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-acetyltansferase, 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 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 tetrapeptide 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 post-translational, 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-C16 (Fig. 1) (formerly referred to as arylomycin C16) (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...
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-C16 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 SpaPse (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-C16, 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 SpaPse activity. Our data suggest that arylomycin A-C16 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 SpaPse. Lastly, the identified strains were confirmed for correct gene deletion by PCR with oligonucleotide primers located at 200 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 (OD590) 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 × 10⁸ CFU/ml. The resulting saturated 40-ml culture was then divided into 10-ml aliquots, and the following concentrations of arylomycin A-C16 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, 4.0, and 16.0 μg/ml, respectively). After incubation with shaking for 1.5 h at 37°C, the optical density was recorded 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) phosphate hydrochloride (TCEP; Sigma) for 30 min at room temperature and then alkylated with 12.5 mM fresh iodoacetamide (IAA). 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 M CaCl₂ 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 (15.9949 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 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 non-tryptic 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-C16 (0, 1, 4, or 8 μg/ml) and then lysed using the RNAProtect bacterial reagent (Qiagen). Lysates were subjected to total RNA isolation using 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 MyQ 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.cambio.ru.nl/locatep-db/cgi-bin/locatepdbh.html), SignalP 3.0 neural network (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 then spread with a solution of arylomycin A-C16 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, SCX818, and SCX108 were grown for MudPIT mass spectrometry to generate stationary-phase cultures (1.5 × 10⁸ CFU/ml). Cultures were treated with DMSO alone or an equal volume of a DMSO solution of arylomycin A-C16 to a final concentration of 2× the respective arylomycin A-C16 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-C16. 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-C16 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-C16 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-C16.

<table>
<thead>
<tr>
<th>Locus</th>
<th>Description</th>
<th>Predicted signal peptides</th>
<th>Spectral count ± SEM</th>
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<td>0× MIC</td>
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SEC-type signal sequence, no cell wall retention signal

00051 | 1-Phosphatidylinositol phosphodiesterase (pk) | MKCCKTLFSLILIVMSGWYSHAA | 592 ± 74 | 71 ± 16 | 126 ± 2 | 77 ± 25 |

00192 | Staphylocoagulase | MKQGISSLGALASSLWDTNKADA | 58 ± 39 | 20 ± 13 | 33 ± 19 | 6 ± 1 |

00256 | Conserved hypothetical protein | MKKT1LMTMMLTTSFSMPSNAQA | 32 ± 4 | 15 ± 7 | 27 ± 12 | 16 ± 4 |

00300 | Lipase (lip2) | MLRQGEKQKGRYKSGVGVSSLATMVFVSSHEAQ | 642 ± 40 | 109 ± 22 | 129 ± 44 | 51 ± 16 |

00354 | Superantigen-like protein | MFKKYDSKNSVLKSLGILHGGYTGTIGPKADA | 21 ± 10 | 12 ± 9 | 17 ± 13 | ND |

00399 | Superantigen-like protein | MKLKNKASALGICTTMTTATQPYKA | 25 ± 1 | 8 ± 2 | 11 ± 4 | 5 ± 2 |

00427 | Autolysin precursor (rlf) | MQRVIAIDGTSASAVATQAQA | 236 ± 56 | 33 ± 24 | 61 ± 40 | 19 ± 11 |

00617 | Conserved hypothetical protein | MKELLTAISACWVMGVGLTVNXTA | 394 ± 35 | 248 ± 90 | 293 ± 73 | 126 ± 17 |

00818 | Thrombinase precursor (nuc) | a^2^SVNSGKOYAKCFREYYTFCISSLTILVTVLVSLSAN | 59 ± 14 | 19 ± 5 | 11 ± 2 | 13 ± 2 |

00897 | Glycosylphosphoryl diester phosphodiesterase (gpQ) | MTN5SKSTFKEAMAATVMTGLSFPTAGA | 180 ± 32 | 58 ± 6 | 79 ± 15 | 63 ± 25 |

