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Yersinia pestis acrAB-tolC in Antibiotic Resistance and Virulence

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The efflux pump AcrAB is important in the antibiotic resistance and virulence of several pathogenic bacteria. We report that deletion of the *Yersinia pestis* AcrAB-TolC homolog leads to increased susceptibility to diverse substrates, including, though unlike in *Escherichia coli*, the aminoglycosides. Neither is the *Y. pestis* pump affected by the efflux pump inhibitor phenylalanine-arginine beta-naphthylamide. In mouse plague models, pump deletion does not have a significant effect on tissue colonization.

Increased activity of efflux pumps is a major route to decreased susceptibility (8, 15, 21, 22, 39, 41) in many bacteria. An important pump in active efflux is AcrAB-TolC (26, 35). TolC is located in the outer membrane and is linked to the inner membrane AcrB through AcrA, thus forming a channel from the cytoplasm to the extracellular space (25). AcrAB-TolC has an extremely broad substrate range but favors lipophilic substrates (25). Efflux pumps are also involved in the pathogenicity of several bacteria, including *Pseudomonas aeruginosa*, *Salmonella enterica* serovar Typhimurium, *Vibrio cholerae*, *Francisella tularensis*, *Klebsiella pneumoniae*, and the plant pathogen *Erwinia amylovora* (3, 27, 30); although not fully understood, the pumps could affect virulence through removal of antibacterial molecules such as bile (25), mammalian steroid hormones (7), and antimicrobial peptides (34, 37, 40) and therefore would aid survival in the host.

To explore the function of the AcrAB-TolC pump in susceptibility of *Yersinia pestis* to antibiotics and virulence, deletion and complemented strains were engineered using splicing by overlap extension, as described previously (11, 19), in the attenuated *Y. pestis* strain KIM 1001 *pgm* (18), a gift from John Goguen (University of Massachusetts Medical School). Sites of genetic manipulation were verified by sequencing. Growth studies showed no difference in the doubling times of the engineered strains compared with that of the wild-type (WT) strain (data not shown).

Drug susceptibility studies were carried out using Etests (AB Biodisk) against antibiotics from different classes, including antibiotics favored in plague treatment and prophylaxis (streptomycin, gentamicin, tetracycline, chloramphenicol, ciprofloxacin, and trimethoprim-sulfamethoxazole) (9). Figure 1 shows the average MIC (from at least three experiments) against selected antibiotics chosen to represent different antibiotic classes. Statistical analysis was performed using one-way analysis of variance (ANOVA) with Tukey's posttest. Deletion of *acrAB* increased susceptibility to all the antibiotics by 50 to 92%. *tolC* deletion increased susceptibility to the aminoglycosides by 52 to 66%, but not to the other drugs (9 to 23% increase in susceptibility), compared to the levels for the *acrAB* strain (Fig. 1; Table 1). Resistance was restored when strains were complemented with ectopically expressed *acrAB* or *tolC* (Table 1). The reason for the variation in the MICs for chloramphenicol between the two *tolC* strains is uncertain, but as it is only a 2-fold difference, this is within the acceptable range for MIC determination. This finding suggests that, of the efflux pumps that interact with TolC, AcrAB is the major one for antibiotic efflux, with the caveat that our results do not take into account changes in susceptibility brought about by membrane perturbation resulting from the absence of pump components. Increased susceptibility to the aminoglycosides was unexpected, as this antibiotic class is not a substrate for the *Escherichia coli* AcrAB-TolC pump (24), although it is a substrate for the homologous *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* complexes (25, 27). A ClustalW alignment of AcrB pri-

