Mechanism of Action of the Arylomycin Antibiotics and Effects of Signal Peptidase I Inhibition

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Clinically approved antibiotics inhibit only a small number of conserved pathways that are essential for bacterial viability, and the physiological effects of inhibiting these pathways have been studied in great detail. Likewise, characterizing the effects of candidate antibiotics that function via novel mechanisms of action is critical for their development, which is of increasing importance due to the ever-growing problem of resistance. The arylomycins are a novel class of natural-product antibiotics that act via the inhibition of type I signal peptidase (SPase), which is an essential enzyme that functions as part of the general secretory pathway and is not the target of any clinically deployed antibiotic. Correspondingly, little is known about the effects of SPase inhibition or how bacteria may respond to mitigate the associated secretion stress. Using genetically sensitized *Escherichia coli* and *Staphylococcus aureus* as model organisms, we examine the activity of arylomycin as a function of its concentration, bacterial cell density, target expression levels, and bacterial growth phase. The results reveal that the activity of the arylomycins results from an insufficient flux of proteins through the secretion pathway and the resulting mislocalization of proteins. Interestingly, this has profoundly different effects on *E. coli* and *S. aureus.* Finally, we examine the activity of arylomycin in combination with distinct classes of antibiotics and demonstrate that SPase inhibition results in synergistic sensitivity when combined with an aminoglycoside.

Most clinically deployed antibiotics interfere with the essential cellular processes of DNA synthesis, RNA synthesis, peptidoglycan synthesis, or membrane polarization (43). While these drugs have revolutionized health care, the emergence of multidrug-resistant pathogens necessitates the identification of new classes of antibiotics (50), especially those that act via novel mechanisms of action and thereby minimize the potential for cross-resistance. Bacterial type I signal peptidase (SPase) has long been appreciated as a potential target for antibacterial therapy, because it is conserved, essential, and located in the relatively accessible outer leaflet of the cytoplasmic membrane (29, 31, 46). SPase functions as part of the general secretory pathway to remove the N-terminal signal peptide from membrane-bound preproteins following their translocation across the cytoplasmic membrane, which affords the mature proteins access to their ultimate destinations in the periplasm, outer membrane, or extracellular milieu (7–9, 38). Therefore, the inhibition of SPase is likely to result in the accumulation of preproteins in the cytoplasmic membrane and the misregulation of essential pathways that utilize these proteins. In addition, depending on the protein-protein interactions involved, SPase inhibition could result in the obstruction of secretion channels, which has been linked to the antibiotic activity of some translational inhibitors (52).

The arylomycins (Fig. 1) are a relatively recently discovered class of natural-product antibiotics that were shown to bind and inhibit SPase *in vitro* (20, 30, 41). Despite the apparent accessibility and essentiality of SPase, initial reports suggested that the arylomycins are active against only a few Gram-positive bacteria, including *Streptococcus pneumoniae*, *Rhodococcus opacus*, and *Brevibacillus brevis* (20, 41), and not against other important Gram-positive pathogens or against any Gram-negative bacteria. However, after reporting the first total synthesis of an arylomycin (35), as well as several derivatives (23, 35, 36, 44, 45), including arylomycin A-C_{16} (Fig. 1) (previously referred to as arylomycin C_{16}), we found that they have potent antibacterial activity against a wide variety of Gram-positive and Gram-negative bacteria. Moreover, we identified a specific binding-site Pro residue that contributes to the natural resistance of the Gram-positive pathogen *Staphylococcus aureus* and the Gram-negative pathogens *Escherichia coli* and *Pseudomonas aeruginosa* (45). Importantly, for both *E. coli* and *S. aureus*, we demonstrated that the Pro mutation contributes to resistance by reducing the affinity with which SPase is bound by the arylomycins, suggesting that arylomycin derivatives that bind SPase with improved affinity could have clinical utility (45). Indeed, several derivatives with improved activity against *S. aureus* have been identified (34, 36).

Due to the novelty of the arylomycin class of antibiotics and of SPase as a target, little is known about their activity, including whether it is bacteriostatic or bactericidal and how it varies as a function of concentration, bacterial density, state of growth, or level of SPase expression. Moreover, it is unknown if the lipid tail contributes to activity, for example, by causing nonspecific membrane depolarization, as has been observed with other lipidated antibiotics (49). Herein, we begin to explore these issues using model strains of *E. coli* and *S. aureus* whose SPases have been rendered sensitive to the arylomycins via a single point mutation that removes the resistance-conferring Pro. The data demonstrate that the antibiotic activity of the arylomycins results from insufficient SPase activity and not from blocked secretion channels or from a nonspecific membrane depolarization caused by their li-
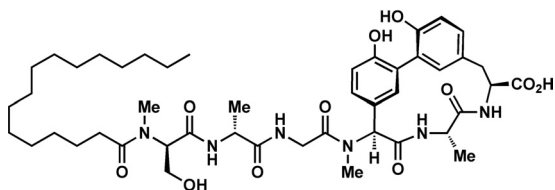
FIG 1 Structure of arylomycin A-C_{16}. Other members of the arylomycin family of natural-product antibiotics are defined by different fatty acid lipid tails or modifications of the central biphenyl core (51).