00987 | Putative cysteine protease precursor (spB) | MNNSSCVRNISMSIMSVISSLGAFANNNKKA | 638 ± 182 | 123 ± 12 | 127 ± 45 | 115 ± 31 |

00988 | Putative glutamyl endopeptidase precursor (spA) | MKGKFLKVLFLSTFYATLTVSSPAANA | 589 ± 127 | 191 ± 43 | 229 ± 100 | 142 ± 43 |

00994 | Bifunctional autolysin precursor (adl) | MKFNYKSLPMVALLTVSSAVTAHQVQA | 1,256 ± 170 | 346 ± 113 | 443 ± 109 | 323 ± 86 |

01114 | Fibrinogen-binding protein (fB) | MKKKIQKSLTTAAAGTTTIAATA | 70 ± 31 | 32 ± 10 | 42 ± 11 | 15 ± 5 |

01227 | Staphopain thiol proteinase | MKRNFKPLAILLSIPSLTPIANA | 406 ± 104 | 85 ± 11 | 114 ± 47 | 42 ± 10 |

02171 | Putative staphylokinase precursor | MKLRSLFLFTVLSSFLSFSTENVES | 519 ± 29 | 127 ± 17 | 116 ± 23 | 80 ± 26 |

02241 | Putative leucokin B subunit like | MKQICKCNLTATLTASFLPITSAF | 1,083 ± 162 | 170 ± 26 | 155 ± 44 | 38 ± 9 |

02316 | Aerolysin/leukocidin family protein | MKKIRVLCLASSLCAAILTAATQANS | 758 ± 432 | 209 ± 121 | 144 ± 57 | 53 ± 11 |

02466 | Hyaluronate lyase (hylA) | MTYIRKWKQLKSLTTLMAVGLTNGEERSVDKQHIAVA | 1,072 ± 94 | 168 ± 31 | 214 ± 26 | 126 ± 23 |

02498 | Truncated MHC class II analog protein | MKLEFKVATLALGISSLGTVGPAHESA | 48 ± 11 | 28 ± 8 | 28 ± 6 | 25 ± 5 |

02706 | Immunoglobulin G-binding protein (sbi) | MKNNKSNQGAVGAATTILATLMISNEA | 143 ± 37 | 58 ± 5 | 63 ± 15 | 38 ± 10 |

02708 | Putative gamma-hemolysin h-gamma-ii subunit (hlgA) | MKMNKSTLASTLVGLAVPIEASA | 41 ± 10 | 12 ± 3 | 12 ± 4 | ND |

02709 | Putative leukocidin s subunit (hlgC) | MLKNNKSTLASTLVGLAVPIEASA | 451 ± 116 | 166 ± 122 | 125 ± 83 | 26 ± 8 |

02710 | Leukocidin I subunit precursor (hlgB) | MKNNKKNKSVASSATLLGNSGTA | 649 ± 52 | 132 ± 19 | 281 ± 53 | 25 ± 10 |

02971 | Putative aureolysin (aur) | VRFSRFAYSTMAALTLSTLSAPA | 73 ± 3 | 16 ± 4 | 26 ± 7 | 18 ± 6 |

02972 | Immunodominant staphylococcal antigen B (sbi) | MNKTSKVCVATATLGIGLTVGTVTENATPSKQAQA | 22 ± 2 | 12 ± 1 | 22 ± 9 | 7 ± 3 |

02979 | N-acetylmuramoyl-l-alanine amidase | MPKNKLLVTVLILVTLYSTPIAYA | 882 ± 76 | 689 ± 87 | 872 ± 192 | 513 ± 140 |