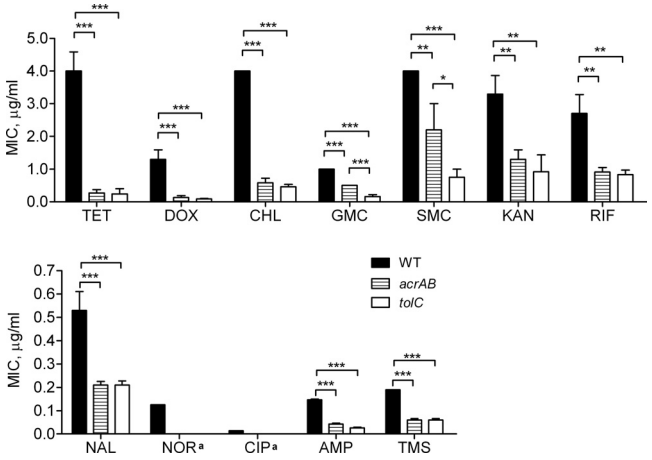

FIG 1 Drug susceptibilities of wild-type KIM 1001 *pgm* and *acrAB* and *tolC* deletion mutants. The data are representative of at least 3 experiments for each antibiotic. In all instances, deletion caused major increases in drug susceptibility. a, norfloxacin and ciprofloxacin MICs could not be shown for the *tolC* and *acrAB* strains, as the boundary of bacterial growth was below the lowest antibiotic concentration of the Etest strip. Asterisks denote significance as calculated by one-way ANOVA with Tukey's posttest, as follows: ∗, *P* value of 0.01 to 0.05; ∗∗, *P* value of 0.001 to 0.01; ∗∗∗, *P* value of <0.001. AMP, ampicillin; CHL, chloramphenicol; CIP, ciprofloxacin; DOX, doxycycline; GMC, gentamicin; KAN, kanamycin; NAL, nalidixic acid; NOR, norfloxacin; RIF, rifampin; SMC, streptomycin; TET, tetracycline; TMS, trimethoprim-sulfamethoxazole.

Received 29 July 2011 **Returned for modification** 25 August 2011
Accepted 6 November 2011
Published ahead of print 14 November 2011
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doi:10.1128/AAC.05338-11

TABLE 1 MICs of various strains as a percentage of WT *Y. pestis* MIC

Strain genotype[a]	% mutant MIC/WT MIC[b]													
	TET	DOX	CHL	GMC	SMC	KAN	RIF	NAL	NOR	CIP	AMP	TMS	Bile	PMB
WT	100	100	100	100	100	100	100	100	100	100	100	100	100	100
lacZ	**100**	**ND**	**100**	**ND**	**118**	**100**	**ND**	**ND**	**88**	**ND**	**ND**	**89**	**100**	**100**
acrAB	**10*****	**8*****	**18*****	**50*****	**49*****	**40*****	**34*****	**36*****	**NA**	**NA**	**35*****	**29*****	**<17*****	**23*****
acrAB lacZ::*acrAB*	95	98	89	107	100	105	ND	103	91	ND	ND	84	100	100
tolC	**10*****	**6*****	**16*****	**19*****	**17*****	**19*****	**31****	**25*****	**NA**	**NA**	**22*****	**29*****	**ND**	**ND**
tolC lacZ*::*kan	**10*****	**4*****	**8*****	**24*****	**16*****	**R**	**ND**	**24*****	**NA**	**NA**	**ND**	**25*****	**<4*****	**0.1*****
tolC::*tolC lacZ*::*kan*	100	ND	100	ND	88	ND	ND	ND	86	ND	ND	83	100	100
tolC/*acrAB*[c]	100 (NS)	77 (NS)	100 (NS)	38***	34***	48 (NS)	91 (NS)	71 (NS)	NA	NA	67 (NS)	100 (NS)	23***	0.4***

[a] Genes were deleted and complemented using splicing by overlap extension. In complemented strains, *acrAB* was inserted into the *lacZ* gene and *tolC* was inserted into the original deletion site after *lacZ* deletion by insertion of a kanamycin resistance gene. Boldface type denotes deletion strains.

[b] MICs used to calculate percentages were an average of at least 3 experiments. Abbreviations: AMP, ampicillin; Bile, OxGall; CHL, chloramphenicol; CIP, ciprofloxacin; DOX, doxycycline; GMC, gentamicin; KAN, kanamycin; NAL, nalidixic acid; NOR, norfloxacin; RIF, rifampin; SMC, streptomycin; TET, tetracycline; TMS, trimethoprim-sulfamethoxazole; PMB, polymyxin B; NA, not available (the MIC was below the lowest value of 0.016 μg/ml on the Etest strip by 2 to 6 mm); ND, not determined; R, complete resistance due to kanamycin resistance gene chromosomal integration. Significance is denoted as follows: **, *P* value of 0.001 to 0.01; ***, *P* value of <0.001; NS, not significant. Boldface type denotes results for deletion strains.

