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The AcrAB-TolC Efflux Pump Contributes to Multidrug Resistance in the Nosocomial Pathogen *Enterobacter aerogenes*

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We identified the genes encoding the AcrA-AcrB-TolC efflux pump in *Enterobacter aerogenes* and constructed *acrAB* and *tolC* mutants from a multidrug-resistant isolate. Both derivatives were more susceptible to antibiotics than the parental strain. Sequence analysis and complementation experiments revealed that the multidrug-resistant isolate is an *acrR* mutant.

For the last decade, *Enterobacter aerogenes*, a commensal gram-negative bacterium of human intestinal flora, has been rapidly emerging as an important nosocomial pathogen (14, 18). Of concern is the increasing frequency of *E. aerogenes* isolates that are resistant to antibiotics and antiseptics (3).

Several types of systems have evolved in gram-negative bacteria to pump deleterious molecules out of the cytosol (13, 16). Among these, the resistance-nodulation-division family of systems bypasses the periplasm and provides efflux across both the inner membrane and the outer membrane (OM). Such systems require three partners: an inner membrane transporter, a periplasmic membrane fusion protein, and an OM channel (13). These pumps utilize the energy of the proton motive force to extrude dyes, detergents, disinfectants, solvents, and antibiotics from the cell. The *Escherichia coli* AcrAB-TolC and the *Pseudomonas aeruginosa* MexAB-OprM efflux systems have been the most extensively studied (13, 16). The AcrAB-TolC pump provides *E. coli* with a natural resistance to bile salts (19).

Active antibiotic efflux has been detected in some *E. aerogenes* multidrug-resistant (MDR) clinical isolates (11). As a first step to identify the efflux systems and their regulation, we cloned the *E. aerogenes* genes involved in such mechanisms by complementation of *E. coli* mutants.

The strains and plasmids used in this work are listed in Table 1. MICs were determined according to the standard twofold dilution method of the French Society for Microbiology (11). EA27 is an MDR isolate that exhibits active efflux of norfloxacin (11). EAEP289, the Kan^s derivative of EA27, was obtained after growth of the isolate in successive cultures in the presence of mitomycin C (0.4 μg/ml). No differences were observed between EA27 and EAEP289 in their plasmid, protein, or lipopolysaccharide profiles (data not shown).

Primers acrR1 (5′-GCGAATAGCGGCAGAGA-3′) and acrR2 (5′-GAGAGCATCAGAACGACCG-3′) were used to amplify *acrR* and its promoter. PCR products were cloned into the *Sma*I site of pJQ254 (17).

Whole-membrane extracts were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 10% gel. Proteins were electroblotted onto nitrocellulose membranes and probed with polyclonal antibodies raised against *E. coli* AcrA (22) or TolC (5), and the immunoblots were developed by colorimetric detection with alkaline phosphatase-conjugated secondary antibodies.

Cloning and identification of the *E. aerogenes acr* and *tolC* loci. We used a genomic library in the form of a Mu dI5166 phage lysate to complement *E. coli* Δ*acrAB* or *tolC* mutants for growth on plates supplemented with 0.1% SDS (6, 10). From pEP676, which complemented the *E. coli* Δ*acrAB* mutant, a 6-kb genomic DNA fragment was cloned into $pBCSK^+$ to generate pEP709. From pEP685, which complemented the *E. coli tolC* mutant, an 8-kb DNA insert was subcloned into $pBCSK^+$ to generate pEP710. Whole-membrane extracts of *E. coli* AG100 ($AcrAB^+$ $TolC^+$), AG100A Δ*acrAB*, AG100A (pEP709), AG100 *tolC*, and AG100 *tolC* bearing pEP710 were analyzed by immunoblotting. An immunoreactive protein was detected in AG100A(pEP709) with antibodies raised against *E. coli* AcrA (22) (Fig. 1A), while an anti-TolC immunoreactive protein was detected in AG100 *tolC*(pEP710) (5) (Fig. 1B).

Sequence analysis of the insert in pEP709 revealed that the 6-kb *E. aerogenes acrRAB* locus organization is identical to that of *E. coli.* First, *acrR* and *acrA* are transcribed divergently. The sequences of the *acrA* promoter and AcrR repressor binding sites identified in *E. coli* are strictly conserved in *E. aerogenes*. Second, the 22-bp *acrA-acrB* intergenic sequences are identical in both species, and thus the *E. aerogenes* genes are probably also transcribed as an operon.

E. aerogenes and *E. coli* AcrR proteins share 78% overall sequence identity in a 215-amino-acid (aa) overlap. The sequence of the predicted N-terminal helix-turn-helix DNA binding motif is even more conserved (90% identity in a 31-aa overlap). Both AcrA proteins exhibit 85% identity in a 399-aa overlap. *E. aerogenes* AcrB and its *E. coli* homolog present 87% sequence identity in a 1,049-aa overlap.