pophilicity. Furthermore, arylomycin activity can be either bacteriostatic or bactericidal, depending on the organism and growth conditions, and the activities against the Gram-negative and Gram-positive organisms are distinctly different, suggesting that secretion plays distinct roles in the viability of these divergent organisms and perhaps suggesting that they have evolved different mechanisms to cope with secretion stress. Finally, while we found that the arylomycins show relatively little synergy or antagonism with most other classes of antibiotics, they do show pronounced synergy with gentamicin, suggesting that SPase inhibitors may be particularly efficacious when coadministered with an aminoglycoside.

MATERIALS AND METHODS

Medium and antibiotics. Bacteria were routinely grown at 37°C on Mueller-Hinton II agar (MHAII) or in cation-adjusted Mueller-Hinton II broth (MHBII) with shaking at 275 rpm. Stock solutions of antibiotics were prepared in water or dimethyl sulfoxide (DMSO) at the following concentrations: arylomycin A-C_{16}, 10 mg/ml (DMSO); polymyxin B, 1 mg/ml (H_2O); vancomycin, 10 mg/ml (H_2O); cephalexin, 1 mg/ml (H_2O); erythromycin, 15 mg/ml (DMSO); tetracycline, 15 mg/ml (DMSO); rifampin, 15 mg/ml (DMSO); gentamicin, 15 mg/ml (H_2O); trimethoprim, 15 mg/ml (DMSO); ciprofloxacin, 1 mg/ml (H_2O). Arylomycin A-C_{16} was synthesized as described previously (35); polymyxin B and vancomycin were obtained from Sigma-Aldrich (St. Louis, MO); all other antibiotics were obtained from MP Biomedicals (Solon, OH). Anhydrotetracycline (aTc; Acros, Morris Plains, NJ) stock solutions were prepared in neat DMSO at a concentration of 100 μg/ml.

Bacterial strains and plasmids. All strains and plasmids used in this study are listed in Table 1. The effects of inhibiting SPase of *E. coli* MG1655 and *S. aureus* NCTC 8325-4 were examined with derivative strains PAS0260 and PAS8001, respectively (45). In these strains, the Pro residue in the SPase responsible for arylomycin resistance was replaced by a residue that confers arylomycin sensitivity; the resulting SPases were LepB(P84L) in *E. coli* strain PAS0260 and SpsB(P29S) in *S. aureus* strain PAS8001. To examine the effect of increased expression of arylomycin-susceptible SPases, *E. coli* strains PAS0275 and PAS0234 were created to allow for the aTc-inducible, ectopic expression of LepB(P84L) and LepB(P84S) from the plasmid pTetBHR2. Briefly, the wild-type *E. coli lepB* coding sequence and upstream Shine-Dalgarno sequence were amplified using primers lepB+RBS_NF_NdeI and lepB_CR_KpnI, and the product was phosphorylated using T4 polynucleotide kinase (New England BioLabs). The DNA was ligated into the vector pTetBHR2 that had been digested with XhoI and XmaI (New England BioLabs) and treated with Klenow fragment DNA polymerase (New England BioLabs). The resulting vector was subjected to QuikChange mutagenesis (Stratagene) with primers described previously (45) to introduce the LepB(P84L) or LepB(P84S) mutations, resulting in plasmids pTetBHR2-LepB(P84L) and pTetBHR2-LepB(P84S), respectively. The plasmid pTetBHR2-LepB(P84L) was subsequently electroporated into PAS0260 to generate PAS0275 to allow overexpression of LepB(P84L). Plasmid pTetBHR2-LepB(P84S) was transformed into PAS0232, which contains the chromo-

somal LepB(P84S) mutation. The P84S mutation in SPase also sensitizes *E. coli* to the arylomycins (45).