[c] *tolC* MIC as a percentage of the *acrAB* MIC.

mary sequences from *E. coli*, *Y. pestis*, and *P. aeruginosa* showed no obvious reason why AcrAB-TolC from *Y. pestis* should efflux aminoglycosides (data not shown).

Bacteria entering host organisms such as humans and rodents use defenses against antimicrobial peptides which are secreted by epithelial cells as part of the innate immune response and by other resident bacteria (2, 5, 12, 13, 17). We used polymyxin B (PMB), as a representative antimicrobial peptide, and determined the susceptibility of *Y. pestis* using plate dilution assays (14), taking the average of at least 3 experiments. The MICs for PMB of the WT (350 μg/ml), *acrAB* (80 μg/ml), and *tolC* (0.35 μg/ml) strains suggest that while PMB is a substrate for AcrAB, the dramatic increase in susceptibility with a *tolC* deletion (0.4% of the AcrAB MIC [Table 1]) indicates that TolC likely acts as the exit duct for other pumps associated with PMB efflux. In *E. coli*, the PMB MIC is far lower (0.03 μg/ml) and only 50% lower than that of the WT for both the *acrAB* and the *tolC* deletion mutants (40). As for the antibiotic MICs, these findings suggest that the related pumps of different bacterial species may have different substrate affinities.

Enteric pathogens must survive passage through bile in the small intestine. Bile is a substrate of AcrAB-TolC in *E. coli*, *V. cholerae*, *F. tularensis*, and *S. enterica* (3, 6, 23, 36). We used plate dilution assays (14) with OxGall (Sigma) to determine the MICs of *Y. pestis* WT, *acrAB*, and *tolC* strains as >9 mg/ml, 1.5 mg/ml, and 0.35 mg/ml, respectively. As for PMB, these findings show that *Y. pestis* AcrAB is not the only bile efflux pump, as the *tolC* deletion mutant causes a further increase in susceptibility. In contrast to PMB, this finding compares well with the *E. coli* TolC pumps, where bile salts are the substrates of several pumps as well as AcrAB-TolC and deletion of *tolC* increases susceptibility above that of the *acrB* or *acrAB* deletion strains (35).

It has been shown that deletion of *tolC* results in an increase in the levels or activity of the transcriptional regulators MarA, SoxS, and Rob (32). Porins are among the many genes that are regulated by these transcription factors (1, 31). It is highly probable that the loss of AcrAB-TolC induces changes in membrane permeability beyond simply loss of efflux which may also affect susceptibility.

Multidrug-resistant clinical strains show a high frequency of mutations in efflux pump genes (8, 15, 21, 22, 39, 41). Dual therapy involving treatment with an antibiotic and an efflux pump inhibitor (EPI) is proposed as a way to restore the efficacy of the antibiotic in resistant strains. We used the broad-spectrum EPI phenylalanine-arginine β-naphthylamide (PAβN), a potentiator of susceptibility to chloramphenicol, tetracycline, macrolides, fluoroquinolones, and aminoglycosides (4, 10, 16), to determine if this would increase the susceptibility of *Y. pestis* to antibiotics. Etest studies on plates with and without 20 μg/ml of PAβN showed that WT susceptibility to nalidixic acid and rifampin in-