Several subclones of pEP710 were constructed, and they enabled us to localize *E. aerogenes tolC* in a 2.2-kb *Sac*II fragment carried by pEP787. Sequence analysis of this fragment revealed that TolC proteins from *E. aerogenes* and *E. coli* share 82% sequence identity in a 495-aa overlap. *E. aerogenes* TolC

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant features[a]	Source or reference
E. coli strains		
AG100	K-12	15
AG100A	AG100 Δ*acrAB*::Kan^r	15
BW5104	Mu-1 Δ*lac169 creB510 hsdR514*	10
LBB1296	AG100 *tolC*::Tn*10*	4
EP661	BW5104 Δ*acrAB*::Kan^r	This study
EP663	BW5104 *tolC*::Tn*10*	This study
E. aerogenes strains		
BW16627	ATCC 15038 *rpsL*	10
BW16662	BW16627 (pREG2-1, pEG5166S)	10
BW16665	BW16627 (pREG2-1, pEG5166S)	10
EA27	MDR clinical isolate; Kan^r Amp^r Chl^r Nal^r Str^r Tet^r	11
EAEP289	Kan^s derivative of EA27	This study
EAEP294	EAEP289 *acrA*::Kan^r (pEP755 integration)	This study
EAEP298	EAEP289 *tolC*::Kan^r (pEP786 integration)	This study
Plasmids		
Mu dI5166	Mini-Mu for in vivo cloning; Chl^r	6
pEP676	Mu dI5166 bearing *acrRAB* on a 6-kb insert	This study
pEP685	Mu dI5166 bearing *tolC* on an 8-kb insert	This study
pBCSK$^+$	High-copy-number vector; Chl^r	Stratagene
pEP709	pBCSK$^+$ bearing *acrRAB* on a 7-kb *Bam*HI-*Hin*dIII fragment from pEP676, 1 kb is from Mu dI5166	This study
pEP710	pBCSK$^+$ bearing *tolC* on a 9-kb *Bam*HI-*Hin*dIII fragment from pEP685, 1 kb is from Mu dI5166	This study
pVIK108	pir-dependent plasmid, oriR6K; Kan^r	7
pEP755	pVIK108 bearing *′acrA′* on a 0.8-kb *Xho*I-*Eco*RI fragment	This study
pEP786	pVIK108 bearing *′tolC′* on a 1-kb *Nru*I-*Sac*I fragment	This study
pBBR1MCS	Medium-copy-number vector; Chl^r	9
pEP787	pBBR1MCS bearing *tolC* on a 2.2-kb *Sac*II fragment oriented opposite P*lac*	This study
pJQ254	High-copy-number vector; Kan^r	17
pEP805	pJQ254 bearing *acrR* from EA27 cloned under P*lac*	This study
pEP806	pJQ254 bearing *acrR* from EA27 cloned opposite P*lac*	This study
pEP808	pJQ254 bearing *acrR* from BW16627 cloned under P*lac*	This study
pEP809	pJQ254 bearing *acrR* from BW16627 cloned opposite P*lac*	This study

[a] Amp^r, Chl^r, Kan^r, Nal^r, Str^r, and Tet^r, resistance to ampicillin, chloramphenicol, kanamycin, nalidixic acid, streptomycin, and tetracycline, respectively.

is slightly smaller as it contains a 6-aa deletion in a cell-surface-exposed loop and a 3-aa deletion in the C-terminal region.

AcrAB and TolC contribute to drug resistance in an MDR *E. aerogenes* isolate. We constructed *acrA* and *tolC* mutants from EAEP289 via chromosomal integration of Kan^r suicide plasmids pEP755 and pEP786, respectively (Table 1). The absence of AcrA or TolC production in the resulting Kan^r clones, EAEP294 and EAEP298, was confirmed by immunoblotting (Fig. 1). EAEP294 and EAEP298 were unable to grow on plates supplemented with the bile salt deoxycholate (data not shown), with SDS, or with novobiocin and showed reduced resistance to all drugs tested (Table 2).

To determine that the observed phenotypes did not result from polar effects on downstream genes, we transformed EAEP294 *acrA*::Kan^r with pEP709 and EAEP298 *tolC*::Kan^r with pEP787. The transformants were selected on Luria broth agar-kanamycin plates containing 0.05% cetyltrimethylammonium bromide, a detergent, since selection on plates containing SDS or chloramphenicol was not efficient. EAEP294(pEP709) and EAEP298(pEP787) were able to grow on plates containing SDS and novobiocin (Table 2). The MICs of all drugs for both transformants were increased 2- to 16-fold, compared to those for the nontransformed strains.

