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Origins of *Yersinia pestis* Sensitivity to the Arylomycin Antibiotics and the Inhibition of Type I Signal Peptidase

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***Yersinia pestis* is the etiologic agent of the plague. Reports of *Y. pestis* strains that are resistant to each of the currently approved first-line and prophylactic treatments point to the urgent need to develop novel antibiotics with activity against the pathogen. We previously reported that *Y. pestis* strain KIM6+, unlike most *Enterobacteriaceae*, is susceptible to the arylomycins, a novel class of natural-product lipopeptide antibiotics that inhibit signal peptidase I (SPase). In this study, we show that the arylomycin activity is conserved against a broad range of *Y. pestis* strains and confirm that it results from the inhibition of SPase. We next investigated the origins of this unique arylomycin sensitivity and found that it does not result from an increased affinity of the *Y. pestis* SPase for the antibiotic and that alterations to each component of the *Y. pestis* lipopolysaccharide—O antigen, core, and lipid A—make at most only a small contribution. Instead, the origins of the sensitivity can be traced to an increased dependence on SPase activity that results from high levels of protein secretion under physiological conditions. These results highlight the potential of targeting protein secretion in cases where there is a heavy reliance on this process and also have implications for the development of the arylomycins as an antibiotic with activity against *Y. pestis* and potentially other Gram-negative pathogens.**

The emergence of multidrug-resistant bacteria, especially Gram-negative pathogens, is a major health concern that can be combated only by the continued development of new antibiotics, particularly ones that act via novel mechanisms of action to limit the potential for cross-resistance. *Yersinia pestis*, a Gram-negative bacterium and the causative agent of plague, is of particular historical significance due to the mortality and social havoc wreaked by at least three major pandemics (1, 2). Today, plague continues to pose a threat, even in developed countries (3), and while *Y. pestis* infections are now treatable with available antibiotics, delays in the initiation of effective therapy, as can be caused by resistance to the employed antibiotic, results in significantly increased mortality (4). Thus, reports of *Y. pestis* strains that are resistant to first-line antibiotic therapies (5–7) are troubling, and the all but certain continued evolution of these strains toward resistance to all available antibiotics threatens to return the plague agent to its historical position as an important pathogen. This threat is unlikely to abate until new, effective antibiotics are discovered and developed; however, no new class of antibiotics with activity against Gram-negative bacteria has been approved in over 40 years (8). Moreover, a recent report by the Infectious Diseases Society of America (IDSA) identified only seven new candidate antibiotics that have progressed into clinical development for the treatment of multidrug-resistant Gram-negative bacilli since 2010 (9).

A general challenge in developing antibiotics against Gram-negative bacteria is the barrier to penetration provided by the lipopolysaccharide (LPS) leaflet of the outer membrane (OM) (10–12). Generally, LPS consists of three parts—the O antigen, the core oligosaccharide, and lipid A—and metal-mediated cross-linking results in the formation of a tightly packed, extended structure. While small hydrophilic molecules may pass through the outer membrane via porins, larger and more hydrophobic molecules must traverse it passively through exposed membrane phospholipids, which are largely precluded by the tight packing of the LPS (10, 11).

The arylomycins are a promising family of natural-product antibiotics that inhibit type I signal peptidase (SPase) (13–15). SPase is highly conserved and essential in both Gram-negative and Gram-positive bacteria because it is required to release proteins from their membrane-bound N-terminal leader sequences after translocation across the cytoplasmic membrane via the Sec or Tat translocation pathway (16, 17). Thus, it was somewhat surprising that the arylomycins were initially reported to possess activity against only a narrow spectrum of Gram-positive bacteria (18, 19). However, a more extensive characterization, made possible by the total synthesis of several arylomycin variants (20–24), including arylomycin A-C_{16} (Fig. 1), revealed that they are active against a broad range of Gram-positive and Gram-negative bacteria and that the majority of the observed resistance was associated with the idiosyncratic presence of a specific proline residue in SPase (14, 25). While this proline appears to universally reduce sensitivity, the absolute values of the sensitivities are species and sometimes even strain specific (14, 25), suggesting that other factors also contribute. Of particular note, despite encoding an SPase with the resistance-conferring proline, *Y. pestis* KIM6+ is sensitive to arylomycin A-C_{16}, with a MIC of 4 μg/ml (14).

Here, we show that the arylomycins have activity against a

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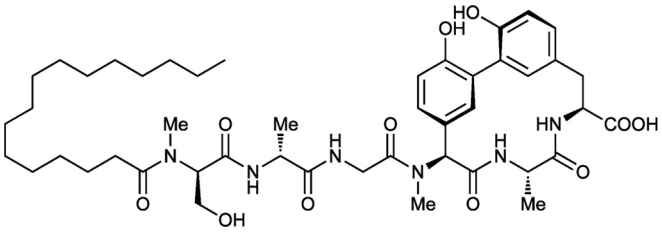

FIG 1 Structure of arylomycin A-C_{16}.

broad range of *Y. pestis* strains and that the activity indeed results from the potent inhibition of SPase. To investigate why *Y. pestis* is sensitive while related *Enterobacteriaceae*, such as *Escherichia coli*, are resistant, we determined the contributions of different factors that are unique to *Y. pestis*. We found that the sensitivity does not result from a greater affinity of the arylomycins for *Y. pestis* SPase and that its unique LPS is insufficient on its own to explain the sensitivity. In contrast, we found that deletion of the gene encoding the highly expressed cell adhesion protein Ail (locus tag y1324; UniProt entry Q8D0Z7) significantly alleviates sensitivity and that overexpression of Ail or *E. coli* maltose-binding protein then restores sensitivity, suggesting that it results from a high secretion burden at physiological temperatures. These results highlight the potential of the arylomycins as antibiotics for the treatment of *Y. pestis*, and potentially other Gram-negative bacteria, especially those that rely on high levels of protein secretion for viability or virulence.

MATERIALS AND METHODS

Medium and antibiotics. Bacteria were routinely grown at 28°C or 37°C, as appropriate, on Mueller-Hinton II agar (MHIIA) or in cation-adjusted Mueller-Hinton II broth (CAMHB). Antibiotic stock solutions were prepared at the following concentrations: arylomycin A-C_{16}, 10 mg/ml (in dimethyl sulfoxide [DMSO]); actinomycin D (in DMSO), 10 mg/ml; deoxycholic acid (in 1:1 CAMHB-H_2O), 250 mg/ml; polymyxin B nonapeptide (PMBN) (in H_2O), 10 mg/ml; L-arabinose (in CAMHB), 50% (wt/vol); tetracycline (in ethyl alcohol [EtOH]), 1 mg/ml; and gentamicin (in H_2O), 1 mg/ml. Arylomycin A-C_{16} was synthesized as described previously (20). PMBN and deoxycholic acid were obtained from Sigma-Aldrich (St. Louis, MO), actinomycin D was obtained from Fisher Scientific (Pittsburgh, PA), L-arabinose and gentamicin sulfate were obtained from MP Biomedicals (Solon, OH), and tetracycline hydrochloride was obtained from Fisher Bioreagents (Fairlawn, NJ).

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1 (also see Table 3). *E. coli* BAS901 is a hyperpermeable strain harboring the *lptD* mutation, which results in defective LPS assembly. In strains PAS0260 and DBS600, the Pro residue in the SPase responsible for arylomycin resistance was replaced by a residue that confers arylomycin sensitivity; the resulting SPases are LepB(P84L) in *E. coli* strain PAS0260 and LepB(P91S) in *Y. pestis* strain DBS600. Strain *Y. pestis* KIM5-pLpxL (which expresses a hexa-acylated lipid A) and its parental strain were kindly provided by Egil Lien (University of Massachusetts Medical School). Strain RS 058, a *Y. pestis* strain that harbors the *E. coli lepB* gene in the pBAD vector, was kindly provided by RQX Pharmaceuticals (La Jolla, CA). The KIM6+ *phoP* knockout strain was kindly provided by NIAID Rocky Mountain Laboratories (Crested Butte, CO).