E. coli strain PAS0162 expresses wild-type SPase solely from a plasmid and requires aTc for SPase expression and wild-type growth rates. To construct this strain, following transformation of *E. coli* MG1655 with pTetBHR2-LepB, the chromosomal copy of *lepB* was removed via allelic exchange (28). Briefly, a disruption cassette was assembled using 3-way assembly PCR, in which approximately 1,000-bp regions upstream and downstream of the gene to be deleted were fused to the kanamycin resistance cassette from plasmid pUC4K in place of the *lepB* gene. The resulting cassette was electroporated into cells expressing SPase from the vector pTetBHR2-LepB and the lambda red recombinase (5), and the resulting chromosomal *lepB* deletion was transferred into *E. coli* MG1655 with pTetBHR2-LepB by P1 transduction. Strain PAS0216 was generated similarly using pTetBHR2-LepB(P84S) to complement the chromosomal deletion.

Susceptibility determination. Antibiotic susceptibilities were determined for PAS0260 or PAS8001 by measuring MICs using the CSLI broth microdilution method (3). Briefly, 2-fold serial dilutions of antibiotics were prepared in 96-well plates containing 100 μl of cation-adjusted MHBII. Bacterial inocula were prepared by suspending colonies grown for 24 h on MHAII to a final density of 1×10^7 CFU per ml in MHBII. Wells containing the antibiotic dilutions were inoculated to a final density of 5×10^5 CFU/ml, and MICs were defined as the lowest arylomycin A-C_{16} concentration at which no visible growth occurred following 24 h of incubation at 37°C. The contribution of increased levels of SPase expression to arylomycin susceptibilities was determined by inoculating 96-well plates containing a checkerboard of 2-fold dilutions of arylomycin A-C_{16} and aTc with *E. coli* strain PAS0275 harboring LepB(P84L) under the control of the aTc-inducible promoter on plasmid pTetBHR2. The contribution of SPase underexpression to the susceptibility of *E. coli* expressing wild-type and P84S SPases was determined similarly except that bacteria were grown on MHAII containing 64 μg/ml aTc. Colonies were then diluted into MHBII without aTc to an initial optical density (OD) of 0.01 and grown to an OD of 0.5 to dilute out the high levels of SPase produced under inducing conditions on solid media. These cultures were used to inoculate 96-well plates containing a checkerboard of 2-fold dilutions of arylomycin A-C_{16} and aTc.

Time-kill assays. Time-kill experiments were performed by suspending bacterial colonies in MHBII and inoculating the suspension into a 50-ml conical tube containing 10 ml of MHBII to a final OD at 600 nm (OD_{600}) of 0.025. Cultures were grown to mid-logarithmic growth phase ($OD_{600} = 0.4$ to 0.5) and diluted with prewarmed MHBII to a final density of 1×10^6 or 1×10^8 CFU/ml for normal and high-density kill curves, respectively (1×10^8 CFU/ml corresponds to an OD_{600} of 0.2 for *E. coli* and 0.1 for *S. aureus*). Diluted cultures (3 ml) were aliquoted into 14-ml culture tubes containing arylomycin A-C_{16} and incubated at 37°C with

TABLE 1 Strains used in this study

| Strain	Relevant characteristics[a]	Reference or source
MG1655	*E. coli* F⁻ λ⁻ *ilvG rbf-50 rph-1*	2
PAS0162	MG1655 ΔLepB::Kanʳ + pTetBHR2-LepB	This work
PAS0216	MG1655 ΔLepB::Kanʳ + pTetBHR2-LepB(P84S)	This work
PAS0232	MG1655 LepB(P84S)::Kanʳ	45
PAS0234	MG1655 LepB(P84S)::Kanʳ + pTetBHR2-LepB(P84S)	This work
PAS0260	MG1655 LepB(P84L)::Kanʳ	45
PAS0275	MG1655 LepB(P84L)::Kanʳ + pTetBHR2-LepB(P84L)	This work
PAS8001	*S. aureus* NCTC 8325 SpsB(P29S)	45

