSSCKSRVFNIISIIMVSMLILSLGAFANNNKAKA	638 ± 182	123 ± 12	127 ± 45	115 ± 31
00988	Putative glutamyl endopeptidase precursor (*sspA*)	MKGKFLKVSSLFVATLTTATLVSSPAANA	589 ± 127	191 ± 43	229 ± 100	142 ± 43
00994	Bifunctional autolysin precursor (*atl*)	MAKKFNYKLPSMVALTLVGSAVTAHQVQA	1,256 ± 170	346 ± 113	443 ± 109	323 ± 86
01114	Fibrinogen-binding protein (*efb*)	MKNKLIAKSLLTLAAIGITTTTIASTADA	70 ± 31	32 ± 10	42 ± 11	15 ± 5
01121	Alpha-hemolysin precursor (*hla*)	MKTRIVSSVTTTLLLGSILMNPVANA	1,684 ± 436	401 ± 85	673 ± 125	270 ± 102
01125	Superantigen-like proten	MNNNITKKIILSTTLLLLGTASTQFPNTPINSSSEAKA	12 ± 2	2 ± 1	3 ± 1	ND
01935	Putative serine protease (*splF*)	MNKNIIIKSIAALTILTSITGVGTTMVEGIQQTAKA	342 ± 82	56 ± 5	62 ± 9	22 ± 9
01936	Putative serine protease (*splE*)	MNKNIIIKSIAALTILTSVTGVGTTVVEGIQQTAKA	257 ± 46	16 ± 1	12 ± 2	4 ± 3
01938	Putative serine protease (*splD*)	MNKNIIIKSIAALTILTSITGVGTTVVDGIQQTAKA	180 ± 39	40 ± 6	43 ± 4	20 ± 7
01939	Putative serine protease (*splC*)	MNKNIVIKSMAALAILTSVTGINAAVVEETQQIANA	99 ± 61	19 ± 2	18 ± 3	11 ± 5
01941	Putative serine protease (*splB*)	MNKNVVIKSLAALTILTSVTGIGTTLVEEVQQTAKA	258 ± 79	55 ± 8	51 ± 9	31 ± 13
01942	Putative serine protease (*splA*)	MNKNVMVKGLTALTILTSLGFAENISNQPHSIAKA	272 ± 122	46 ± 36	38 ± 24	10 ± 4
01949	Putative intracellular serine protease	MKIIKRAIISLIILSLLISITMSNASA	12 ± 4	ND	ND	ND
01954	Leukotoxin (*lukD*)	MKMKKLVKSSVASSIALLLLSNTVDA	279 ± 16	45 ± 10	80 ± 12	27 ± 8
02127	Staphopain thiol proteinase	MKRNFPKLIALSLIFSLSVTPIANA	406 ± 104	85 ± 11	114 ± 47	42.3 ± 10
02171	Putative staphylokinase precursor	MLKRSLLFLTVLLLLFSFSSITNEVSA	519 ± 29	127 ± 17	116 ± 23	80 ± 26
02241	Putative leukocidin F subunit like	MIKQLCKNITICTLALSTTFTVLPATSFA	1,083 ± 162	170 ± 26	155 ± 44	38 ± 9
02243	Aerolysin/leukocidin family protein	MKNKKRVLIASSLSCAILLLSAATTQANS	758 ± 432	209 ± 121	144 ± 57	53 ± 11
02260	Delta-hemolysin precursor (*hld*)	MSCLILRIFILIKEGVISMA	29 ± 8	10 ± 2	14 ± 4	9 ± 5
02463	Hyaluronate lyase (*hysA*)	MTYRIKKWQKLSTITLLMAGVITLNGGEFRSVDKHQIAVA	1,072 ± 94	168 ± 31	214 ± 26	126 ± 23
02466	Truncated MHC class II analog protein	MKLKSFVTATLALGLLSTVGAALPSHEASA	48 ± 11	28 ± 8	28 ± 6	25 ± 5
02706	Immunoglobulin G-binding protein (*sbi*)	MKNKYISKLLVGAATITLATMISNGEAKA	143 ± 37	58 ± 5	63 ± 15	38 ± 10
02708	Putative gamma-hemolysin h-gamma-ii subunit (*hlgA*)	MIKNKILTATLAVGLIAPLANPFIEISKA	41 ± 10	12 ± 3	12 ± 4	ND
02709	Putative leukocidin s subunit (*hlgC*)	MLKNKILTTTLSVSLLAPLANPLLENAKA	451 ± 116	166 ± 112	125 ± 83	26 ± 8
02710	Leukocidin f subunit precursor (*hlgB*)	MKMNKLVKSSVATSMALLLLSGTANA	649 ± 52	132 ± 19	281 ± 53	25 ± 10
02971	Putative aureolysin (*aur*)	VRKFSRYAFTSMAALTLLSTLSPAALA[c]	73 ± 3	16 ± 4	26 ± 7	18 ± 6
02972	Immunodominant staphylococcal antigen B (*isaB*)	MNKTSKVCVAATLALGTLIGVTVVENSAPTSKQAQA	22 ± 2	12 ± 1	22 ± 9	7 ± 3
02979	*N*-acetylmuramoyl-L-alanine amidase	MPKNKILIYLLSTTLVLPTLVSPTAYA	882 ± 76	689 ± 87	872 ± 192	513 ± 140
03006	Lipase (*lip1*)	MKSQNKYSIRKFSVGASSILIATLLFLSGGQAQA	482 ± 127	100 ± 14	120 ± 36	65 ± 15
Cell wall retention signal						
00069	Protein A (*spa*)	MKKKNIYSIRKLGVGIASVTLGTLLISGGVTPAANA	28 ± 5	20 ± 5	25 ± 9	3 ± 2
00094	Surface protein D (*sasD*)	MKKLATVGSLIVTSTLVFSSMPFQNAHA	298 ± 23	70 ± 8	65 ± 6	44 ± 11
01652	Penicillin-binding protein 3 (*pbp3*)	–	51 ± 5	15 ± 1	27 ± 5	23 ± 5
02742	Putative amino acid transporter	MKKIKYILVVFVLSLTVLSGCSLPGLGS	11 ± 2	3 ± 1	5 ± 2	ND
02798	Surface protein G (*sasG*)	[aa14]FLSNKLNKYSIRKFTVGTASILIGSLMYLGTQQEAEA	11 ± 2	4 ± 2	5 ± 1	2 ± 1
Transmembrane domains						
00728	Lipoteichoic acid synthase (*ltaS*)	[aa137]ILIFKTKWLDTKAFSKKFVPVVMAASVALFFLNLAFA	452 ± 44	156 ± 43	172 ± 43	90 ± 22
02885	*O*-acetyltransferase (*oatA*)	[aa365]FKAFAFLPKKKGQFARTVLVILLLVPSIVVLSGQFDA	36 ± 8	10 ± 3	11 ± 4	10 ± 6

[a] Data shown are the averages and standard errors of the means of the spectral counts observed for each protein at varying arylomycin concentrations in three independent, biological samples. ND, not detected.