Cloning and expression of SPase. The primers used in this study are listed in Table 2. Plasmids for the expression of the wild-type *Y. pestis* SPase were constructed by amplifying the DNA corresponding to the soluble C-terminal fragment of the enzyme and cloning it into the previously constructed pET23b expression vector containing full-length *E. coli* SPase (14) using the polymerase incomplete primer extension method (26) with the primer pairs vP_EclepBpET-1/vP_EclepBpET-2 and iP_YplepB-3/ip_YplepB-4. This resulted in the replacement of the homologous *E. coli* SPase soluble C-terminal fragment and the generation of a chimeric construct consisting of an N-terminal His tag, several residues of *E. coli* SPase, and, finally, the soluble, active fragment of *Y. pestis* SPase (pET23b-YplepB). To express the P91S variant of this construct, the LepB(P91S) mutation was introduced into pET23b-YplepB using site-directed mutagenesis by inverse PCR with Phusion polymerase (New England BioLabs) and the primer pair 5′_QC_P91S_protein/3′_QC_P91S_protein.

His-tagged *Y. pestis* SPase proteins were expressed in BL21(DE3) containing pET23b-YplepB and pET23b-YplepB(P91S) and purified as described previously (14). Briefly, expression was induced with IPTG (isopropyl-β-D-thiogalactopyranoside), and the protein was recovered using Ni-nitrilotriacetic acid (NTA) Superflow resin (Qiagen) in the presence of 1% Elugent detergent (CalBioChem). Fractions were analyzed by SDS-PAGE to identify those with single-band purity, which were then combined, concentrated, and used in subsequent binding assays.

***In vitro* K_D measurements.** Binding assays were done essentially as described previously (13, 14) using fluorescence quenching of arylomycin. Briefly, a 384-well plate was prepared, with wells containing a constant SPase concentration of 50 nM and variable concentrations of drug (ranging from 2 to 8,000 nM) and a control well containing 125 nM the inhibitor saturated with a 6-fold molar excess of enzyme. The assay buffer for both the *E. coli* and *Y. pestis* proteins was as follows: 100 mM NaCl, 20 mM Tris-HCl (pH 7.4), 1 mM EDTA, 1% *n*-octyl-β-glucopyranoside (Anatrace). The plates were incubated in the dark at room temperature for 240 (±30) min. The fluorescence intensity (excitation [λ_{ex}], 280 nm; emission [λ_{em}], 405 nm) was corrected for the intrinsic fluorescence intensity of free protein and inhibitor, and the resulting amplitude as a function of the inhibitor concentration was fitted to the quadratic equation describing two-state equilibrium binding to yield equilibrium dissociation constant (K_D) values. The K_D values, as well as the total protein concentration, were determined by direct fitting of the data using a nonlinear least-squares fitting algorithm (27). The reported K_D values are the averages of the results of at least three independent experiments and are reported with their standard deviations (SD). The precision with which the K_D is determined using the nonlinear least-squares method is typically taken to be within a factor of 2 (27). The binding data are also presented graphically using semilog plots, where the data points and error bars represent the average values and standard deviations from the results of independent experiments.

Construction of mutant *Y. pestis* strains. The *Y. pestis* LepB(P91S) mutant strain DBS600 was constructed via allelic exchange using previously described methods (28). Briefly, the point mutation was introduced using overlap PCR with the primer pairs 5′_lepB_flanking_ApaI/3′_P91S_mutant_strain and 5′_P91S_mutant_strain/3′_ lepB_flanking_XbaI, and the amplified DNA was cloned into the ApaI-XbaI site of the suicide vector pKNG101. The construct was propagated in and recovered from the *E. coli pir*$^+$ DH5α strain BW27343. The recovered construct was transformed into electrocompetent *Y. pestis* KIM6+ prepared using a previously reported method (29). Colonies in which allelic exchange occurred were selected on 5% sucrose.

The full-length *ail* deletion in strain DBS600, which expresses LepB(P91S), and insertion of a kanamycin resistance cassette flanked by FLP recognition sequences to generate strain DBS601 were accomplished using lambda Red-mediated recombination as described by Datsenko and Wanner (30). The PCR products used to construct gene replacements were generated using the template plasmid pKD4 and the oligonucleotide primers 5′_ail_KAN and 3′_ail_KAN. The PCR product was purified using the DNA Clean and Concentrator kit (Zymo Research, Irvine, CA) and treated with DpnI. Electrocompetent DBS600 carrying plasmid pKD46, encoding the Red recombinase, were prepared using the same method described above (29) with the modification of induction with 0.2% L-arabinose for 2 h prior to harvest. Competent cells were electro-

TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
Strains		
BW27343	*E. coli* DH5α *pir*$^+$	CGSC
MG1655	*E. coli* K-12 F$^-$ λ$^-$ *ilvG rbf-50 rph-1*	75–77
PAS0260	MG1655 LepB(P84L)::Kan^r	14
KIM6+	*Y. pestis* pCD1$^-$ pMT1$^+$ pPCP1$^+$ *pgm*$^+$	78
DBS600	KIM6+ expressing LepB(P91S)	This study
ATCC 23715	*Y. enterocolitica* serotype 8	79
BAS901	*E. coli lptD4213*) mutant	80
RS 058	KIM6+ containing pBAD (*E. coli* K-12 *lepB*)	RQX Pharmaceuticals
D21	*E. coli* F$^-$ *proA23 lac-28 tsx-81 trp-30 his-51 rpsL173*(*strR*) *ampCp-1*	81, CGSC
D21e7	*E. coli* F$^-$ *proA23 lac-28 tsx-81 trp-30 his-51 rpsL173*(*strR*) *rfa-1 ampCp-1*	82, CGSC
D21e19	*E. coli* F$^-$ *proA23 lac-28 tsx-81 trp-30 his-51 rpsL173*(*strR*) *rfa-11 ampCp-1*	82, CGSC
D21f1	*E. coli* F$^-$ *proA23 lac-28 tsx-81 trp-30 his-51 rpsL173*(*strR*) *rfa-21 rfa-1 ampCp-1*	81, CGSC
D21f2	*E. coli* F$^-$ *proA23 lac-28 tsx-81 trp-30 his-51 rpsL173*(*strR*) *rfa-31 rfa-1 ampCp-1*	81, CGSC
KIM5	*Y. pestis* KIM6 pCD1Ap pMT1$^+$ pPCP1$^+$ *pgm* mutant Ap^r	83
KIM5-pLpxL	KIM5 containing pBR322(*E. coli* K-12 *lpxL*)	E. Lien (University of Massachusetts Medical School) (53)
KIM6+ Δ*phoP*	KIM6+ lacking *phoP*	Rocky Mountain Laboratories
DBS601	KIM6+ expressing LepB(P91S)::Kan^r lacking *ail*	This study
DBS601- pBAD/His B	DBS601 complemented with pBAD/HisB (empty)	This study
DBS601- pBAD/His B-*ail*	DBS601 complemented with pBAD/HisB vector (*Y. pestis* KIM6+ *ail*)	This study
DBS601- pBAD/His B-*malE*	DBS601 complemented with pBAD/HisB vector (*E. coli* K-12 *malE*)	This study
Plasmids		
pET23b	Expression vector; T7 promoter; His_6 tag	EMD Millipore
pET23b-EclepB	pET23b harboring *E. coli* K-12 *lepB*	14
pET23B-YplepB	pET23b harboring a chimeric *E. coli* K-12/*Y. pestis* KIM6+ *lepB* gene	This study
pET23b- YplepB(P91S)	pET23b harboring a mutated, chimeric *E. coli* K-12/*Y. pestis* KIM6+ *lepB* gene that encodes the P91S substitution	This study
pKNG101	Suicide vector; *sacB*	28
pKNG101- YplepB(P91S)	pKNG101 harboring a mutated *Y. pestis* KIM6+ *lepB* gene that encodes the P91S substitution	This study
pKD4		30; Addgene plasmid no. 45605
pKD46		30; CGSC
pCP20		30; CGSC
pBAD/His B	Expression vector; *ara*BAD promoter	Invitrogen
pBAD/His B-*ail*	pBAD/HisB harboring *Y. pestis* KIM6+ *ail*	This study
pBAD/His B-*malE*	pBAD/HisB harboring *E. coli* K-12 *malE*	This study