[a] Kanʳ, kanamycin resistance.
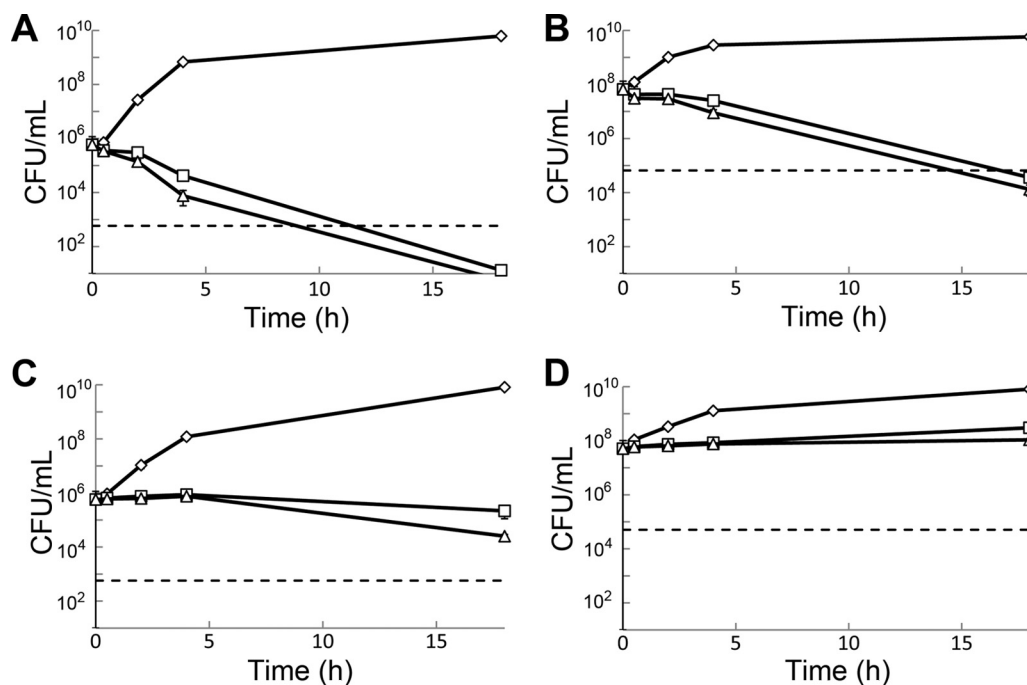
FIG 2 Viability of exponentially growing *E. coli* PAS0260 at normal density (A) and high density (B) and exponentially growing *S. aureus* PAS8001 at normal density (C) and high density (D) in the presence of arylomycin A-C_{16} at concentrations 2-fold (open squares) or 8-fold (open triangles) above the MIC compared to that of the vehicle control (open diamonds). A 3-log kill is used as the definition of bactericidal activity and is represented by dashed lines.

shaking at 275 rpm. Bacterial viability was quantified by plating serial dilutions onto MHAII and determining viable CFU after 24 h. Additionally, to increase the detection limit at the final 18-h time point, 1 ml of each culture was pelleted, washed once in phosphate-buffered saline (PBS), and plated onto MHAII. Time-kill assays were also performed with *S. aureus* in the presence of 0.5 μg/ml (2 times the MIC) of the bacteriostatic translational inhibitor tetracycline. Time-kill assays with stationary-phase cells were performed similarly. Briefly, MHBII (10 ml) was inoculated with colonies suspended to a final OD_{600} of 0.025, and the cultures were grown for 18 h at 37°C with shaking at 275 rpm. The resulting saturated cultures were pelleted and washed twice in PBS, then diluted into PBS to a density of approximately 1×10^6 CFU/ml and incubated for 30 min at 37°C with shaking to provide time to acclimate to the nutrient-depleted conditions. Cultures were then aliquoted into 14-ml culture tubes containing arylomycin A-C_{16}, and CFU were quantified as described above.

Interactions between arylomycin A-C_{16} and other antibiotics. Interactions between antibiotics were determined by calculating fractional inhibitory concentration (FIC) indexes from microdilution checkerboard MIC experiments (14, 33). The FIC of an antibiotic is the concentration that kills when used in combination with another antibiotic divided by the concentration that has the same effect when used alone, and the FIC index is defined as the sum of the FICs of the two antibiotics. FIC indexes of ≤0.5 reflect significant synergism, and those that are ≥2 reflect significant antagonism (10, 14). Because interactions between two antibiotics can vary depending on the relative concentration of each agent, the minimum and maximum FIC indexes are reported for each combination of agents. Checkerboard analyses for each antibiotic combination were performed five times to ensure the significance of the results (33), and the average and standard deviation of each FIC index are reported.

RESULTS

Time-kill studies. To examine whether the arylomycins have bacteriostatic or bactericidal activity, time-kill experiments were performed with actively growing cultures of the arylomycin-suscep-

tible strains *E. coli* PAS0260 and *S. aureus* PAS8001 (Fig. 2). Each of these strains differs from its corresponding wild-type strain by a single amino acid substitution that converts the resistance-conferring Pro to either Leu (*E. coli* strain PAS0260) or Ser (*S. aureus* strain PAS8001), resulting in increased arylomycin A-C_{16} sensitivity (45). In the absence of arylomycin A-C_{16}, the growth of these strains is identical to that of the respective wild-type strains. Unless otherwise noted, experiments were conducted with *E. coli* PAS0260 and *S. aureus* PAS8001.