[b] Sequences shown are the complete N-terminal signal peptides up to the predicted cleavage site unless otherwise indicated (longer sequences have been N-terminally truncated for clarity as denoted by the superscripted amino acid position). Signal peptide cleavage sites were predicted as described in Materials and Methods. –, no signal peptide identified by *in silico* sequence analysis.

[c] Signal peptide shown is based on the GTG initiation site located 33 nucleotides upstream of the annotated ATG codon of the SAOUHSC_02971 sequence.

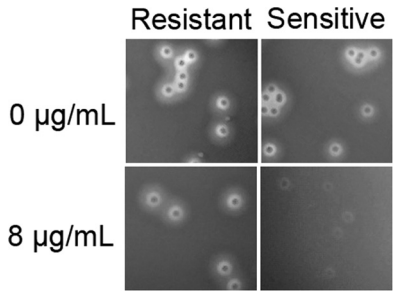

FIG 2 δ-Hemolysin secretion in single colonies of arylomycin-resistant (NCTC 8325) and arylomycin-sensitive (PAS8001) *S. aureus*. The concentration of arylomycin present is indicated on the left. See Materials and Methods for details.

ever, neither has the C-terminal LPXTG sequence required for retention (72, 90). Also among these proteins is SasD. In some *S. aureus* strains, SasD has a C-terminal LPAAG signal that might mediate cell wall retention, and in some cases it has been observed on the cell surface (23); however, the SasD ortholog in the strain employed here is truncated and lacks the potential retention signal. Accordingly, SasD has been detected in the media in another study employing a derivative strain of NCTC 8325, where its presence was shown to depend on a nonessential component of the Sec pathway (SecG) (98).

δ-Hemolysin was also found among the canonically secreted proteins. While this hemolysin has been known to be secreted for decades, the literature consensus is that it is secreted in a Sec- and SPase-independent manner (29, 46, 60, 72). To confirm the proteomics result, we performed an assay for δ-hemolysin activity using sheep blood agar (Fig. 2). As expected, we found that single colonies of *S. aureus* NCTC 8325 (arylomycin resistant) grown on sheep blood agar plates containing β-hemolysin were surrounded by a clear zone of hemolysis due to the action of δ-hemolysin, and no differences were observed when the arylomycin was present. However, colonies of arylomycin-sensitive *S. aureus* PAS8001 showed a similar zone of clearance only when the arylomycin was absent. In the presence of arylomycin A-C_{16}, the zone was turbid, consistent with the absence of δ-hemolysin and thus with the inhibition of its secretion.

Four of the 46 proteins whose presence in the media was decreased by the inhibition of SPase are predicted to be associated with the cell wall (Table 1). Two, protein A and SasG, are predicted to be cell wall associated by virtue of possessing, in addition to canonical Sec-type signal peptides, a C-terminal LPXTG cell wall retention signal. One, the class B high-molecular-weight penicillin binding protein, Pbp3, appears to be anchored to the cell wall via a noncleavable signal peptide (32) and has been observed experimentally in a cell surface fraction (39); however, other studies have also reported finding Pbp3 in the extracellular fraction (91, 99). Finally, SAOUHSC_02742 is a putative substrate binding component of an ATP-binding cassette (ABC) transporter and is predicted to be a lipoprotein processed by SPase II, based on both domain homology (SMART database) and LipoP 1.0.

The extracellular domains of two five-transmembrane proteins, LtaS and OatA, were also detected in the media at decreasing levels with increasing SPase inhibition (Table 1). In each case, we detected only peptides corresponding to the C-terminal domains, consistent with cleavage between the C-terminal-most transmembrane domain and the extracellular domain. Indeed, SignalP 3.0 predicts an SPase cleavage site in this region of both of these proteins (SignalP 3.0 HMM signal peptide probability scores of 1.000 and 0.899 for LtaS and OatA, respectively).

To confirm that the arylomycin-dependent decrease of all 46 of the proteins identified in the extracellular fraction was due to inhibited secretion and not altered transcription, we examined the corresponding mRNA levels as a function of added arylomycin A-C_{16} by qRT-PCR (see Table S3 in the supplemental material). None of the mRNA levels were significantly decreased relative to the control cells grown in the absence of arylomycin. These data are consistent with the conclusion that the decreased detection of these proteins results from the inhibition of SPase and not from altered transcription.

Finally, in addition to the 46 proteins whose levels in the media were decreased by the addition of the arylomycin, three proteins, PrsA (SAOUHSC_01972), HtrA (SAOUHSC_01838), and SAOUHSC_01761, were detected at increased levels with added arylomycin. Based on LocateP and SignalP, PrsA is predicted to be lipid anchored, while SAOUHSC_01761 and HtrA are predicted to be N-terminally anchored. At 8× the MIC, the levels of PrsA, HtrA, and SAOUHSC_01761 were found at 59 ± 12-, 42 ± 11-, and 30 ± 8-fold-increased levels, respectively, relative to that of the no-inhibitor control. Examination of the corresponding mRNA transcripts by qRT-PCR under identical conditions revealed that the transcript levels of each gene increased in a dose-dependent manner, up to 16-, 2-, and 4-fold for PrsA, HtrA, and SAOUHSC_01761, respectively, in the presence of 8 μg/ml of the inhibitor. While correlations between transcript and protein levels are difficult to predict, the data suggest that at least some of the increased protein levels were due to arylomycin-dependent induction.

Despite repeated attempts, we were unable to construct the *prsA* deletion strain. Thus, to further explore the role of induced genes, we constructed and characterized the chromosomal deletion mutants of *htrA* and SAOUHSC_01761 in the PAS8001 background (101). Neither mutant showed any apparent growth or cytological defects in the absence of arylomycin A-C_{16} (data not shown). In addition, gene deletion had only modest effects on the arylomycin A-C_{16} MIC, increasing the MIC by 2-fold in the *htrA* mutant and decreasing it by 2-fold in the SAOUHSC_01761 deletion strain. Finally, using one-dimensional (1D) PAGE analysis, we examined the profile of stationary-phase protein secretion in the wild-type and mutant strains (Fig. 3). The deletion of *htrA* resulted in no significant differences in the absence or presence of SPase inhibition. In contrast, while the deletion of SAOUHSC_01761 resulted in no differences in the absence of the arylomycin, it resulted in a significant reduction in protein secretion in its presence. These results suggest that SAOUHSC_01761 is required to maintain protein secretion in the face of SPase inhibition.