porated with the purified and DpnI-treated PCR product, plated on LB agar containing 35 μg/ml of kanamycin, and grown at 30°C. To verify deletion of *ail* and replacement with the kanamycin cassette, colony PCR was conducted using the primers 5′_ail_KAN_conf and 3′_ail_KAN_conf. The pKD46 plasmid was cured by growth at 37°C.

Complementation plasmid construction. Plasmids for the expression of the *Y. pestis ail* and *E. coli malE* genes were constructed by amplifying the corresponding DNA from *Y. pestis* KIM6+ or *E. coli* K-12 MG1655 using the primer set 5′_pBAD_ail_KpnI/3′_pBAD_ail_HindIII or 5′_pBAD_malE_KpnI/3′_pBAD_malE_HindIII and cloning into the KpnI and HindIII sites of pBAD-HisB to generate pBAD/His B-*ail* and pBAD/HisB-*malE*. These plasmids were electroporated into DBS601 [LepB(P91S) Δ*ail*] to yield DBS601-pBAD/HisB-*malE* and DBS601-pBAD/HisB-*ail*. As a control strain, the empty pBAD/His B vector was also electroporated into DBS601 to yield DBS601-pBAD/HisB.

Susceptibility determinations. Antibiotic susceptibilities were determined for the strains listed in Table 1 by measuring MICs using the CLSI broth microdilution method (31). Briefly, 2-fold serial dilutions of antibiotics were prepared in 96-well plates containing 100 μl of CAMHB. Bacterial inocula were prepared by suspending colonies grown for 48 h (for *Y. pestis*) and 16 to 20 h (for all other strains) to a final density of 1×10^7 CFU per ml in CAMHB. Wells containing the antibiotic dilutions were inoculated to a final density of 5×10^5 CFU/ml, and the MICs were defined as the lowest drug concentration at which no visible growth occurred following 24 to 48 h (for *Y. pestis*) and 16 to 20 h (for all other strains) of incubation at 20°C, 28°C, or 37°C, as appropriate. All susceptibility determinations were performed at least in triplicate.

MICs were determined for the panel of virulent *Y. pestis* isolates listed in Table 3 by the broth microdilution method in 96-well plates according to CLSI guidelines. Bacterial inocula for *Y. pestis* strains were prepared by suspending in CAMHB colonies from isolates grown aerobically at 35°C on sheep blood agar (SBA) plates for 42 to 48 h. The suspended cultures were diluted with CAMHB to a bacterial cell density of 10^5 CFU/ml using 0.5 McFarland standard. Antibiotics were serially diluted 2-fold in 50 μl of CAMHB, and 50 μl of the adjusted bacterial dilution was added to each well of the 96-well plate. The plates were incubated at 35°C. MICs were determined visually at 42 to 48 h and also by optical density (OD) (600 nm; SpectroMax M2 plate reader; Molecular Devices). Susceptibility determinations were performed in triplicate.

Arabinose-induced SPase overexpression. The contribution of increased SPase expression to arylomycin susceptibility was determined by inoculating strain RS 058 into 96-well plates containing a checkerboard of 2-fold dilutions of arylomycin A-C_{16} and L-arabinose. To induce overexpression of the *E. coli* SPase in this *Y. pestis* strain, colonies were first

TABLE 2 Primers used in this study

Primer name	Sequence (5′→3′)
vP_EclepBpET-1	ACCGGTTTCCAGCCAGCCAGGCTTCGGCGCAACCTTTTTC
vP_EclepBpET-2	TGGCCAACGGGTGTGCGCTTAAGTCGCATTGGCGGCATCCAT
iP_YplepB-3	TGGCTGGAAACCGGTGCCTCTATCTTCCCGGTGCTGGCCTTG
iP_YplepB-4	CACACCCGTTGGCCATTCACCTTCTTGCTTTTCAAAACTC
5′_QC_P91S_protein	GTTCGTTTATTTACGAGTCATTCCAGATCCCTTCTGG
3′_QC_P91S_protein	CCAGAAGGGATCTGGAATGACTCGTAAATAAACGAAC
5′_lepB_flanking_ApaI	TTGGTTGGGCCCGGCGATAAAGTTAAGGTTATGAGTACCGGCC
3′_lepB_flanking_XbaI	AATCTAGAGGCCCGCATACGACTCATATCCCC
5′_P91S_mutant_strain	CGTGCGTTCGTTTATTTACGAGTCATTCCAGATCCCTTCTGGTTCGATGATGCC
3′_P91S_mutant_strain	GGCATCATCGAACCGAAGGGATCTGGAATGACTCGTAAATAAACGAACGGACG
5′_ail_KAN_pKD4	TGTCAGATATTTGTTAATATTTGGCTGGCCACTTTAGTCTGTGTAGGCTGGAGCTGCTTC
3′_ail_KAN_pKD4	GAAACCACCATTATGGTGGGTTTTCATGGTTAGGAGGACGCATATGAATATCCTCCTTAG
5′_ail_KAN_conf	TTCGAATTTATTCCGCGAGGCAGT
3′_ail_KAN_conf	ACGCGGTGAAGGACGGATTCT
5′_pBAD_ail_KpnI	TTGGTTGGTACCGTGGTTACTGTATTAGGTATTGTT
3′_pBAD_ail_HindIII	AACCAAAAGCTTTTAGAACCGGTAACCCG
5′_pBAD_malE_KpnI	TTGGTTGGTACCATGAAAATAAAAACAGGTGC
3′_pBAD_malE_HindIII	AACCAAAAGCTTTTACTTGGTGATACGAGTCTG

inoculated into CAMHB supplemented with 0.2% L-arabinose and 5 mM $CaCl_2$ and incubated at 37°C with shaking for 2 to 3 h. The cultures were diluted to a final density of 1×10^7 CFU per ml in fresh CAMHB and inoculated into test wells to a final density of 5×10^5 CFU/ml. The plate was read for growth following 24 h of incubation at 37°C. KIM6+ transfected with an empty pBAD vector was used as a control. The checkerboard analyses between arylomycin A-C_{16} and L-arabinose were performed at least in triplicate.