TABLE 2 Effect of PAβN on drug susceptibility of the various strains

Strain[a]	% MIC +PaβN/−PAβN (SD)[b]									
	TET	KAN	SMC	CHL	CIP	NOR	NAL	RIF	TMS	AMP
Y. pestis										
WT	100 (0)NS	100 (0)NS	83 (19)NS	69 (24)NS	*179 (20)**	*134 (25)*NS	**46 (8)****	**50 (0)*****	101 (41)NS	*116 (27)*NS
acrAB strain	**50 (0)*****	40 (9)NS	60 (20)NS	**69 (6)***	NA	NA	**31 (5)****	**50 (0)*****	101 (28)NS	NA
tolC lacZ::*kan* strain	**41 (9)***	R	78 (19)NS	**31 (17)***	NA	NA	**28 (5)****	**25 (0)*****	56 (10)NS	NA
E. coli MG1655	*172 (86)*NS	*164 (97)*NS	*300 (0)****	**13 (0)*****	*228 (67)***	*253 (48)***	**23 (4)****	**4 (2)*****	69 (6)NS	*117 (76)*NS

[a] Genes were deleted and a kanamycin resistance gene was inserted into the *lacZ* gene of the *tolC* strain using splicing by overlap extension.

[b] Standard deviations were calculated from 3 experiments. Significance is denoted as follows: *, *P* value of 0.01 to 0.05; **, *P* value of 0.001 to 0.01; ***, *P* value of <0.001; NS, not significant. R denotes complete resistance due to the kanamycin resistance gene integrated into the *lacZ* gene. NA denotes that data were not available as the boundary of bacterial growth was below that of the lowest Etest antibiotic concentration with and/or without PAβN supplementation. Numbers in bold show significant percent changes less than or equal to 50%. Numbers in italics show percent changes where susceptibility decreased in the presence of PAβN.

TABLE 3 Checkerboard fractional inhibitory concentration results and comparison of MICs obtained from checkerboard and Etest assays at 20 μg/ml PAβN on *E. coli* strain MG1655[a]

	Etest			CB			
Antibiotic	MIC (μg/ml)	MIC+ (μg/ml)	% difference	MIC (μg/ml)	MIC+ (μg/ml)	% difference	FIC
NAL	1.67 (0.29)	0.38 (0.00)	23	3.3 (1.4)	0.31 (0)	9	0.078 (0.016)
CIP	0.003 (0.001)	0.007 (0.001)	233	0.032 (0.000)	0.043 (0.018)	134	1.80 (0.58)

[a] MIC denotes MIC in the absence of PAβN; MIC+ denotes MIC in the presence of 20 μg/ml PAβN. Numbers in parentheses show standard deviations from three experiments. The percent difference was calculated as MIC+/MIC × 100. CB, checkerboard; FIC, fractional inhibitory concentration.

creased only by ~50%. Susceptibility to other antibiotics was not affected. For comparison, we tested the effect of PAβN on the drug susceptibility of the *E. coli* MG1655 strain and found a 77 to 96% increase in susceptibility for chloramphenicol, nalidixic acid, and rifampin (Table 2). These findings are similar to results reported previously (33). For norfloxacin, ciprofloxacin, streptomycin, and ampicillin, there was, in fact, an unexpected decrease in susceptibility of *E. coli* in the presence of PAβN (Table 2).

Etests on the *acrAB* and *tolC* strains showed increases in susceptibility to tetracycline, nalidixic acid, chloramphenicol, and rifampin (50 to 75% [Table 2]) in the presence of PAβN, suggesting that PAβN is effluxed by AcrAB in the WT strain, and with the loss of the pump in the deletion strains, PAβN is able to inhibit other pumps. Further, unlike WT *Y. pestis*, *acrAB* or *tolC* strains were not able to grow at 50 or 100 μg/ml PAβN, suggesting that in the absence of AcrAB or TolC, PAβN is lethal.

Together, these findings suggest that *Y. pestis* AcrAB-TolC plays a role in efflux of PAβN but is minimally inhibited by it. In contrast, the *E. coli* AcrAB pump and the *P. aeruginosa* MexAB pump efflux PAβN but are also inhibited by it (4, 20, 21, 42).