DISCUSSION

SPase plays an essential role during the secretion of most proteins from both Gram-positive and Gram-negative bacteria, and because the arylomycins selectively inhibit SPase, these natural products should allow for the direct experimental characterization of the SPase-dependent secretome of different bacteria. While our initial efforts toward this goal were limited by the insensitivity of many interesting bacteria to the arylomycins, we

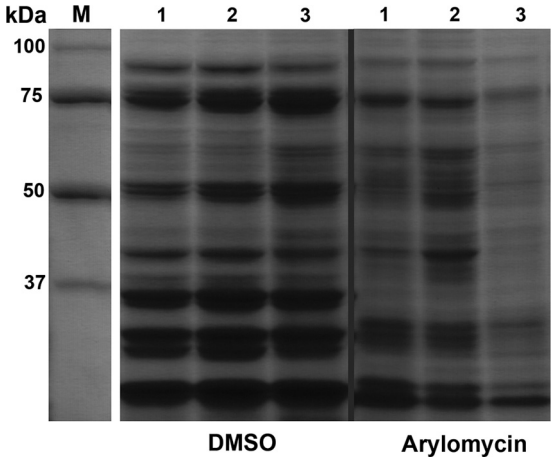

FIG 3 Dependence of protein secretion on HtrA and SAOUHSC_01761. Protein in the three left lanes was isolated from stationary-phase cells incubated in the absence of arylomycin A-C_{16} (but in the presence of DMSO vehicle) while those in the three right lanes were isolated from cells incubated in the presence of 0.5× their MIC (individually adjusted for each strain) of arylomycin A-C_{16}. Lanes: 1, PAS8001; 2, SCXA81 (Δ*htrA*); 3, SCXA108 (Δ*SAOUHSC_01761*); M, molecular weight marker.

recently determined that it is possible to sensitize most bacteria, including *E. coli*, *Pseudomonas aeruginosa*, and *S. aureus*, to the arylomycins via mutation of a specific SPase residue from Pro to Ser or Leu (101). In the present study, we used such a mutant of *S. aureus*, along with a MudPIT MS-based analytical approach, to perform a global analysis of the secretome of this important human pathogen. We identified 46 proteins whose presence in the media decreased in an arylomycin A-C_{16} dose-dependent manner and thus appear to be processed by SPase (Table 1).

Comparison with previous studies. It is instructive to compare the results of the current study with those published by Ravipaty and Reilly (84), as both approaches are in principle capable of characterizing the entire secretome. Ravipaty and Reilly identified 52 proteins in the extracellular media of methicillin-resistant *S. aureus* COL that lacked their predicted N-terminal signal peptide, thus suggesting that they had been processed by SPase. Of these 52 proteins, *S. aureus* NCTC 8325 (the parent of the mutant strain used in the current study) encodes a homolog of 48, and of these we identified 39 in the extracellular fraction, but arylomycin A-C_{16} inhibited the secretion of only 28. Thus, the corresponding orthologs of 11 proteins identified in the Ravipaty and Reilly study were not identified in the current study (LytM, SdrH, SceD, SsaA, SsaA2, IsaA, SAOUHSC_00284, SAOUHSC_0041, SAOUHSC_00671, and SAOUHSC_00918, SAOUHSC_01005). Conversely, 18 proteins identified in the current study were not identified by Ravipaty and Reilly. Of these 18, one does not have a homolog in *S. aureus* COL (staphylokinase), nine were not detected by Ravipaty and Reilly in the media (staphylocoagulase, HysA, OatA, SasG, Pbp3, intracellular serine protease SAOUHSC_01949, putative amino acid transporter SAOUHSC_02742, and two superantigen-like proteins, SAOUHSC_0039 and SAOUHSC_01125), four were detected but assumed not to have been processed by SPase due to a consensus in the literature that they are translated without a signal peptide or are associated with the membrane (Hld, protein A, SasD, and LtaS), and four were detected only as peptide fragments (SspA, SspB, HlgC, and Aur).

While it is likely that at least some of the differences can be attributed to differences in the strains (52, 86, 119) or growth conditions employed, they may also represent different biases of the two techniques. The arylomycin-based approach may not detect proteins that are recognized by SPase with a particularly high affinity (see below), while the approach of Ravipaty and Reilly may not detect proteins that are further proteolyzed after secretion or whose signal peptides are misannotated. Thus, it appears that a combination of both approaches is most likely to allow for the full and rigorous characterization of the bacterial secretome.

Does SPase specificity contribute to the secretome? An advantage of the inhibition-based approach is that it inherently provides biochemical data. Indeed, we found that the secretion of different proteins shows distinctly different susceptibilities to arylomycin-mediated inhibition. For example, at the lowest inhibitor concentrations, inhibition relative to the no-inhibitor control ranged from 2- to 16-fold, while at the highest, it ranged from 3- to 60-fold. These various levels of inhibition may reflect differences in the affinities with which each preprotein is recognized by SPase (with those recognized with the lowest affinities being more inhibited). Analysis of the primary sequence of the signal peptides revealed no obvious differences; however, the efficiency of processing a signal peptide is known to be preprotein context dependent (96). Variable susceptibility to arylomycin-mediated inhibition was also observed for the secretion of different proteins in *S. epidermidis* (82). Thus, it seems likely that there is a hierarchy in the affinity with which SPase binds its different substrates and that this is a general property of the SPases from different organisms. Under conditions where SPase activity is limiting, this hierarchy could provide a previously unappreciated mechanism of SPase control over the secretome.

The *S. aureus* 8325 secretome, its potential contribution to virulence, and the potential antivirulence activity of an arylomycin-based antibiotic. *S. aureus* is thought to secrete a core set of virulence factors that are critical for pathogenicity (8, 24, 45, 53, 59, 69, 73, 99), including proteases, lipases, elastases, nucleases, hyaluronidase, and collagenase, as well as a variety of cytolytic toxins such as the hemolysins and leukotoxins. Moreover, the genomes of different strains also encode additional virulence factors, such as pyrogenic toxin superantigens (SAgs), whose secretion is thought to be associated with specific diseases. Thus, the identification of the virulence factors actually processed by SPase and secreted into the extracellular environment is expected to help understand the diseases caused by *S. aureus* and how they might be treated.

The major proteolytic enzymes secreted by *S. aureus* are thought to include a serine glutamyl endopeptidase (referred to as V8 or SspA), two cysteine proteases, staphopain A (SspB2 or ScpA) and staphopain B (SspB), and the zinc metalloprotease, aureolysin (Aur) (6), and the secretion of all four is inhibited by arylomycin A-C_{16}. Moreover, in a variety of strains, including 8325, a cluster of an additional six genes has been identified that are predicted to encode serine proteases with Sec-type signal sequences and that have also been speculated to be virulence factors (80, 85). We found that the secretion of all six is inhibited by arylomycin A-C_{16}. We also found that the secretion of SAOUHSC_01949, which possesses a clear Sec-type signal sequence but which is currently (mis)annotated as a putative intracellular serine protease, is inhibited by the arylomycin.

S. aureus carries an arsenal of four different hemolysins and several other cytolytic toxins. We found that the secretion of α-,

γ-, and δ-hemolysin was inhibited by arylomycin A-C_{16}. Interestingly, δ-hemolysin is annotated as a 26-amino-acid peptide (UniProt [108] entry Q2FWM8), and the general consensus in the literature for years has been that this peptide is translated and secreted without the aid of SPase or the Sec pathway (29, 46, 60). Nonetheless, the Kegg database annotates it as a 45-amino-acid prepeptide with a 19-residue signal peptide (51). The observation that the accumulation of δ-hemolysin in the media is inhibited by the arylomycin demonstrates that at least some of it is synthesized as the prepeptide whose secretion requires SPase and likely the Sec pathway, as well.