TABLE 3 Strain identifiers and MICs of arylomycin A-C_{16} and ciprofloxacin for the 30-member *Y. pestis* panel[a]

Y. pestis strain	MIC (μg/ml)	
	Arylomycin A-C_{16} (MIC_{50} = 8; MIC_{90} = 32)	Ciprofloxacin (MIC_{50} = 0.03; MIC_{90} = 0.03)
CO92	8	0.03
C12	4	0.06
Antiqua	8	0.015
Pestoides B	4	0.015
Pestoides Fmp1	1	0.015
Yeo154	8	0.03
Angola	8	0.015
Java9	8	0.03
M111(74)	8	0.03
LaPaz	8	0.03
195P mp1	8	0.03
T26 mp3	4	0.03
KIM 10	32	0.03
Pest E	8	0.015
RFPBM 19	8	0.03
PeXu 429	2	0.03
Yokohama	8	0.03
Nicholisk 41	32	0.03
Nairobi	16	0.015
South Park	16	0.03
Cambodia	16	0.03
27	8	0.03
31	8	0.03
390	1	0.015
590	8	0.03
25	32	0.06
316	>64	0.06
366	8	0.015
Harbin 35	4	0.008
Pest C	8	0.015

[a] MIC_{50} and MIC_{90} are the drug concentrations that inhibit growth of 50% and 90% of the isolates tested in this panel, respectively.

Arabinose-induced complementation. To determine the effect of ectopic expression of *E. coli* MalE and *Y. pestis* Ail on the MICs of DBS601 [LepB(P91S) Δ*ail*], colonies of DBS601, DBS601-pBAD/HisB, DBS601-pBAD/HisB-*malE*, and DBS601-pBAD/HisB-*ail* were inoculated into CAMHB supplemented with 4 mM $CaCl_2$ (plus 50 μg/ml ampicillin for the pBAD/HisB-containing strains) and incubated at 37°C with shaking overnight. The cultures were diluted 1:40 in corresponding fresh medium and incubated at 37°C with shaking for 2 to 3 h. The cultures were diluted to a final density of 1×10^7 CFU per ml in fresh CAMHB and inoculated into test wells to a final density of 5×10^5 CFU/ml. Testing was conducted in 96-well plates containing serial 2-fold dilutions of arylomycin A-C_{16} and an L-arabinose concentration of 0, 0.2, or 2% and performed at least in triplicate. The plates were read for growth following 24 h of incubation at 37°C.

Arylomycin A-C_{16}–polymyxin B nonapeptide checkerboard analysis. The contribution of increased membrane permeability to arylomycin susceptibility was determined by inoculating 96-well plates containing a checkerboard of 2-fold dilutions of arylomycin A-C_{16} and PMBN with *E. coli* strain PAS0260 harboring LepB(P84L), *E. coli* strain BAS901, *Y. pestis* KIM6+, *Y. pestis* strain DBS600 harboring LepB(P91S), *Y. pestis* KIM5, and *Y. pestis* KIM5-pLpxL. Bacterial inocula were prepared by suspending colonies grown for 48 h (for *Y. pestis*) and 16 to 20 h (for all other strains) to a final density of 1×10^7 CFU per ml in CAMHB. All test wells were inoculated to a final density of 5×10^5 CFU/ml, and the plate was read for growth following 24 h of incubation at either 28°C or 37°C, as appropriate. The checkerboard analyses between arylomycin A-C_{16} and PMBN were performed at least in triplicate.

Synergy studies with protein synthesis inhibitors. The interaction of arylomycin and tetracycline or gentamicin was determined by inoculating the strains to be tested into 96-well plates containing a checkerboard of 2-fold dilutions of arylomycin A-C_{16} and either tetracycline or gentamicin. Bacterial inocula were prepared by suspending colonies grown for 48 h to a final density of 1×10^7 CFU per ml in CAMHB. All test wells were inoculated to a final density of 5×10^5 CFU/ml, and the plate was read for growth following 24 h of incubation at 37°C, as appropriate. The checkerboard analyses were performed at least in triplicate. Antibiotic interactions were determined by measuring the fractional inhibitory concentration (FIC) index of the microdilution checkerboard consisting of two

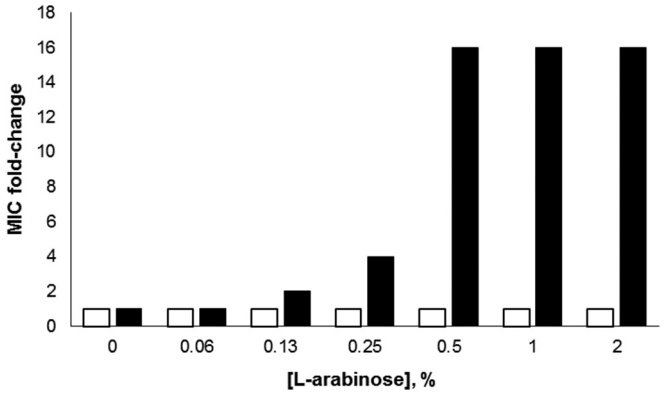

FIG 2 Arylomycin A-C_{16} MIC change for *Y. pestis* RS 058 (black bars), which contains pBAD-EcLepB, and a mock-transfected control *Y. pestis* containing pBAD-empty (white bars) as a function of increasing L-arabinose. The MICs for RS 058 and the mock-transfected *Y. pestis* in the absence of L-arabinose were 8 and 2 μg/ml, respectively. For RS 058, the arylomycin A-C_{16} MICs at 0.5 to 2% L-arabinose were >64 μg/ml. To calculate the fold change, the MICs were taken to be 128 μg/ml.

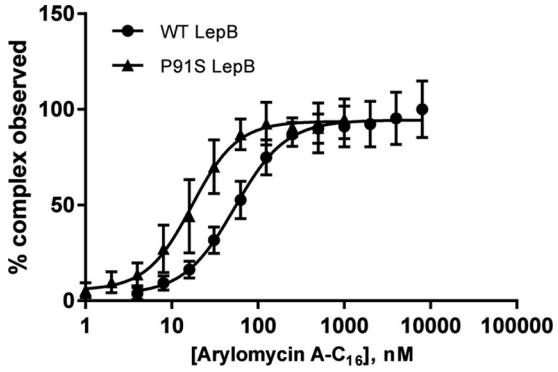

FIG 3 Equilibrium binding curves for wild-type and sensitized P91S *Y. pestis* LepB. The error bars represent SD.

drugs (for simplicity, drugs A and B). The FIC is the MIC of drug A or B in combination divided by the MIC of that drug alone, and the FIC index is defined as follows: $\Sigma FIC_{drug\ A} + FIC_{drug\ B}$. A FIC index of ≤0.5 denotes synergy, whereas a FIC index of ≥4 represents antagonism (32). The minimum and maximum FIC indexes are reported for each drug combination.

Determination of protein localization. The levels of membrane and periplasmic proteins were determined by isolating the cold-shocked cell and periplasmic fractions from cells grown with and without arylomycin A-C_{16} using a previously reported protocol with some modification (33). Briefly, cells were grown with shaking at 37°C overnight in tryptic soy broth (TSB) supplemented with 4 mM $CaCl_2$ to an OD of ~0.3. The cells were pelleted, resuspended in fresh medium, grown with or without arylomycin A-C_{16} (0.5× MIC) with shaking at 37°C for an additional 3 h, and then pelleted at 1,300 × *g* for 10 min at 4°C. The supernatant was discarded, and the pellet was gently resuspended in 1/4 the original culture volume of ice-cold sucrose buffer (50 mM Tris, pH 7.4, 1 mM EDTA, 20% [wt/vol] sucrose) and incubated on ice for 10 min. The resulting suspension was centrifuged at 5,200 × *g* for 10 min at 4°C. The supernatant was discarded, and the pellets were resuspended in 1/10 the original culture volume of ice-cold 5 mM $MgCl_2$. The incubation and centrifugation steps were repeated to yield the cold osmotic shock solution that contained the periplasmic proteins (supernatant) and the shocked-cell fraction that contained the membrane-bound proteins (pellet). The periplasmic fraction was precipitated with 10% trichloroacetic acid overnight and washed with cold 90% acetone, and the resulting protein pellet was resuspended in 50 mM NH_4HCO_3, 1% SDS. The shocked-cell fraction was treated with DNase and lysozyme and lysed by three cycles of freezing-thawing. The protein concentrations of the samples were determined using the Micro BCA Protein Assay kit (Life Technologies, Grand Island, NY) and were normalized by OD.