At a cell density of 1×10^6 CFU/ml, we observed rapid inhibition of growth for both PAS0260 and PAS8001 at arylomycin concentrations 2- and 8-fold above their respective MICs, and this inhibition lasted the entire 18-h course of the experiment. During the initial 4 h of exposure, no decrease in the viability of the *S. aureus* cultures was observed, and the majority of cells remained viable even after 18 h of exposure to the lower concentration of arylomycin. Only a 20-fold decrease in viability was observed at the higher concentration after 18 h. In contrast, the viability of *E. coli* cultures was reduced 10- to 100-fold during the initial 4 h, with a slightly greater effect at higher arylomycin concentrations. Moreover, the cultures of *E. coli* were almost completely sterilized (10^5-fold reduction in CFU/ml) following the prolonged exposure. Increasing the density of the inocula to 1×10^8 CFU/ml had only modest effects on the kinetics of killing, and at 18 h the viability of *S. aureus* was unchanged, whereas the viability of *E. coli* cultures decreased more than 10^3-fold. Similar to the results observed with lower-density cultures, increasing the concentration of arylomycin had at most a modest effect on the activity against higher-density cultures.

Activity against quiescent cells. To determine whether the arylomycins have bactericidal activity against nongrowing cells,
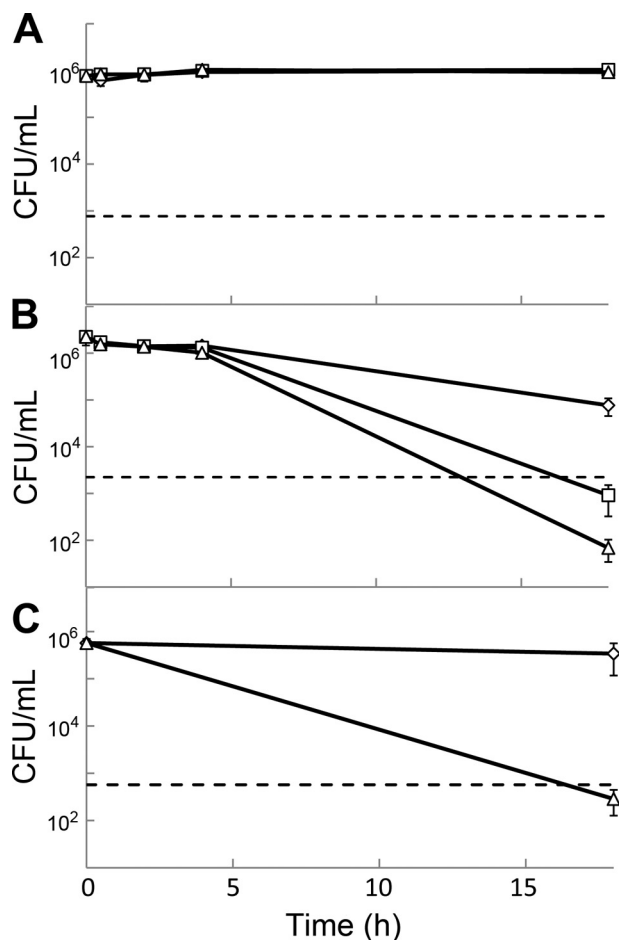
FIG 3 Viability of stationary-phase cultures of arylomycin-susceptible *E. coli* and *S. aureus* strains. Stationary-phase cultures of *E. coli* (A) and *S. aureus* (B) were washed and diluted into PBS and treated with vehicle controls (open diamonds) or with arylomycin A-C$_{16}$ at concentrations 2-fold (open squares) or 8-fold (open triangles) above the respective MICs. Cultures of *S. aureus* whose growth had been arrested by the addition of tetracycline were treated with vehicle controls (open diamonds) or with arylomycin A-C$_{16}$ at 8 times the MIC (open triangles) (C). A 3-log kill is used as the definition of bactericidal activity and is represented by dashed lines.

cultures of *E. coli* maintained full viability in the presence or absence of arylomycin, suggesting that active growth is required for bactericidal activity. In contrast, while the viability of *S. aureus* cultures decreased slightly (approximately 30-fold) in the absence of arylomycin A-C$_{16}$, a reduction of over 3 orders of magnitude was observed in the presence of arylomycin A-C$_{16}$ at both 2 and 8 times the MIC. To examine whether the observed sensitivity of *S. aureus* results from poor tolerance of prolonged suspension in PBS, we characterized the effect of arylomycin A-C$_{16}$ on rich-medium cultures of *S. aureus* treated with the bacteriostatic translational inhibitor tetracycline (Fig. 3C). While tetracycline alone had almost no effect on the viability of *S. aureus*, tetracycline in combination with arylomycin A-C$_{16}$ resulted in a greater-than-10^3-fold reduction in viability.