In addition to the hemolysins, we found that the secretion of four members of the leukocidin/hemolysin family of toxins is inhibited. This includes both subunits of LukS-LukF (Panton-Valentine leukocidin), two proteins with ~50% homology to LukS and LukF (SAOHUSC_02241 and SAOHUSC_02243, respectively) (16, 70), and the D subunit of the LukE-LukD leukotoxin.

The secretion of SAgs helps *S. aureus* circumvent the host immune response by binding to T cell receptor (TCR) and class II major histocompatibility complex (MHC) and thus stimulating a massive proliferation of antigen-independent T cells and cytokines, which results in tissue damage and shock-like syndromes. Nearly all SAgs are encoded on mobile genetic elements, and thus their presence and secretion are strain dependent (74, 113). The *S. aureus* strain used in the present analysis appears to encode at least 14 Sags (31), 11 of which are predicted to be N-terminally anchored by LocateP. The remaining three (SAOHUSC_00399, SAOUHSC_01125, and SAOUHSC_01125) are predicted to be encoded with N-terminal signal sequences, and indeed, we found that each is secreted in an SPase-dependent manner.

The secretion of several additional virulence factors was also inhibited by the arylomycin, including two lipases (Lip1 and Lip2, SAOUHSC_00300 and SAOUHSC_03006, respectively), the autolysins Sle and Atl (SAOUHSC_00427 and SAOUHSC_00994, respectively), an *N*-acetylmuramoyl-L-alanine amidase (SAOUHSC_02979), 1-phosphatidylinositol phosphodiesterase (Plc; SAOUHSC_00051), glycerophosphoryl diester phosphodiesterase (GlpQ; SAOUHSC_00897), hyaluronate lyase (HysA; SAOUHSC_02463, hysA), staphylokinase (SAOUHSC_02171), thermonuclease (Nuc; SAOUHSC_00818), staphylocoagulase (SAOUHSC_00192), fibronectin binding protein (Efb; SAOUHSC_01114), immunodominant staphylococcal antigen B (IsaB; SAOUHSC_02972), and the immunoglobulin G-binding protein (Sbi; SAOUHSC_ 02706) (47, 48, 57, 66, 67, 77, 92, 106, 112). In addition, we identified two hypothetical proteins that are secreted in an SPase-dependent manner, SAOUHSC_00256 and SAOUHSC_00617, suggesting that they should be investigated as potential virulence factors.

In addition to these canonically secreted proteins, several known or likely cell surface-associated proteins were identified, including protein A, SasG, Pbp3, and a putative component of an amino acid transporter (SAOUHSC_02742). With the exception of Pbp3, each protein has a likely Sec-type signal peptide with an apparent SPase cleavage site, and both Pbp3 and SAOUHSC_02466 were detected previously in the media of growing *S. aureus* (17, 91, 99). However, these proteins were generally detected at relatively low levels in the untreated control samples, suggesting that their presence in the media may result from proteolytic release via an extracellular protease (a process referred to as shaving) (99). Thus, their reduced levels in the presence of arylomycin A-C_{16} may result directly from their reduced presence on the surface (with the exception of Pbp3) and/or indirectly, from reduced levels of a protease required for their shaving whose secretion is dependent on SPase. The results do not exclude the possibility that a significant fraction of the proteins remains bound to the cell wall.

In addition to being a useful chemical biology probe of SPase-mediated secretion, the arylomycins are promising antibiotics (89, 100, 101). Clearly, the secretion of many of the virulence factors required for *S. aureus* pathogenicity are inhibited by the arylomycin, suggesting that an arylomycin-based therapeutic might reduce virulence while simultaneously eradicating an infection. This is in stark contrast to several other antibiotics that have been shown to induce the production of virulence factors (15, 35, 58, 75, 78, 97). While the full manifestation of the potential broad-spectrum activity of the arylomycins requires their optimization to overcome the reduced target affinity afforded by the resistance-conferring Pro residue naturally present in many clinically important bacteria (87), the fact that significant levels of inhibition are apparent with most of the virulence factors at 0.5× the MIC suggests that even at sub-MIC levels the arylomycins might have antivirulence activities.

Atypical SPase cleavage sites. LtaS and OatA each have five transmembrane domains/helices with no obvious Sec-type N-terminal signal sequence but were each identified as SPase substrates. LtaS is a polyglycerol phosphate synthase involved in the synthesis of lipoteichoic acid, which is a component of the *S. aureus* envelope (33, 34). It was recently demonstrated that SPase processes LtaS in *S. aureus* (115), which our results confirm, and along with our previous demonstration that SPase processes LtaS in *S. epidermidis* (82) suggests that SPase-mediated cleavage of LtaS is general and possibly physiologically significant. OatA is an *O*-acetyltransferase and an integral membrane protein that confers the high level of resistance to lysozyme observed in the staphylococci by *O*-acylating peptidoglycan muramic acid (13). To our knowledge, there was no previous experimental data suggesting that OatA is processed by SPase. While it is not possible to rigorously exclude the possibility that these proteins are processed by other extracellular proteases whose translocation depends on SPase, the presence of SPase recognition sequences, and the consistency of the size of the fragment detected with cleavage at these sites, suggests that they themselves are SPase substrates. The identification of two transmembrane proteins that are likely processed by SPase, with cleavage sites embedded within the protein, adds to a growing list of such proteins (3, 4, 40, 61, 82, 83, 103) and strongly suggests that the physiological functions of SPase extend outside its role in secretion.

Increased secretion of several proteins as a bacterial response to the secretion stress resulting from the inhibition of SPase. The arylomycin-mediated induction of HtrA, PrsA, and SAOUHSC_01761 suggests that they could be part of a response to the inhibition of SPase. Deletion of SAOUHSC_01761 resulted in a minor sensitization of *S. aureus* to arylomycin A-C_{16}. However, while its deletion did not significantly affect stationary-phase protein secretion in the absence of the arylomycin, it did significantly reduce the level of secreted protein in the presence of the arylomycin. Little is known about SAOUHSC_01761, other than its being induced by vancomycin treatment (along with both *htrA* and *prsA*) (68) or deletion of the gene encoding serine/threonine kinase Stk1, which interestingly also results in elevated levels of hemolysin secretion (18). It seems likely that SAOUHSC_01761 is required for efficient secretion

when SPase or other components of the secretion machinery are inhibited or otherwise compromised.

HtrA is conserved in many Gram-positive bacteria, where it may be cell surface associated or processed and secreted (2), as well as in Gram-negative bacteria, where it is also known as DegP and is localized to the periplasm. HtrA is a bifunctional enzyme with both chaperone and protease activities and it is thought to be involved in the folding and maturation of secreted proteins as well as in the degradation of proteins that misfold during secretion (22, 30, 64, 81, 94, 102), and at least in *Bacillus subtilis*, *htrA* is induced by secretion stress (2, 44, 63). Deletion of *htrA* conferred only a marginal sensitization to the arylomycin and did not significantly affect stationary-phase protein secretion in the presence or absence of the inhibitor. This is consistent with previously reported results that identified a second HtrA-like protein in *S. aureus* (SAOUHSC_00958, also referred to as *htrA2*) which may compensate for some of the function of HtrA (86). Interestingly, inhibiting cell wall synthesis in *S. aureus* 8325 by treatment with oxacillin, bacitracin, or D-cycloserine induces *htrA* expression (109), but as with the arylomycin, deletion of *htrA* does not confer sensitivity to these antibiotics.