RESULTS

Susceptibility of *Y. pestis* to arylomycin A-C_{16}. We first characterized the susceptibility of *Y. pestis* to arylomycin A-C_{16} as a function of SPase activity by determining the sensitivity of a KIM6+ derivative strain (RS 058) that overexpresses *E. coli* SPase in *trans* under arabinose induction. A mock-transfected KIM6+ strain was assessed in parallel for comparison. The addition of arabinose alone did not significantly affect growth for either strain, and the addition of arabinose did not affect the arylomycin MIC of the mock-transfected strain. However, the arylomycin MIC values showed a clear concentration dependence for RS 058, with complete resistance (>64 μg/ml) observed at high arabinose concentrations (Fig. 2). These results confirm that the activity of the arylomycins against *Y. pestis* results from the inhibition of SPase.

Having verified the on-target activity of arylomycin with KIM6+, we sought to determine its spectrum of activity against a broad range of other strains using a panel of 30 natural isolates representing all three biovars and including strains CO92, Antiqua, KIM10, and Angola (Table 3). As a control, MIC values for ciprofloxacin were determined and found to range between 0.008 and 0.06 μg/ml, as expected. The arylomycin A-C_{16} MIC_{50} and MIC_{90} values for the panel were 8 and 32 μg/ml, respectively, demonstrating the conservation of arylomycin susceptibility across this broad range of *Y. pestis* strains.

To quantitatively compare the sensitivity of *Y. pestis* to that of *E. coli*, which is naturally resistant (thus precluding a quantitative measurement of sensitivity), we constructed a *Y. pestis* strain harboring the sensitivity-conferring SPase point mutation P91S (strain DBS600) for comparison with the analogously sensitized *E. coli* strain (P84L; strain PAS0260), which has an MIC of 1 to 2 μg/ml (14). The mutant *Y. pestis* strain has an arylomycin A-C_{16} MIC of 0.03 μg/ml, which is reduced 128-fold compared to the parental *Y. pestis* strain. Comparison with PAS0260 reveals that *Y. pestis* is 30- to 60-fold more sensitive than *E. coli* to arylomycin A-C_{16}.

The activity of the arylomycins against *Y. pestis* does not result from increased affinity for SPase. To investigate whether the sensitivity of *Y. pestis* relative to *E. coli* is caused by a greater affinity of its SPase for the arylomycins, we determined equilibrium binding constants using micelle-reconstituted recombinant SPase. Wild-type *Y. pestis* SPase and the arylomycin-sensitized P91S mutant were expressed in *E. coli* as chimeric constructs with the N-terminal 66 residues of the *E. coli* protein. These constructs preserve the entire catalytic domain of the *Y. pestis* enzyme but incorporate the *E. coli* signal sequence and membrane anchor, which our experience suggested would facilitate expression in *E. coli*.

Using a fluorescence-quenching assay, the binding constants ($K_D \pm$ SD) for arylomycin A-C_{16} were found to be 31 ± 14 nM for the wild-type *Y. pestis* protein and 6 ± 2 nM for the P91S variant (Fig. 3). These dissociation constants are similar to those determined for the *E. coli* protein and its corresponding P84S mutant

TABLE 4 MICs of actinomycin D, arylomycin A-C_{16}, and deoxycholic acid for Gram-negative strains determined at 37, 28, and 20°C

Temp (°C)	Strain	MIC (μg/ml)		
		Actinomycin D	Arylomycin A-C_{16}	Deoxycholic acid
37	*E. coli* MG1655	>64	>64	>50,000
	Y. enterocolitica ATCC 23715	64	>64	>50,000
	Y. pestis KIM6+	4	4	6,250
	Y. pestis KIM6+ LepB(P91S) (DBS600)	4	0.03	6,250
	E. coli lptD4213 (BAS901)	0.06	16	500
	E. coli LepB(P84L) (PAS0260)	64	2	>50,000
	Y. pestis $KIM6^+$ Δ*phoP*	8	16	25,000
	Y. pestis KIM5	8	4	3,100
	Y. pestis KIM5-pLpxL	16	8	25,000
	Y. pestis KIM6+ LepB(P91S) Δ*ail* (DBS601)	16	1	12,500
28	*Y. enterocolitica* ATCC 23715	64	>128	>50,000
	Y. pestis KIM6+	32	16	50,000
	Y. pestis KIM6+ LepB(P91S) (DBS600)	32	0.06	50,000
	E. coli lptD4213 (BAS901)	–[a]	16	–
	E. coli LepB(P84L) (PAS0260)	>64	2	>50,000
	Y. pestis $KIM6^+$ Δ*phoP*	32	>64	50,000
	Y. pestis KIM5	>64	16	50,000
	Y. pestis KIM5-pLpxL	>64	16	50,000
	Y. pestis KIM6+ LepB(P91S)Δ*ail* (DBS601)	32	1	50,000
20	*Y. pestis* KIM6+	>64	>64	50,000
	Y. pestis KIM6+ LepB(P91S) (DBS600)	–	1	–
	E. coli lptD4213 (BAS901)	–	16	–
	E. coli LepB(P84L) (PAS0260)	>64	2	>50,000
	Y. pestis $KIM6^+$ Δ*phoP*	>64	>64	50,000
	Y. pestis KIM5	32	>64	50,000
	Y. pestis KIM5-pLpxL	–	>64	–
	Y. pestis KIM6+ LepB(P91S)Δ*ail* (DBS601)	64	1	50,000

[a] –, not determined.

(60 ± 16 nM and 5.7 ± 1.0 nM, respectively) (14; see Fig. S1 in the supplemental material). These results demonstrate that the difference in susceptibility between *Y. pestis* and *E. coli* does not result from altered SPase affinity for the arylomycins.

The arylomycin susceptibility of *Y. pestis* is temperature dependent. *Y. pestis* is able to survive and propagate in insect and mammalian hosts due to its ability to regulate its membrane characteristics and proteome expression in response to environmental cues, such as temperature and cation (i.e., calcium or iron) concentration (34–37). To test whether such factors are important for arylomycin sensitivity, we determined arylomycin A-C_{16} MICs with *Y. pestis* KIM6+ at 20, 28, and 37°C (Table 4). We observed an inverse correlation between the MIC and the temperature, with the lowest MIC (4 μg/ml) at 37°C and the highest (>64 μg/ml) at 20°C. Supplementation of the medium with calcium or iron did not have a significant effect on the arylomycin MIC at any of the temperatures examined (data not shown). A similar trend was observed with the hydrophobic antibiotic actinomycin D and the hydrophobic bile salt deoxycholic acid. These data suggest that the sensitivity of *Y. pestis* to the arylomycins may result from temperature-dependent alterations of the pathogen's membrane, as is thought to be the case with hydrophobic agents (38), or from changes in secreted proteins.

The unique LPS of *Y. pestis* provides at most a small contribution to arylomycin sensitivity. In general, the LPS barrier of Gram-negative bacteria is thought to provide an effective barrier that prevents large and hydrophobic molecules from passing through the outer membrane. Interestingly, *Y. pestis* is unique in that it possesses "rough," or R-type, LPS that lacks the O antigen (39), and it has been shown to be more susceptible to hydrophobic agents than other *Yersinia* species (38). Moreover, *Y. pestis* has been shown to modify its LPS as a function of temperature, and such modifications have been shown to impart to *Y. pestis* a temperature-dependent susceptibility to several agents that is similar to that observed with arylomycin A-C_{16} (38, 40).