03006 | Lipase (lip1) | MKQSNKYSIRKFVSGASSILGLITLSSGQQQA | 482 ± 127 | 104 ± 14 | 120 ± 36 | 65 ± 15 |

Cell wall retention signal

00069 | Protein A (spa) | MKKNNYSIRKLVGQYASLTVLGTLTSSGPTPA | 28 ± 5 | 20 ± 5 | 25 ± 9 | 3 ± 2 |

00094 | Surface protein D (sadD) | MKKATLGVSSVTLLVSSMPFQNAHA | 298 ± 23 | 70 ± 8 | 65 ± 6 | 44 ± 11 |

01652 | Penicillin-binding protein 3 (pbp3) | MKKNNKNNKSVASSATLLGNSGTA | 51 ± 5 | 15 ± 1 | 27 ± 5 | 23 ± 5 |

02742 | Putative amino acid transporter | MKKLYKLVVFVFLSVLTSCLSGPLGLS | 11 ± 2 | 3 ± 1 | 5 ± 2 | ND |

02798 | Surface protein G (sadG) | a^2^FLSNKNKYIRKFVGTASLGLSMYLGTQFQAEA | 11 ± 2 | 4 ± 2 | 5 ± 1 | 2 ± 1 |

Transmembrane domains

00728 | Lipoteichoic acid synthase (itaS) | a^2^ILIFTKWKDLTAKFSSKFKFVFPVMAASLVALFILNALFA | 452 ± 44 | 156 ± 43 | 172 ± 43 | 90 ± 22 |

00885 | O-acetyltransferase (otaT) | a^2^PKAFAPFKPPKQAPRTVYLVSLESIVLSGQQA | 36 ± 8 | 10 ± 3 | 11 ± 4 | 10 ± 6 |

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

**Note:**
- 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.
- Signal peptide shown is based on the GTG initiation site located 33 nucleotides upstream of the annotated ATG codon of the SAOUHSC_02971 sequence.
SPase and Protein Secretion in *S. aureus*

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...
recently determined that it is possible to sensitize most bacteria, including *E. coli*, *Pseudomonas aeruginosa*, and *S. aureus*, to the aryloymcins 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 aryloymycin A-C<sub>16</sub> 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* whose signal peptides are misannotated. Thus, it appears that a number of the proteins not detected by SPase in the current study (see below) are further proteolyzed after secretion. The approach of Ravipaty and Reilly may 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 aryloymycin-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 aryloymycin-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 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 aryloymycin-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 SpmA), two cysteine proteases, staphopain A (SpaB or SpdB) and spathopain B (SpaB), and the zinc metalloprotease, aureolysin (Aur) (6), and the secretion of all four is inhibited by aryloymycin A-C<sub>16</sub>. 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 aryloymycin A-C<sub>16</sub>. 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 aryloymycin.

*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 O2FWM8), 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 (SAOUHSC_02241 and SAOUHSC_02243, respectively) (16, 70), and the D subunit of the LukE-LukD leukotoxin.

The secretion of SAg's 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 SAg's 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 SAg's (31), 11 of which are predicted to be N-terminally anchored by LocateP. The remaining three (SAOUHSC_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_00306, respectively), the autolysins Sle and Atl (SAOUHSC_00427 and SAOUHSC_00994, respectively), an N-acetylmuramyl-l-alanine amidase (SAOUHSC_02979), 1-phosphadiyninositol phosphodiesterase (Plc; SAOUHSC_00051), glycerophosphoryl diester phosphodiesterase (GlpQ; SAOUHSC_00897), hyaluronate lyase (HysA; SAOUHSC_02463, hysA), staphylokinase (SAOUHSC_02171), thermonuclease (Nuc; SAOUHSC_00188), staphylococcal nuclease (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× MIC suggests that even at sub-MIC levels the arylomycins might have anti-virulence 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-acetytransferase and an integral membrane protein that confers the high level of resistance to lysosome observed in the staphylococci by O-acetylating 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, 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.

May 2012 Volume 194 Number 10 jb.asm.org 2683
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} analog 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.

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