Contribution of SPase expression levels. To determine whether SPase expression levels affect arylomycin susceptibility, arylomycin MICs were determined for *E. coli* underexpressing wild-type (arylomycin-resistant) SPase (PAS0162) or overexpressing either of the arylomycin-sensitive P84L (PAS0234) or P84S (PAS0275) mutants (Table 2). The controlled underexpression of wild-type SPase failed to yield detectable arylomycin sensitivities (MIC > 64 μg/ml), even at levels that reduced the growth rates by over 80% (see Fig. S1 in the supplemental material). However, underexpression of the sensitive P84S mutant resulted in a decrease of the arylomycin A-C$_{16}$ MIC of up to 8-fold, whereas overexpression of either P84L or P84S mutants resulted in an 8-fold increase of the MIC, demonstrating that target overexpression can yield at least modest levels of resistance. Antibiotics whose activity decreases with target overexpression typically demonstrate dominant resistance when both resistant and susceptible alleles of the target are expressed. Consistently, an *E. coli* strain expressing a wild-type SPase chromosomally and the susceptible (P84L) SPase ectopically was completely resistant to arylomycin A-C$_{16}$ (Table 2).

Antibiotic interactions. To examine how the presence of other antibiotics affects the MIC of arylomycin, checkerboard MIC experiments were performed with PAS0260 and PAS8001 and minimal and maximal FIC indexes were determined (Table 3; see Fig. S2 in the supplemental material). Against *E. coli*, we observed no significant interactions between arylomycin A-C$_{16}$ and erythromycin, polymyxin B, trimethoprim, or ciprofloxacin. In contrast, arylomycin A-C$_{16}$ showed mild synergism with cephalexin, pronounced synergism with rifampin and gentamicin, and antagonism with the translational inhibitor tetracycline. Against *S. aureus*, tetracycline, erythromycin, and vancomycin each interacted

PAS0260 and PAS8001 cultures were grown to stationary phase in rich media (MHBII), diluted in PBS to a density of 1 × 10^6 CFU/ml, and then treated with various concentrations of arylomycin A-C$_{16}$ for a period of 18 h (Fig. 3A and B). Under these conditions,

TABLE 2 Effects of SPase expression levels on the arylomycin A-C$_{16}$ sensitivity of *E. coli*

aTc concn (ng/ml)	Arylomycin A-C$_{16}$ MIC (mg/ml) for strain[a]:						
	MG1655 (NEV/Wt)	PAS0162 (Wt/NCS)	PAS0260 (NEV/P84L)	PAS0275 (P84L/P84L)	PAS0232 (NEV/P84S)	PAS0234 (P84S/P84S)	PAS0216 (P84S/NCS)
0	>64	>64	1	2	8	16	1
4	>64	>64	1	2	8	>64	2
8	>64	>64	1	4	8	>64	4
16	>64	>64	1	8	8	>64	64
32	>64	>64	1	16	8	>64	>64
64	>64	>64	1	16	8	>64	>64
128	>64	>64	1	16	8	>64	>64

[a] For each strain, ectopic/chromosomal SPases are in parentheses. Wt, wild-type SPase; P84L and P84S, arylomycin-sensitive mutants (see the text); NEV, no SPase expression vector; NCS, no chromosomal SPase.

TABLE 3 MICs of antibiotics alone and FIC indexes[a] of antibiotics in combination with arylomycin A-C_{16}

	E. coli PAS0260			S. aureus PAS8001		
Antibiotic	MIC (μg/ml)	FIC$_{min}$	FIC$_{max}$	MIC (μg/ml)	FIC$_{min}$	FIC$_{max}$
Cephalexin	12	0.70 ± 0.19	1.14 ± 0.07	4	0.65 ± 0.06	1.38 ± 0.42
Gentamicin	1	0.55 ± 0.11	0.91 ± 0.25	0.5	0.46 ± 0.12	0.83 ± 0.22
Tetracycline	1.5	1.10 ± 0.06	2.68 ± 0.89	0.25	0.73 ± 0.06	1.36 ± 0.40
Erythromycin	128	0.90 ± 0.21	1.28 ± 0.14	1	0.79 ± 0.26	1.15 ± 0.06
Trimethoprim	0.25	0.90 ± 0.21	1.58 ± 0.50	2	1.05 ± 0.03	2.55 ± 1.02
Ciprofloxacin	0.023	0.80 ± 0.11	1.23 ± 0.06	0.25	0.63 ± 0.08	1.39 ± 0.38
Rifampin	24	0.50 ± 0.00	1.03 ± 0.22	0.008	1.06 ± 0.00	2.85 ± 1.25
Polymyxin B	1	0.78 ± 0.30	1.15 ± 0.32	—	—	—
Vancomycin	—[b]	—	—	2	0.80 ± 0.11	1.23 ± 0.06
Arylomycin A-C_{16}	1	—	—	4	—	—

[a] FIC$_{min}$ and FIC$_{max}$, minimum and maximum FIC indexes observed, respectively. Values are averages ± standard deviations.
[b] —, not determined.

additively with arylomycin A-C_{16}, while rifampin and trimethoprim showed pronounced antagonism. There was mild synergism between arylomycin A-C_{16} and ciprofloxacin or cephalexin with *S. aureus*, and as with *E. coli*, pronounced synergism between arylomycin A-C_{16} and gentamicin.