To begin to explore the potential contribution of the LPS barrier to arylomycin sensitivity, we first examined a panel of Gram-negative bacteria with different LPS characteristics. We also examined the responses of the panel to the effects of added PMBN, which selectively permeabilizes LPS (41), using a checkerboard analysis with serial 2-fold dilutions of arylomycin A-C_{16} and PMBN (Fig. 4) (the PMBN MICs for all strains tested were >256 μg/ml, and thus, PMBN has no activity on its own at the concentrations employed). *E. coli* BAS901, which lacks LPS altogether due to an *lptD* mutation, is hypersensitive to arylomycin A-C_{16}, and PMBN coadministration does not increase its sensitivity further, consistent with the complete absence of an LPS barrier. Conversely, PMBN coadministration dramatically sensitizes *E. coli* PAS0260 to arylomycin A-C_{16} (as well as *E. coli* MG1655 [20]), consistent with the intact *E. coli* LPS acting as a barrier to arylomycin penetration. The effect of PMBN on *E. coli* PAS0260 is independent of temperature. At 37°C, PMBN had a modest effect on the sensitivity of *Y. pestis* KIM6+ and its arylomycin-sensitized P91S SPase strain, but the effect was more pronounced at 28°C.

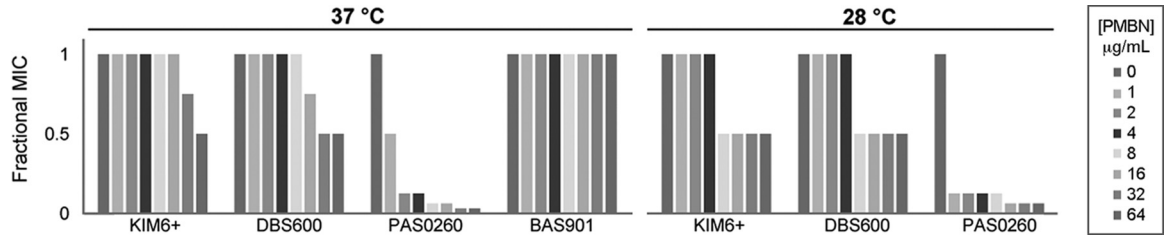

FIG 4 Median fractional arylomycin A-C_{16} MICs as a function of increasing PMBN concentrations at 37°C and 28°C. The fractional arylomycin A-C_{16} MIC is equal to the MIC of arylomycin A-C_{16} in the presence of the tested PMBN concentration divided by the MIC of arylomycin A-C_{16} alone. The shading of the bars indicates the PMBN concentration tested (1 to 64 μg/ml in a 2-fold dilution series). The strains tested (the MICs in the absence of PMBN are shown in parentheses) were *Y. pestis* KIM6+ (4 μg/ml at 37°C; 16 μg/ml at 28°C), *Y. pestis* expressing P91S LepB (DBS600) (0.03 μg/ml at 37°C; 0.06 μg/ml at 28°C), *E. coli* expressing P84L LepB (PAS0260) (2 μg/ml at 37°C and 28°C), and the *E. coli lptD4213* mutant (BAS901) (16 μg/ml). To standardize the MIC values among the different strains, the changes in the MICs are reported as fractional arylomycin A-C_{16} MICs, which are defined as the MIC of arylomycin A-C_{16} in the presence of the tested PMBN concentration divided by the MIC of arylomycin A-C_{16} alone.

These data suggest that the *Y. pestis* LPS is a somewhat less efficient barrier to arylomycin penetration than is that of *E. coli*, or possibly that PMBN is less efficient at disrupting the LPS of *Y. pestis*.

Like *Y. pestis* in general, *E. coli* MG1655 (and its P84L SPase derivative, PAS0260) does not express the LPS O antigen (in this case due to a defect in the *rfb* gene cluster [42]); thus, the resistance of these strains suggests that the loss of the O antigen is unlikely to be the cause of *Y. pestis* sensitivity. To further support this conclusion, we examined *Yersinia enterocolitica*, which downregulates O antigen expression at higher temperatures (43). Consistent with the unimportance of the O antigen, this strain was resistant to arylomycin A-C_{16} at both 28 and 37°C (Table 4).

While there is typically one major variant of the LPS core oligosaccharide when *Y. pestis* is cultured at 37°C, modifications are made when *Y. pestis* is cultured at lower temperatures, resulting in the production of up to four different core isoforms (44–48; reviewed in reference 39). However, different subspecies of *Y. pestis* have been shown to have different core structures (46), and the general susceptibility of *Y. pestis* spp. to arylomycin (see above) suggests that core modifications do not substantially contribute to the observed sensitivity. To more systematically address the contribution of the LPS core to arylomycin permeability, we examined the sensitivities of a panel of isogenic *E. coli* mutants with well-defined core truncations: strain D21 lacks the O antigen but retains the entire LPS core, strain D21e7 contains only the heptose portion of the core plus a glucose residue, strain D21e19 is similar to strain D21e7 but contains an additional galactose residue, strain D21f1 contains only the Kdo and heptose portions of the core, and strain D21f2 contains only the Kdo residue. Despite the varying LPS truncations, each of these strains is resistant to arylomycin A-C_{16} (MIC > 128 μg/ml). Thus, as with the loss of the O antigen, it is unlikely that the LPS core of *Y. pestis* is itself sufficient to account for sensitivity to the arylomycins.

Y. pestis modifies its LPS with 4-amino-4-deoxy-L-arabinose (Ara4N), which is mediated by the two-component response regulator PhoPQ (49). Moreover, the Ara4N content increases with decreased temperature, and these modifications have been linked to resistance to cationic antimicrobial peptides (49–51). To examine the contribution of Ara4N modification to arylomycin sensitivity, we examined the arylomycin A-C_{16} sensitivity of a *Y. pestis phoP* knockout strain (KIM6+ Δ*phoP*). The loss of *phoP* results in reduced susceptibility to arylomycin A-C_{16} (Table 4). The addition of 10 mM $MgCl_2$ to the growth media, which is known to repress expression of *phoPQ* (52), also resulted in reduced arylomycin susceptibility of wild-type *Y. pestis*, but not the *phoP* deletion strain or *E. coli* strain BAS901 or PAS0260 (Table 5). Regardless of the specific mechanism, it is clear that the PhoP function actually sensitizes *Y. pestis* to the arylomycins, and because the Ara4N content and susceptibility in the context of the *phoP* deletion show opposite dependencies on temperature, LPS modification with Ara4N is unlikely to underlie the arylomycin sensitivity of *Y. pestis*.