Treatment with arylomycin A-C_{16} caused a particularly pronounced induction of *prsA*, whose transcript and protein levels increased up to 16- and 60-fold, respectively. PrsA is an extracytoplasmic membrane-anchored lipoprotein that is thought to function as a chaperone during the posttranslocation folding of secreted proteins (38, 94, 99), including enzymes important for cell wall synthesis (43). Despite our inability to delete *prsA* in the *S. aureus* NCTC 8325-derived strain used in the present study, and the well-known essentiality of *prsA* in *Bacillus subtilis* (43, 94), it is not essential in a variety of other Gram-positive bacteria, including *Lactococcus lactis* (26) and *Streptococcus pyogenes* (65) or in several other strains of *S. aureus*, including NCTC 8325 itself (10, 21). It is interesting to speculate that our inability to delete *prsA* in *S. aureus* PAS8001 is related to the SPase mutation that renders it sensitive to the arylomycin. Whatever the reason, our inability to delete *prsA* precluded a more direct test of its role in the response to SPase inhibition, but it is interesting to note that several lines of evidence connect it with HtrA. For example, mutation of *prsA* in *B. subtilis* induces expression of *htrA* (42, 44), and the two proteins appear to collaborate for the efficient secretion of at least some proteins in *Streptococcus pyogenes* (22, 65).

Conclusion. Arylomycin A-C_{16}, along with the genetically sensitized strain of *S. aureus*, has allowed for the identification of the proteins whose secretion depends on the activity of SPase, as well as the identification of proteins that appear to mediate the cellular response to its inhibition. With regard to the latter, while HtrA, PrsA, and SAOUHSC_01761 appear to mediate important components of the response, the detailed mechanism, as well as the contributions of increased transcription, stability, or processing, needs to be evaluated further. With regard to the characterization of the secretome, it is likely that a variety of the SPase substrates identified, including δ-hemolysin, LtaS, and OatA, would not have been identified using other proteomics approaches, as either the signal sequence had been misannotated or because they are processed despite the absence of a signal sequence. Importantly, the demonstration that LtaS and OatA are processed by SPase despite not possessing a signal sequence, or even being secreted proteins, further supports the suggestion that SPase has important functions outside its well-known role in the general secretory pathway. Finally, the large array of virulence factors whose secretion is inhibited by the arylomycin in both *S. aureus* and *S. epidermidis* (82), and likely other pathogens as well, further attests to the promise of this natural product scaffold as a unique antibiotic that actually reduces virulence while eradicating an infection.

ACKNOWLEDGMENTS

Funding was provided by the Office of Naval Research (grant no. N000140810478 to F.E.R.) and the National Institutes of Health (grant no. AI081126 to F.E.R.).

REFERENCES

1. **Adhikari RP, Arvidson S, Novick RP.** 2007. A nonsense mutation in *agrA* accounts for the defect in *agr* expression and the avirulence of *Staphylococcus aureus* 8325-4 *traP*::*kan*. Infect. Immun. **75**:4534–4540.
2. **Antelmann H, et al.** 2003. The extracellular proteome of *Bacillus subtilis* under secretion stress conditions. Mol. Microbiol. **49**:143–156.
3. **Antelmann H, et al.** 2001. A proteomic view on genome-based signal peptide predictions. Genome Res. **11**:1484–1502.
4. **Antelmann H, et al.** 2005. The extracellular and cytoplasmic proteomes of the nonvirulent *Bacillus anthracis* strain UM23C1-2. Proteomics **5**:3694–3695.
5. **Arnaud M, Chastanet A, Debarbouille M.** 2004. New vector for efficient allelic replacement in naturally nontransformable, low-GC-content, Gram-positive bacteria. Appl. Environ. Microbiol. **70**:6887–6891.
6. **Arvidson S.** 2000. Extracellular enzymes. *In* Fischetti VA (ed), Gram-positive pathogens. American Society of Microbiology, Washington, DC.
7. **Bae T, Schneewind O.** 2003. The YSIRK-G/S motif of staphylococcal protein A and its role in efficiency of signal peptide processing. J. Bacteriol. **185**:2910–2919.
8. **Bartlett AH, Hulten KG.** 2010. *Staphylococcus aureus* pathogenesis: secretion systems, adhesins, and invasins. Pediatr. Infect. Dis. J. **29**:860–861.
9. **Becher D, et al.** 2009. A proteomic view of an important human pathogen—towards the quantification of the entire *Staphylococcus aureus* proteome. PLoS One **4**:e8176.
10. **Begun J, Sifri CD, Goldman S, Calderwood SB, Ausubel FM.** 2005. *Staphylococcus aureus* virulence factors identified by using a high-throughput *Caenorhabditis elegans*-killing model. Infect. Immun. **73**: 872–877.
11. **Bendtsen JD, Kiemer L, Fausboll A, Brunak S.** 2005. Non-classical protein secretion in bacteria. BMC Microbiol. **5**:58.
12. **Bendtsen JD, Nielsen H, von Heijne G, Brunak S.** 2004. Improved prediction of signal peptides: SignalP 3.0. J. Mol. Biol. **340**:783–795.
13. **Bera A, Herbert S, Jakob A, Vollmer W, Gotz F.** 2005. Why are pathogenic staphylococci so lysozyme resistant? The peptidoglycan *O*-acetyltransferase OatA is the major determinant for lysozyme resistance of *Staphylococcus aureus*. Mol. Microbiol. **55**:778–787.
14. **Bernardo K, et al.** 2002. Identification of *Staphylococcus aureus* exotoxins by combined sodium dodecyl sulfate gel electrophoresis and matrix-assisted laser desorption/ionization-time of flight mass spectrometry. Proteomics **2**:740–746.
15. **Bisognano C, Vaudaux P, Rohner P, Lew DP, Hooper DC.** 2000. Induction of fibronectin-binding proteins and increased adhesion of quinolone-resistant *Staphylococcus aureus* by subinhibitory levels of ciprofloxacin. Antimicrob. Agents Chemother. **44**:1428–1437.
16. **Boyle-Vavra S, Daum RS.** 2007. Community-acquired methicillin-resistant *Staphylococcus aureus*: the role of Panton-Valentine leukocidin. Lab. Invest. **87**:3–9.
17. **Burlak C, et al.** 2007. Global analysis of community-associated methicillin-resistant *Staphylococcus aureus* exoproteins reveals molecules produced in vitro and during infection. Cell. Microbiol. **9**:1172–1190.
18. **Burnside K, et al.** 2010. Regulation of hemolysin expression and virulence of *Staphylococcus aureus* by a serine/threonine kinase and phosphatase. PLoS One **5**:e11071.
19. **Carvalho PC, Yates JR III, Barbosa VC.** 2010. Analyzing shotgun proteomic data with PatternLab for proteomics. Curr. Protoc. Bioinformatics **13**:13. 13.1–15.
20. **Cash P, Argo E.** 2009. Analysis of bacterial proteins by 2DE. Methods Mol. Biol. **519**:131–144.
21. **Chaudhuri RR, et al.** 2009. Comprehensive identification of essential