DISCUSSION

Protein secretion is an essential process in all bacteria, and the general secretory pathway, consisting of the SecA motor protein and the SecYEG channel, is universally conserved in bacteria (7–9, 38). Due to its essential role in this process (24, 37), as well as its relatively accessible location, SPase has long engendered interest as a potential antibiotic target (29, 31, 46). The arylomycin class of natural products is the only known class of inhibitors of SPase that exhibit significant antibiotic activity. While this activity is limited in many organisms due to a mutation in SPase, the arylomycins are active against representative organisms from four bacterial phyla, including Gram-negative and Gram-positive pathogens, suggesting that with optimization, the arylomycins could have broad-spectrum activity (45), and indeed several derivatives with increased potency against *S. aureus* have recently been identified (34, 36). To better understand the effects of arylomycin-mediated SPase inhibition, we examined how its activity depends on its concentration, bacterial growth and density, SPase expression levels, and the presence of unrelated classes of antibiotics.

To begin to understand how cells arrested with arylomycin A-C_{16} cope with the stresses associated with SPase inhibition, we examined the effect of arylomycin A-C_{16} on cell viability as a function of exposure time. Against rapidly growing *S. aureus*, arylomycin A-C_{16} is largely bacteriostatic, but under nutrient-depleted conditions or following growth arrest by tetracycline, exposure to arylomycin A-C_{16} induces a greater-than-10^3-fold decrease in viability. It is possible that, even in the presence of tetracycline at twice the MIC or during nutrient starvation, cells still produce proteins at some reduced level and that the secretion of these proteins is essential to tolerating these stresses. Alternatively, tetracycline or nutrient starvation may prevent the production of some protein(s) required to tolerate the inhibition of SPase. Consistent with the latter possibility, we recently demonstrated that arylomycin A-C_{16} induces the expression of three proteins in *S. aureus*, PrsA, HtrA, and SAOUHSC_01761 (40). Although little is known about SAOUHSC_01761, PrsA and HtrA are conserved in

Gram-positive bacteria and are thought to facilitate the folding and maturation of secreted proteins and the degradation of proteins that misfold during secretion (4, 12, 15, 17, 25, 32, 39, 42, 48). Thus, the activity of the arylomycins may be bacteriostatic against *S. aureus* due to the production of these, and possibly other, proteins that help mitigate the stress associated with inhibited protein secretion.

Interestingly, the effect of arylomycin A-C_{16} on viability is notably different with *E. coli*, where bactericidal activity against rapidly growing cells is observed, but not under nutrient-deprived conditions. It has recently been shown that inhibition of ribosomes involved in cotranslation/translocation induces blockage of SecYEG secretion channels and activates a specific FtsH-mediated cell death pathway (52). Similarly, if SPase directly associates with the SecYEG secretion channel, this pathway could be responsible for the observed bactericidal activity. In this case, increased levels of SPase expression should increase toxicity, as observed with other antibiotics that generate toxic products upon binding their targets (18). In contrast, we find that under- or overexpressing an arylomycin-susceptible SPase in *E. coli* increases or decreases sensitivity, respectively. Moreover, the resistance phenotype is dominant in cells simultaneously expressing both susceptible and resistant variants of SPase. These data argue against an FtsH-mediated process and suggest that the bactericidal activity of the arylomycins results from protein mislocalization in the cytoplasmic membrane. Indeed, in Gram-negative bacteria, which must maintain a protein-rich periplasm and outer membrane, SPase processes a far greater number of proteins than in Gram-positive organisms (47). It remains to be determined whether cell death ultimately results from an excessive accumulation of unprocessed preproteins in the membrane, possibly eventually causing depolarization, or from misregulation of essential periplasmic and outer-membrane processes. Regardless of the observed differences between *E. coli* and *S. aureus*, when bactericidal activity is observed in either organism, the kinetics of cell death are slow and relatively independent of arylomycin A-C_{16} concentration. This is consistent with the antibiotic activity resulting solely from the inhibition of SPase and argues against the nonspecific membrane depolarization mechanisms that have been associated with other classes of lipopeptide antibiotics (49).