At 37°C, lipid A of *Y. pestis* primarily exists in a tetra-acylated form, while at lower temperatures there is a shift toward the production of the hexa-acylated variant, which is more similar to that produced by other enterobacteria and is thought to result in lower permeability due to increased packing (39). To explore the contribution of lipid A acylation to arylomycin sensitivity, we extended our studies to *Y. pestis* KIM5-pLpxL, an engineered *Y. pestis* strain that expresses the *E. coli* acyltransferase LpxL and almost exclusively expresses hexa-acylated lipid A at all temperatures (53). At 37°C, *Y. pestis* KIM5-pLpxL was 2-fold less sensitive to arylomycin A-C_{16} than the KIM5 parental strain (Table 4). This is similar to the effect observed with actinomycin D but less than that observed with deoxycholic acid at 37°C. This suggests that, while the acylation state of *Y. pestis* lipid A is important for deoxycholic acid penetration through the LPS, it is less important for actinomycin D or arylomycin A-C_{16}. In addition, coadministration of PMBN resulted in a very modest increase in arylomycin sensitivity (Fig. 5). While the effects on sensitivity and synergy

TABLE 5 Arylomycin A-C_{16} MICs in the presence of 10 mM Mg^{2+}

Strain	MIC (μg/ml)		
	37°C	28°C	20°C
Y. pestis			
KIM6+	16	>128	>128
KIM6+ Δ*phoP*	16	>128	>128
LepB(P91S) (DBS600)	0.125	0.25	2
KIM5	16	>128	>128
KIM5 pLpxL	16	16	>128
E. coli			
lptD4213 (BAS901)	16	16	8
LepB(P84L) (PAS0260)	2	4	4

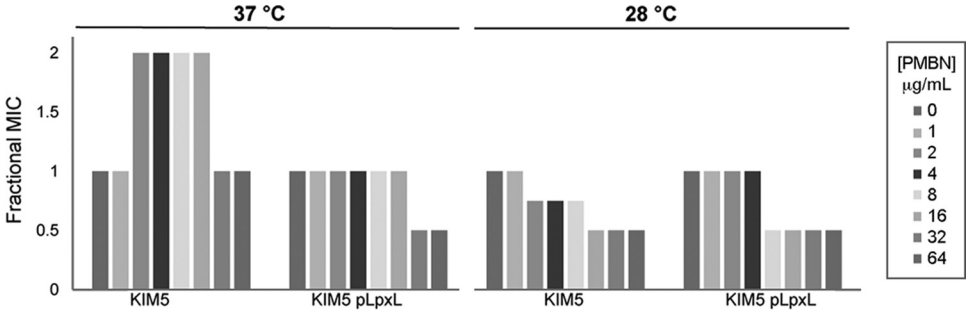

FIG 5 Median fractional arylomycin A-C_{16} MICs as a function of increasing PMBN concentrations at 37°C and 28°C for *Y. pestis* KIM5 and KIM5-pLpxL. The arylomycin A-C_{16} MICs in the absence of PMBN were 4 μg/ml (37°C) and 16 μg/ml (28°C) for KIM5 and 8 μg/ml (37°C) and 16 μg/ml (28°C) for KIM5-pLpxL. The fractional arylomycin A-C_{16} MIC is equal to the MIC of arylomycin A-C_{16} in the presence of the tested PMBN concentration divided by the MIC of arylomycin A-C_{16} alone. The shading of the bars indicates the PMBN concentration tested (1 to 64 μg/ml in a 2-fold dilution series).

with PMBN are small, they are reproducible, suggesting that the reduced acylation state of *Y. pestis* lipid A may underlie the slightly reduced efficiency of the *Y. pestis* LPS barrier to arylomycin penetration at 37°C. Nonetheless, the data demonstrate that core alterations in LPS structure on their own are not the dominant contributors to *Y. pestis* sensitivity to the arylomycins.

The arylomycin susceptibility of *Y. pestis* results from a temperature-dependent increase in protein secretion. There are significant increases in the secretion of several proteins in *Y. pestis* at 37°C relative to lower temperatures (36, 37, 54, 55), and exacerbation of the associated secretion burden by inhibiting SPase could underlie the sensitivity to the arylomycins at physiological temperatures. Consistent with this possibility, reducing translation via mutation or the addition of translational inhibitors has been shown to rescue defects in the *E. coli* general secretory pathway (56). To begin to test this possibility, we first performed synergy studies with tetracycline, which inhibits translation without causing mistranslation, and with gentamicin, which causes mistranslation and the toxic accumulation of aberrant peptides in the cytoplasmic membrane (57) (Table 6). Antibiotic interactions were determined by measuring the FIC index using a microdilution checkerboard assay consisting of combinations of arylomycin A-C_{16} and either tetracycline or gentamicin. An FIC index of $\leq$0.5 denotes synergy, whereas an FIC index of $\geq$4 represents antagonism (32). The minimum (FIC_{min}) and maximum (FIC_{max}) FIC indexes measured are reported for each drug combination.

Based on the FIC indexes, the inhibition of protein synthesis effected by tetracycline cotreatment significantly rescues *Y. pestis* from the effects of arylomycin, and the mitigation of arylomycin's effects observed with *Y. pestis* is much greater than that observed for the sensitized PAS0260 *E. coli* strain expressing SPase with the P84L mutation (Table 6) (58). However, the FIC indexes for *Y. pestis* with gentamicin cotreatment do not differ significantly from those reported for PAS0260 (Table 6) (58). These data suggest that with the inhibition of SPase, *Y. pestis* is hypersensitive to the accumulation of normal proteins in the cytoplasmic membrane.

Y. pestis significantly increases the secretion of four proteins at 37°C relative to lower temperatures: the cell adhesion protein Ail (y1324) and the three hypothetical small β-barrel OM proteins (y1795, y2167, and y4083) (36). All four proteins are predicted (by SignalP version 3.0 [59]) to be translated with signal peptides, suggesting that their increased expression may create a high demand for SPase activity. Ail is a secreted protein, and at 37°C, it accounts for approximately 25% of the *Y. pestis* outer membrane proteome (60). Thus, to determine the extent to which this temperature-dependent secretion accounts for the increased sensitivity of *Y. pestis* relative to *E. coli*, we deleted *ail* in the arylomycin-sensitized strain DBS600, yielding strain DBS601 (Table 4). Deletion of *ail* resulted in 30- and 16-fold increases in the arylomycin MIC at 37°C and 28°C, respectively, and no change at 20°C. The increases in MICs that result from the deletion of *ail* result in the *Y. pestis* and *E. coli* strains (DBS600 and PAS0260, respectively) showing identical sensitivities. Moreover, synergy studies with tetracycline revealed that upon deletion of *ail*, *Y. pestis* and *E. coli* have indistinguishable FICs (Table 6). Finally, complementation with the plasmid-borne and L-arabinose-inducible *ail* or *E. coli malE* resulted in resensitization to arylomycin (Table 7). These data suggest that the increased secretion burden experienced by *Y. pestis* underlies virtually all of its increased sensitivity relative to *E. coli*.

To further support the conclusion that the altered sensitivities result from changes in the secretion burden, we assessed protein levels in a lysed cold-shocked cell fraction (which contained the cytoplasmic membrane) and the periplasmic fraction as a function of *ail* deletion and arylomycin addition (Fig. 6). As expected, the deletion of *ail* reduced the levels of proteins found in both fractions. Moreover,

TABLE 6 MICs of antibiotics alone and fractional inhibitory concentration indexes of antibiotics in combination with arylomycin A-C_{16} at 37°C[a]

				Y. pestis								
	E. coli PAS0260			KIM6+			DBS600			DBS601		
Antibiotic	MIC	FIC_{min}	FIC_{max}	MIC	FIC_{min}	FIC_{max}	MIC	FIC_{min}	FIC_{max}	MIC	FIC_{min}	FIC_{max}
Gentamicin	1	0.58 ± 0.29	1.09 ± 0.05	2	0.60 ± 0.06	1.07 ± 0.06	–	–	–	–	–	–
Tetracycline	1	0.90 ± 0.23	2.35 ± 0.25	2	1.70 ± 0.59	8.42 ± 0.14	1	1.55 ± 0.59	5.50 ± 2.00	2	1.04 ± 0.02	2.25 ± 0.50

[a] The FIC values are averages ± standard deviations. The MIC values are in micrograms per milliliter. –, not determined.