Staphylococcus aureus genes using transposon-mediated differential hybridisation (TMDH). BMC Genomics **10**:291.
22. **Cole JN, et al.** 2007. Role of group A *Streptococcus* HtrA in the maturation of SpeB protease. Proteomics **7**:4488–4498.
23. **DeDent A, Bae T, Missiakas DM, Schneewind O.** 2008. Signal peptides direct surface proteins to two distinct envelope locations of *Staphylococcus aureus.* EMBO J. **27**:2656–2668.
24. **Dinges MM, Orwin PM, Schlievert PM.** 2000. Exotoxins of *Staphylococcus aureus.* Clin. Microbiol. Rev. **13**:16–34.
25. **Driessen AJ, Nouwen N.** 2008. Protein translocation across the bacterial cytoplasmic membrane. Annu. Rev. Biochem. **77**:643–667.
26. **Drouault S, et al.** 2002. The peptidyl-prolyl isomerase motif is lacking in PmpA, the PrsA-like protein involved in the secretion machinery of *Lactococcus lactis*. Appl. Environ. Microbiol. **68**:3932–3942.
27. **du Plessis DJ, Nouwen N, Driessen AJ.** 2011. The Sec translocase. Biochim. Biophys. Acta **1808**:851–865.
28. **Eng JK, McCormack AL, Yates JR III.** 1994. An approach to correlate tandem mass spectral data of peptides with amino acid sequences in a protein database. J. Mass Spectrom. **5**:976–989.
29. **Fitton JE, Dell A, Shaw WV.** 1980. The amino acid sequence of the delta haemolysin of *Staphylococcus aureus.* FEBS Lett. **115**:209–212.
30. **Foucaud-Scheunemann C, Poquet I.** 2003. HtrA is a key factor in the response to specific stress conditions in *Lactococcus lactis.* FEMS Microbiol. Lett. **224**:53–59.
31. **Gillaspy AF, et al.** 2006. The *Staphylococcus aureus* NCTC 8325 genome, p 381–412. *In* Fischetti VA, Novick RP, Ferretti J, Portnoy D, and Rood J (ed), Gram-positive pathogens. American Society for Microbiology, Washington, DC.
32. **Goffin C, Ghuysen JM.** 1998. Multimodular penicillin-binding proteins: an enigmatic family of orthologs and paralogs. Microbiol. Mol. Biol. Rev. **62**:1079–1093.
33. **Grundling A, Schneewind O.** 2007. Genes required for glycolipid synthesis and lipoteichoic acid anchoring in *Staphylococcus aureus.* J. Bacteriol. **189**:2521–2530.
34. **Grundling A, Schneewind O.** 2007. Synthesis of glycerol phosphate lipoteichoic acid in *Staphylococcus aureus.* Proc. Natl. Acad. Sci. U. S. A. **104**:8478–8483.
35. **Hallander HO, Laurell G, Lofstrom G.** 1966. Enhancement of staphylococcal pathogenicity in the presence of penicillin. Acta Pathol. Microbiol. Scand. **68**:463–464.
36. **Hecker M, Becher D, Fuchs S, Engelmann S.** 2010. A proteomic view of cell physiology and virulence of *Staphylococcus aureus.* Int. J. Med. Microbiol. **300**:76–87.
37. **Hecker M, Engelmann S, Cordwell SJ.** 2003. Proteomics of *Staphylococcus aureus*—current state and future challenges. J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. **787**:179–195.
38. **Heikkinen O, et al.** 2009. Solution structure of the parvulin-type PPIase domain of *Staphylococcus aureus* PrsA—implications for the catalytic mechanism of parvulins. BMC Struct. Biol. **9**:17.
39. **Hempel K, et al.** 2010. Quantitative cell surface proteome profiling for SigB-dependent protein expression in the human pathogen *Staphylococcus aureus* via biotinylation approach. J. Proteome. Res. **9**:1579–1590.
40. **Hirose I, et al.** 2000. Proteome analysis of *Bacillus subtilis* extracellular proteins: a two-dimensional protein electrophoretic study. Microbiology **146**(Pt 1):65–75.
41. **Höltzel A, et al.** 2002. Arylomycins A and B, new biaryl-bridged lipopeptide antibiotics produced by *Streptomyces* sp. Tü 6075. II. Structure elucidation. J. Antibiot. (Tokyo) **55**:571–577.
42. **Hyyrylainen HL, et al.** 2001. A novel two-component regulatory system in *Bacillus subtilis* for the survival of severe secretion stress. Mol. Microbiol. **41**:1159–1172.
43. **Hyyrylainen HL, et al.** 2010. Penicillin-binding protein folding is dependent on the PrsA peptidyl-prolyl cis-trans isomerase in *Bacillus subtilis.* Mol. Microbiol. **77**:108–127.
44. **Hyyrylainen HL, Sarvas M, Kontinen VP.** 2005. Transcriptome analysis of the secretion stress response of *Bacillus subtilis.* Appl. Microbiol. Biotechnol. **67**:389–396.
45. **Imamura T, et al.** 2005. Induction of vascular leakage through release of bradykinin and a novel kinin by cysteine proteinases from *Staphylococcus aureus.* J. Exp. Med. **201**:1669–1676.
46. **Janzon L, Lofdahl S, Arvidson S.** 1989. Identification and nucleotide sequence of the delta-lysin gene, *hld*, adjacent to the accessory gene regulator (*agr*) of *Staphylococcus aureus.* Mol. Gen. Genet. **219**:480–485.