As there is a slight but significant decrease in the susceptibility of certain antibiotics in the presence of PAβN (ciprofloxacin, norfloxacin, and streptomycin), checkerboard assays were performed with *E. coli* MG1655 at 5×10^5 CFU/ml in 96-well microtiter plates. A series of 7 twofold dilutions were made such that the MIC of the antibiotic in question was approximately the median concentration of the series. A series of 7 twofold dilutions of PAβN was also made, the highest concentration being 80 μg/ml and the lowest in the series being 1.25 μg/ml. FIC thresholds used were ≤0.5 to denote synergy, >0.5 to ≤4 to denote indifference, and >4 to denote antagonism. Three separate checkerboard assays were performed. FICs were calculated as the sum of the combination MIC divided by the MIC for each compound and are shown in Table 3. Synergy was found for nalidixic acid, and indifference was found for ciprofloxacin. MIC values were higher overall in the microtiter format then by Etest, and this difference was more marked for ciprofloxacin than for nalidixic acid but the trend of decreased susceptibility in the presence of PAβN for ciprofloxacin and increased susceptibility of nalidixic acid held. These data support our Etest findings. A literature search was done to compare our findings with those of other studies, particularly with regard to fluoroquinolones, as the initial study on PAβN on *P. aeruginosa* (21) saw an 8-fold increase in susceptibility to fluoroquinolone and levofloxacin. Other studies show that the effect of PAβN is dependent on strain and antibiotic (4, 29, 33) for both *E. coli* and *P. aeruginosa* and that the fold increase in susceptibility varies from 1 to 8. Pasquali and Manfreda (29) also show that *E. coli* strain ATCC 25922 had MICs of 0.016 to <0.06 μg/ml in the absence and presence of 80 μg/ml PAβN. This shows that there is variation between strains as to the effect on PAβN and that our findings do not contradict the findings from other groups.

Since the homologs of the AcrAB-TolC pump in other pathogens are important in virulence (3, 27, 30), we studied the contribution of *acrAB* in organ colonization in pneumonic and septicemic models of plague and the contribution of *tolC* in the pneumonic plague model. Seven- to 8-week-old female BALBc mice were infected intranasally or intravenously with 20 and 10 times the 50% lethal dose (LD_{50}), respectively (28, 38). Four (intravenous) or 5 (intranasal) days postinfection, mice were euthanized; tissues were harvested, homogenized, serially diluted, and plated for CFU. Strikingly, no difference was seen for the *tolC* strain (results not shown). While a trend toward lower colonization levels by the *acrAB* mutant was observed, it was not significant (Fig. 2). These findings suggest that unlike in other pathogens, *Y.*

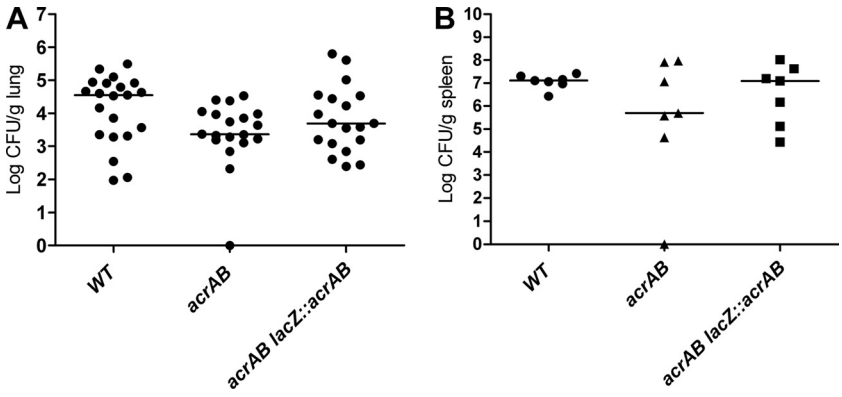

FIG 2 Intranasal (A) and intravenous (B) mouse infections. Each symbol in the graphs shows the results for one mouse. The horizontal bar denotes the median.

pestis AcrAB does not play a significant role in establishing infections in pneumonic or septicemic plague in mice.

ACKNOWLEDGMENTS

We thank Hortensia Garcia-Rolan for performing the intravenous injections in the mouse model of septicemia.

This work was supported in part by the New England Regional Center of Excellence (NERCE) and Biodefense and Emerging Infectious Diseases (BEID) and PHS grant AI 56021.

We thank AB Biodisk for providing Etests for this work.

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