TABLE 7 MICs of arylomycin A-C_{16} as a function of added L-arabinose determined for *Y. pestis* P91S LepB Δ*ail* (DBS601) complemented with various constructs at 37°C

Construct	MIC (μg/ml) at L-arabinose level (%):		
	0	0.2	2
No vector	1	1	1
pBAD/His B (empty)	1	1	0.5
pBAD/His B-*malE*	1	1	0.125
pBAD/His B-*ail*	1	0.5	0.03

in the presence of Ail, the addition of arylomycin A-C_{16} increased the level of proteins in the shocked-cell fraction and decreased it in the periplasmic fraction, consistent with arylomycin sensitivity resulting from protein mislocalization. In contrast, in the absence of Ail, the addition of arylomycin had a diminished effect on the level of protein found in either fraction, consistent with resistance resulting from reduced protein mislocalization.

DISCUSSION

Gram-negative bacteria are emerging as a particularly troubling threat due to the evolution of resistance to available antibiotics and the challenge of developing new antibiotics. Protein secretion is an essential process in all bacteria, and the general secretory pathway is conserved across both Gram-positive and Gram-negative species (61–64). Interestingly, compared to Gram-positive organisms, a far greater number of proteins are processed by SPase in Gram-negative bacteria, which must maintain a protein-rich periplasm and outer membrane (65, 66), suggesting that targeting SPase may be a particularly effective strategy to combat these pathogens. Our previous demonstration that the arylomycin class of antibiotics, which act by inhibiting SPase, has the potential for broad-spectrum activity (14, 25, 58, 67), including against Gram-negative pathogens, suggests that they might be interesting for development as antibiotics. However, this will require an understanding of the varying susceptibilities of different bacteria and their physiological origins. Previously, we demonstrated that a specific SPase allele is responsible for much of the variations in sensitivity (14, 25), but variations among different species, or even within species, that possess the same SPase allele demonstrates that other factors must also contribute.

Among the *Enterobacteriaceae* tested that have SPases possessing the predicted arylomycin-resistant SPase allele, *Y. pestis* KIM6+ was unique in its susceptibility to the arylomycins (14). We have now shown that the arylomycins in fact show at least reasonable activity against a broad range of *Y. pestis* strains, including representative strains from all three classical biovars (antiqua, medievalis, and orientalis). This broad and at least relatively potent activity contrasts sharply with their activities against other *Enterobacteriaceae*, such as *E. coli*, which are typically resistant (14). While SPase is highly conserved, there are sequence differences among bacteria, and a trivial explanation for the higher sensitivity of *Y. pestis* is that arylomycins bind to its SPase with higher affinity. However, based on a comparison of the arylomycin dissociation constant with that determined previously for the *E. coli* protein (14), this is not the case.

A hint as to the factor(s) that might underlie the atypical arylomycin sensitivity of *Y. pestis* is its temperature dependence. We determined that the increased sensitivity of *Y. pestis* to the arylomycins is manifest only at higher (physiological) temperatures, with full resistance observed at 20°C. Interestingly, *Y. pestis* possesses an atypically truncated, or rough, LPS, and it has been shown to modify its LPS as a function of temperature. The ability of LPS to prevent large hydrophobic antibiotics from accessing periplasmic or cytoplasmic targets in many bacteria, the sensitization caused by chemical or genetic perturbations of the LPS with many of the same bacteria, and the fact that the observed temperature-dependent sensitivity of *Y. pestis* to arylomycin A-C_{16} appears to mirror changes in its LPS suggested that the atypical LPS of *Y. pestis* may be the cause of the arylomycin sensitivity. Indeed, the addition of PMBN, which disrupts LPS, had a limited effect on the susceptibility of *Y. pestis* to the arylomycin compared to *E. coli*, suggesting either that the *Y. pestis* LPS is a less efficient barrier to arylomycin penetration or that it is less efficiently disrupted by PMBN. However, little or no change in sensitivity to the arylomycin was observed upon alteration or deletion of the LPS O antigen or core or alteration of lipid A via acylation or with Ara4N. This suggests that while the unusual truncated, or rough, LPS of *Y. pestis* is somewhat less efficient at excluding the arylomycins, it is unlikely in and of itself to be the cause of the increased sensitivity of *Y. pestis* relative to *E. coli*.

A final possibility examined was that the known temperature-dependent increases in protein secretion (36) exacerbate the effects of arylomycin-mediated SPase inhibition at physiological temperatures. To test this hypothesis, we deleted the nonessential gene encoding the Ail protein, which is secreted and constitutes 25% of the outer membrane proteome. We found that deletion of *ail* reduced arylomycin sensitivity and synergy with tetracycline to levels indistinguishable from those of *E. coli* and that ectopic expression of *ail* or *E. coli malE* in the Δ*ail* strain restored sensitivity. In sensitized *E. coli*, we have previously shown that arylomycin A-C_{16} is bactericidal and that this activity appeared to result from protein mislocalization in the cytoplasmic membrane and either excessive accumulation of unprocessed preproteins, and perhaps membrane depolarization, or the misregulation of essential periplasmic and outer membrane processes (58). Similarly, it seems likely that the secretion-dependent sensitization of *Y. pestis* to arylomycin results from the accumulation of unprocessed proteins in the cytoplasmic membrane or from competition with the processing of essential proteins. This model is also consistent with the overexpression of SPase reducing the sensitivity of wild-type *Y. pestis*. However, we cannot exclude the possibility that the secretion burden and a slightly reduced LPS barrier act synergistically. Regardless of the detailed mechanism, the results demonstrate that the demands associated with the high levels of secretion induced at 37°C exacerbate the effects of SPase inhibition and ac-

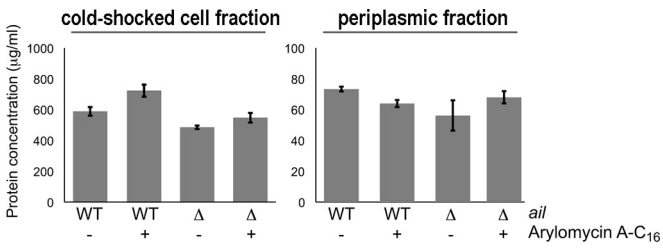

FIG 6 Determination of protein content in the cold-shocked cell and periplasmic fractions of *Y. pestis* DBS600 and its Δ*ail* derivative, DBS601, with and without arylomycin A-C_{16} treatment (0.5× MIC).

count for most of the differences in arylomycin sensitivity between *Y. pestis* and *E. coli*.

Y. pestis has adapted to the different environments required for survival in its insect vector and in its mammalian host (68), in part by regulating the proteins secreted in response to different environmental cues (34–37). The increased secretion of Ail at 37°C is thought to facilitate survival in its mammalian host by mediating resistance to complement and adherence to epithelial cells (69, 70). However, reliance on this protein secretion appears to render *Y. pestis* more susceptible to SPase inhibition and thus to the arylomycins. Interestingly, the viability and/or virulence of many bacteria in different contexts depends on secretion in response to particular environmental cues (71–74), and the secretion of many of these proteins is mediated by the general secretory pathway and SPase. Because these cues are often not present during routine analysis of antibiotic susceptibility, the effect of SPase inhibition may be commonly underestimated. Thus, it seems likely that in the actual context of an infection, the arylomycins may possess a more potent and broad-spectrum activity than predicted based on MICs, especially against Gram-negative bacteria, which possess a single SPase and where high levels of secretion are typically required for the protein-rich periplasm and outer membrane of these organisms (65, 66). Along with the previously demonstrated activity of the arylomycins against many Gram-positive bacteria (25, 67), this reinforces the idea that they may be promising candidates for further development as therapeutics.

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