47. **Jefferson K.** 2010. Characterization of the *Staphylococcus aureus* immunodominant surface antigen B, IsaB. Virginia Commonwealth University, Richmond, VA.
48. **Jin T, et al.** 2004. *Staphylococcus aureus* resists human defensins by production of staphylokinase, a novel bacterial evasion mechanism. J. Immunol. **172**:1169–1176.
49. **Jones RC, Deck J, Edmondson RD, Hart ME.** 2008. Relative quantitative comparisons of the extracellular protein profiles of *Staphylococcus aureus* UAMS-1 and its *sarA*, *agr*, and *sarA agr* regulatory mutants using one-dimensional polyacrylamide gel electrophoresis and nanocapillary liquid chromatography coupled with tandem mass spectrometry. J. Bacteriol. **190**:5265–5278.
50. **Juncker AS, et al.** 2003. Prediction of lipoprotein signal peptides in Gram-negative bacteria. Protein Sci. **12**:1652–1662.
51. **Kanehisa M, Goto S, Furumichi M, Tanabe M, Hirakawa M.** 2010. KEGG for representation and analysis of molecular networks involving diseases and drugs. Nucleic Acids Res. **38**:D355–D360.
52. **Karlsson A, Arvidson S.** 2002. Variation in extracellular protease production among clinical isolates of *Staphylococcus aureus* due to different levels of expression of the protease repressor *sarA*. Infect. Immun. **70**:4239–4246.
53. **Karlsson A, Saravia-Otten P, Tegmark K, Morfeldt E, Arvidson S.** 2001. Decreased amounts of cell wall-associated protein A and fibronectin-binding proteins in *Staphylococcus aureus sarA* mutants due to up-regulation of extracellular proteases. Infect. Immun. **69**:4742–4748.
54. **Kawano Y, et al.** 2000. Rapid isolation and identification of staphylococcal exoproteins by reverse phase capillary high performance liquid chromatography—electrospray ionization mass spectrometry. FEMS Microbiol. Lett. **189**:103–108.
55. **Kawano Y, et al.** 2001. Proteolytic cleavage of staphylococcal exoproteins analyzed by two-dimensional gel electrophoresis. Microbiol. Immunol. **45**:285–290.
56. **Krogh A, Larsson B, von Heijne G, Sonnhammer EL.** 2001. Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. J. Mol. Biol. **305**:567–580.
57. **Kullik I, Giachino P, Fuchs T.** 1998. Deletion of the alternative sigma factor sigmaB in *Staphylococcus aureus* reveals its function as a global regulator of virulence genes. J. Bacteriol. **180**:4814–4820.
58. **Kuroda H, Kuroda M, Cui L, Hiramatsu K.** 2007. Subinhibitory concentrations of beta-lactam induce haemolytic activity in *Staphylococcus aureus* through the SaeRS two-component system. FEMS Microbiol. Lett. **268**:98–105.
59. **Larkin EA, Carman RJ, Krakauer T, Stiles BG.** 2009. *Staphylococcus aureus*: the toxic presence of a pathogen extraordinaire. Curr. Med. Chem. **16**:4003–4019.
60. **Lee KY, Birkbeck TH.** 1984. *In vitro* synthesis of the delta-lysin of *Staphylococcus aureus.* Infect. Immun. **44**:434–438.
61. **Lequette Y, Lanfroy E, Cogez V, Bohin JP, Lacroix JM.** 2008. Biosynthesis of osmoregulated periplasmic glucans in *Escherichia coli*: the membrane-bound and the soluble periplasmic phosphoglycerol transferases are encoded by the same gene. Microbiology **154**:476–483.
62. **Letunic I, Doerks T, Bork P.** 2009. SMART 6: recent updates and new developments. Nucleic Acids Res. **37**:D229–D332.
63. **Lulko AT, et al.** 2007. Production and secretion stress caused by overexpression of heterologous alpha-amylase leads to inhibition of sporulation and a prolonged motile phase in *Bacillus subtilis.* Appl. Environ. Microbiol. **73**:5354–5362.
64. **Lyon WR, Caparon MG.** 2004. Role for serine protease HtrA (DegP) of *Streptococcus pyogenes* in the biogenesis of virulence factors SpeB and the hemolysin streptolysin S. Infect. Immun. **72**:1618–1625.
65. **Ma Y, et al.** 2006. Identification and characterization of bicistronic *speB* and *prsA* gene expression in the group A *Streptococcus*. J. Bacteriol. **188**:7626–7634.
66. **Makris G, Wright JD, Ingham E, Holland KT.** 2004. The hyaluronate lyase of *Staphylococcus aureus*—a virulence factor? Microbiology **150**:2005–2013.
67. **Marques MB, Weller PF, Parsonnet J, Ransil BJ, Nicholson-Weller A.** 1989. Phosphatidylinositol-specific phospholipase C, a possible virulence factor of *Staphylococcus aureus.* J. Clin. Microbiol. **27**:2451–2454.
68. **McCallum N, Spehar G, Bischoff M, Berger-Bachi B.** 2006. Strain dependence of the cell wall-damage induced stimulon in *Staphylococcus aureus.* Biochim. Biophys. Acta **1760**:1475–1481.
69. **McGavin MJ, Zahradka C, Rice K, Scott JE.** 1997. Modification of the