The interactions between two antibiotics can often provide mechanistic insight into their activities that is not apparent when

measuring their independent effects on growth (11, 53). Antibiotics that inhibit pathways that mediate the deleterious effects of SPase inhibition are expected to antagonize arylomycin A-C$_{16}$ activity. In contrast, agents whose mechanisms of action increase the cellular dependence on SPase are expected to result in synergistic interactions with arylomycin A-C$_{16}$. Indeed, by chemical (16; M. Gallant et al., WIPO patent application WO/2011/112441 [http://patentscope.wipo.int/search/en/WO2011112441]) and genetic (21) methods, SPase inhibition in methicillin-resistant *S. aureus* (MRSA) has recently been shown to be synergistic with the activity of the carbapenem class of antibiotics, suggesting that increased protein secretion plays a critical role in the MRSA phenotype. With notable exceptions, we find that arylomycin A-C$_{16}$ shows no interaction or mild antagonism with antibiotics that inhibit DNA, RNA, or protein synthesis. Interestingly, antagonism, where it was observed, was always manifest as an increase in the arylomycin A-C$_{16}$ MIC in the presence of sublethal concentrations of the second antibiotic (see Fig. S2 in the supplemental material). Sublethal inhibition of DNA, RNA, or protein synthesis induces specific stress response pathways (6), and facets of these responses might help alleviate arylomycin-induced stress. Reduced growth rates induced by these agents could also minimize the production of proteins that require translocation across the cytoplasmic membrane and thereby reduce the burden on the general secretion pathway. Similar antagonism between DNA and protein synthesis inhibitors has been attributed to an inability of cells to properly downregulate protein production in response to DNA damage (1). In contrast to the antagonism observed with *S. aureus*, the simultaneous inhibition of RNA synthesis and secretion results in synergistic sensitivity in *E. coli*. Although this synergism could result from an increased penetrance of rifampin following arylomycin-mediated membrane damage, similar interactions were not observed with the hydrophobic antibiotic erythromycin, suggesting that specific target-mediated effects may be involved. The modest synergy observed with arylomycin A-C$_{16}$ and cell wall synthesis inhibitors is consistent with the significant number of secreted proteins that are known to be required for cell wall biosynthesis (22, 27). However, vancomycin, a Gram-positive organism-specific inhibitor of cell wall biosynthesis, does not interact synergistically with the arylomycins, demonstrating that more-nuanced mechanisms are likely involved.

The strongest interaction with both *E. coli* and *S. aureus* was a synergy between arylomycin and the bactericidal aminoglycoside gentamicin. The aminoglycosides bind the 30S ribosomal subunit, and while the antibiotic activity of the aminoglycosides results in part from the inhibition of protein synthesis, they also induce mistranslation, which results in the production of toxic peptides (26). Indeed, aminoglycoside-induced mistranslation of *E. coli* membrane proteins has been shown to induce malfunctions in the electron transport complex, which in turn induce oxidative damage and a periplasmic membrane stress response (19). Thus, the observed synergy between the arylomycin and the aminoglycoside could result from the compounded stress of mistranslated integral membrane proteins and the accumulation of unprocessed preproteins. The mechanism of arylomycin and aminoglycoside synergy warrants further study, since the use of combinations of antibiotics is attracting increasing attention as a method to improve efficacy and decrease susceptibility to resistance. The latter may be particularly important for antibiotics inhibiting single targets that may be prone to single-point-mutation-mediated resistance (43).

The mechanism of arylomycin activity is complex and not yet fully understood. For example, the activity of the arylomycin against both *E. coli* and *S. aureus* is relatively independent of culture density, which is curious given that arylomycin presumably localizes to the bacterial membrane such that higher densities of bacteria should dilute its concentration. Clearly, the parameters affecting arylomycin activity, including its localization, need to be further defined. Despite our incomplete understanding, it appears likely that the antibacterial activity of the arylomycins results from the accumulation of preproteins in the cytoplasmic membrane and/or the depletion of essential proteins in the periplasm, outer membrane, or extracellular milieu and that Gram-negative and Gram-positive bacteria respond very differently to these perturbations. This likely reflects the fundamentally different roles of secretion in these pathogens and, along with previously published phylogenetic data which suggest that *E. coli* and *S. aureus* may each have encountered naturally produced arylomycins in the past (45), might also suggest that these bacteria evolved different ways of coping with the associated secretion stress. Moreover, the data suggest that, if developed as a therapeutic, the arylomycin scaffold may have promising activity, especially when coadministered with an aminoglycoside.

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