Staphylococcus aureus fibronectin binding phenotype by V8 protease. Infect. Immun. **65**:2621–2628.
70. **Mesrati I, Saidani M, Ennigrou S, Zouari B, Ben Redjeb S.** 2010. Clinical isolates of Pantone-Valentine leucocidin- and gamma-haemolysin-producing *Staphylococcus aureus*: prevalence and association with clinical infections. J. Hosp. Infect. **75**:265–268.
71. **Nakano M, et al.** 2002. Two-dimensional analysis of exoproteins of methicillin-resistant *Staphylococcus aureus* (MRSA) for possible epidemiological applications. Microbiol. Immunol. **46**:11–22.
72. **Navarre WW, Ton-That H, Faull KF, Schneewind O.** 1998. Anchor structure of staphylococcal surface proteins. II. Cooh-terminal structure of muramidase and amidase-solubilized surface protein. J. Biol. Chem. **273**:29135–29142.
73. **Novick R.** 2000. Pathogenicity factors and their regulation, p 392–407. *In* Fischetti VA (ed), Gram-positive pathogens. American Society for Microbiology, Washington, DC.
74. **Novick RP.** 2003. Autoinduction and signal transduction in the regulation of staphylococcal virulence. Mol. Microbiol. **48**:1429–1449.
75. **Ohlsen K, et al.** 1998. Effects of subinhibitory concentrations of antibiotics on alpha-toxin (hla) gene expression of methicillin-sensitive and methicillin-resistant *Staphylococcus aureus* isolates. Antimicrob. Agents Chemother. **42**:2817–2823.
76. **Paetzel M, Karla A, Strynadka NC, Dalbey RE.** 2002. Signal peptidases. Chem. Rev. **102**:4549–4580.
77. **Palma M, Shannon O, Quezada HC, Berg A, Flock JI.** 2001. Extracellular fibrinogen-binding protein, Efb, from *Staphylococcus aureus* blocks platelet aggregation due to its binding to the alpha-chain. J. Biol. Chem. **276**:31691–31697.
78. **Panos GZ, Betsi GI, Falagas ME.** 2006. Systematic review: are antibiotics detrimental or beneficial for the treatment of patients with *Escherichia coli* O157:H7 infection? Aliment. Pharmacol. Ther. **24**:731–742.
79. **Pocsfalvi G, et al.** 2008. Proteomic analysis of exoproteins expressed by enterotoxigenic *Staphylococcus aureus* strains. Proteomics **8**:2462–2476.
80. **Popowicz GM, et al.** 2006. Functional and structural characterization of Spl proteases from *Staphylococcus aureus.* J. Mol. Biol. **358**:270–279.
81. **Poquet I, et al.** 2000. HtrA is the unique surface housekeeping protease in *Lactococcus lactis* and is required for natural protein processing. Mol. Microbiol. **35**:1042–1051.
82. **Powers ME, et al.** 2011. Type I signal peptidase and protein secretion in *Staphylococcus epidermidis.* J. Bacteriol. **193**:340–348.
83. **Pugsley AP.** 1993. The complete general secretory pathway in Gram-negative bacteria. Microbiol. Rev. **57**:50–108.
84. **Ravipaty S, Reilly JP.** 2010. Comprehensive characterization of methicillin-resistant *Staphylococcus aureus* subsp. *aureus* COL secretome by two-dimensional liquid chromatography and mass spectrometry. Mol. Cell. Proteomics **9**:1898–1919.
85. **Reed SB, et al.** 2001. Molecular characterization of a novel *Staphylococcus aureus* serine protease operon. Infect. Immun. **69**:1521–1527.
86. **Rigoulay C, et al.** 2005. Comparative analysis of the roles of HtrA-like surface proteases in two virulent *Staphylococcus aureus* strains. Infect. Immun. **73**:563–572.
87. **Roberts TC, Schallenberger MA, Liu J, Smith PA, Romesberg FE.** 2011. Initial efforts toward the optimization of arylomycins for antibiotic activity. J. Med. Chem. **54**:4954–4963.
88. **Roberts TC, Smith PA, Cirz RT, Romesberg FE.** 2007. Structural and initial biological analysis of synthetic arylomycin A_2. J. Am. Chem. Soc. **129**:15830–15838.
89. **Roberts TC, Smith PA, Romesberg FE.** 2011. Synthesis and biological characterization of arylomycin B antibiotics. J. Nat. Prod. **74**:956–961.
90. **Roche FM, et al.** 2003. Characterization of novel LPXTG-containing proteins of *Staphylococcus aureus* identified from genome sequences. Microbiology **149**:643–654.
91. **Rogasch K, et al.** 2006. Influence of the two-component system SaeRS on global gene expression in two different *Staphylococcus aureus* strains. J. Bacteriol. **188**:7742–7758.
92. **Rosenstein R, Gotz F.** 2000. Staphylococcal lipases: biochemical and molecular characterization. Biochimie **82**:1005–1014.
93. **Rusch SL, Kendall DA.** 2007. Interactions that drive Sec-dependent bacterial protein transport. Biochemistry **46**:9665–9673.
94. **Sarvas M, Harwood CR, Bron S, van Dijl JM.** 2004. Post-translocational folding of secretory proteins in Gram-positive bacteria. Biochim. Biophys. Acta **1694**:311–327.
95. **Schimana J, et al.** 2002. Arylomycins A and B, new biaryl-bridged lipopeptide antibiotics produced by *Streptomyces* sp. Tu 6075. I. Taxonomy, fermentation, isolation and biological activities. J. Antibiot. (Tokyo) **55**:565–570.
96. **Shen LM, et al.** 1991. Use of site-directed mutagenesis to define the limits of sequence variation tolerated for processing of the M13 procoat protein by the *Escherichia coli* leader peptidase. Biochemistry **30**:11775–11781.
97. **Shibl AM.** 1983. Effect of antibiotics on production of enzymes and toxins by microorganisms. Rev. Infect. Dis. **5**:865–875.
98. **Sibbald MJ, et al.** 2010. Synthetic effects of *secG* and *secY2* mutations on exoproteome biogenesis in *Staphylococcus aureus.* J. Bacteriol. **192**:3788–3800.
99. **Sibbald MJ, et al.** 2006. Mapping the pathways to staphylococcal pathogenesis by comparative secretomics. Microbiol. Mol. Biol. Rev. **70**:755–788.
100. **Smith PA, Powers ME, Roberts TC, Romesberg FE.** 2011. *In vitro* activities of arylomycin natural-product antibiotics against *Staphylococcus epidermidis* and other coagulase-negative staphylococci. Antimicrob. Agents Chemother. **55**:1130–1134.
101. **Smith PA, Roberts TC, Romesberg FE.** 2010. Broad spectrum antibiotic activity of the arylomycin natural products is masked by natural target mutations. Chem. Biol. **17**:1223–1231.
102. **Sriraman K, Jayaraman G.** 2008. HtrA is essential for efficient secretion of recombinant proteins by *Lactococcus lactis.* Appl. Environ. Microbiol. **74**:7442–7446.
103. **Thony-Meyer L, James P, Hennecke H.** 1991. From one gene to two proteins: the biogenesis of cytochromes b and c1 in *Bradyrhizobium japonicum.* Proc. Natl. Acad. Sci. U. S. A. **88**:5001–5005.
104. **Tjalsma H, et al.** 2004. Proteomics of protein secretion by *Bacillus subtilis*: separating the "secrets" of the secretome. Microbiol. Mol. Biol. Rev. **68**:207–233.
105. **Tjalsma H, Dijl JM.** 2005. Proteomics-based consensus prediction of protein retention in a bacterial membrane. Proteomics **5**:4472–4482.
106. **Torres VJ, et al.** 2010. *Staphylococcus aureus* fur regulates the expression of virulence factors that contribute to the pathogenesis of pneumonia. Infect. Immun. **78**:1618–1628.
107. **Traber KE, et al.** 2008. *agr* function in clinical *Staphylococcus aureus* isolates. Microbiology **154**:2265–2274.
108. **UniProt Consortium.** 2011. Ongoing and future developments at the Universal Protein Resource. Nucleic Acids Res. **39**:D214–D219.
109. **Utaida S, et al.** 2003. Genome-wide transcriptional profiling of the response of *Staphylococcus aureus* to cell-wall-active antibiotics reveals a cell-wall-stress stimulon. Microbiology **149**:2719–2732.
110. **Wang P, Dalbey RE.** 2010. *In vitro* and *in vivo* approaches to studying the bacterial signal peptide processing. Methods Mol. Biol. **619**:21–37.
111. **Washburn MP, Wolters D, Yates JR III.** 2001. Large-scale analysis of the yeast proteome by multidimensional protein identification technology. Nat. Biotechnol. **19**:242–247.
112. **Watanabe S, et al.** 2005. Structural comparison of ten serotypes of staphylocoagulases in *Staphylococcus aureus.* J. Bacteriol. **187**:3698–3707.
113. **Wilson GJ, et al.** 2011. A novel core genome-encoded superantigen contributes to lethality of community-associated MRSA necrotizing pneumonia. PLoS Pathog. **7**:e1002271.
114. Wooldridge K (ed). 2009. Bacterial secreted proteins: secretory mechanisms and role in pathogenesis. Caister Academic Press, Norwich, United Kingdom.
115. **Wormann ME, Reichmann NT, Malone CL, Horswill AR, Grundling A.** 2011. Proteolytic cleavage inactivates the *Staphylococcus aureus* lipoteichoic acid synthase. J. Bacteriol. **193**:5279–5291.
116. **Zhou M, Boekhorst J, Francke C, Siezen RJ.** 2008. LocateP: genome-scale subcellular-location predictor for bacterial proteins. BMC Bioinform. **9**:173.
117. **Ziebandt AK, et al.** 2004. The influence of *agr* and sigmaB in growth phase dependent regulation of virulence factors in *Staphylococcus aureus.* Proteomics **4**:3034–3047.
118. **Ziebandt AK, et al.** 2010. Proteomics uncovers extreme heterogeneity in the *Staphylococcus aureus* exoproteome due to genomic plasticity and variant gene regulation. Proteomics **10**:1634–1644.
119. **Ziebandt AK, et al.** 2001. Extracellular proteins of *Staphylococcus aureus* and the role of SarA and sigma B. Proteomics **1**:480–493.