Overexpression of *acrR* reduces drug resistance in an MDR *E. aerogenes* isolate. The *acr* and *tolC* loci of EA27 were PCR amplified and sequenced. Comparison of the EA27 and

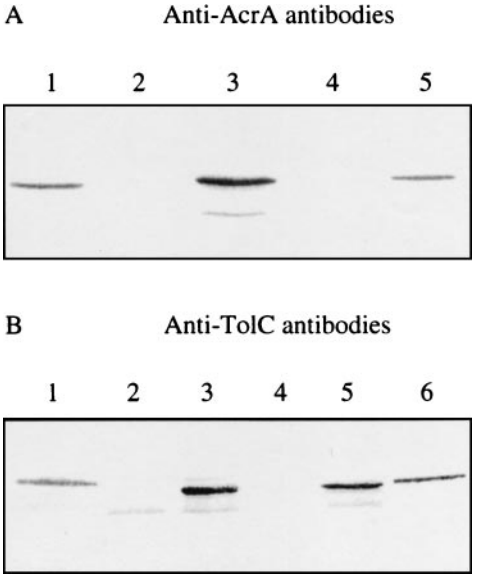

FIG. 1. Immunoblots of whole-membrane extracts with antibodies raised against *E. coli* AcrA (A) or *E. coli* TolC (B). (A) Lane 1, *E. coli* AG100 (AcrA$^+$); lane 2, *E. coli* AG100A (AcrA$^-$); lane 3, *E. coli* AG100A(pEP709); lane 4, *E. aerogenes* EAEP294 (AcrA$^-$); lane 5, *E. aerogenes* EAEP289 (AcrA$^+$). (B) Lane 1, *E. coli* AG100 (TolC$^+$); lane 2, *E. coli* LBB1296 (TolC$^-$); lane 3, *E. coli* LBB1296(pEP710); lane 4, *E. aerogenes* EAEP298 (TolC$^-$); lane 5, *E. coli* LBB1296(pEP787); lane 6, *E. aerogenes* EAEP289 (TolC$^+$).

TABLE 2. Susceptibilities of *E. aerogenes* strains to antimicrobials

Strain and relevant phenotype(s)	MIC (μg/ml) of[a]:						Growth[b]	
	CHL	NOR	CIP	TET	MC	AF	SDS	NOV
BW16627 AcrA$^+$ TolC$^+$	4	<0.125	<0.125	1	1	64	+	−
EA27 AcrA$^+$ TolC$^+$	>256	256	32	8	2	256	+	+
EAEP289 AcrA$^+$ TolC$^+$	>256	256	32	8	4	256	+	+
EAEP294 AcrA$^-$ TolC$^+$	32	64	16	<0.25	<0.125	32	−	−
EAEP298 AcrA$^+$ TolC$^-$	32	16	4	<0.25	<0.125	32	−	−
EAEP294(pEP709) AcrA$^+$ TolC$^+$	>256	256	32	8	2	256	+	+
EAEP298(pEP787) AcrA$^+$ TolC$^+$	>256	128	32	8	1	256	+	+
EA289(pJQ254) AcrR$^-$	>256	256	32	8	4	256	+	+
EA289(pEP805) AcrR$^-$	>256	256	32	8	4	256	+	+
EA289(pEP806) AcrR$^-$	>256	256	64	16	4	256	+	+
EA289(pEP808); AcrR overproducer	128	64	8	2	1	64	−	+
EA289 (pEP809); AcrR producer	>256	256	32	8	4	256	+	+

[a] Abbreviations: CHL, chloramphenicol; NOR, norfloxacin; CIP, ciprofloxacin; TET, tetracycline; MC, mitomycin C; AF, acriflavine.
[b] Growth (+) or absence of growth (−) on LBA plates containing 0.1% SDS or 30 μg of novobiocin (NOV) per ml.

BW16627 *tolC* sequences indicated no difference in the promoter regions. The detected 31 base changes generated only 11 aa substitutions, and most of them were conservative or involved residues located in loops based on the *E. coli* TolC tridimensional structure (8).

The 21 base substitutions observed in the EA27 *acr* locus sequence were silent. However, a frameshift mutation due to a 1-bp deletion was detected in codon 47 of *acrR*. To confirm that EAEP289 is an *acrR* null mutant, we transformed it with pJQ254 (17) bearing *acrR* amplified from the BW16627 (AcrR$^+$) or EA27 (AcrR$^-$) genome. In pEP805 and pEP808, *acrR* is in the same orientation as P*lac*, while in pEP806 and pEP809, *acrR* is cloned opposite P*lac*. EAEP289 containing pJQ254, pEP805, pEP806, or pEP809 exhibited identical resistance levels for all the antimicrobials tested (Table 2). In contrast, EAEP289(pEP808) was SDS susceptible and its susceptibility to all drugs was reduced fourfold. However, EAEP289(pEP808) was able to grow on Luria broth agar-novobiocin plates, suggesting that residual pump activity was sufficient to discharge novobiocin. In EAEP289(pEP808), overproduced AcrR may repress *acrAB* transcription, while in EAEP289(pEP809), AcrR production may be insufficient to repress *acrAB*. This could result from *acrR* autoregulation.

In *E. coli*, upon derepression of *marA* transcription, the MarA regulator mediates MDR by activating *acrAB* and *tolC* and by downregulating the synthesis of the major OmpF porin (1, 2). EA27 does not synthesize the major *E. aerogenes* porin (11), and complementary sequence data obtained in this laboratory indicated the absence of a mutation in the EA27 *mar* locus. Our results suggest that in a porin-deficient *E. aerogenes* strain, *acrAB* derepression is sufficient to generate high resistance levels in the absence of the MarA activator. These observations are in accordance with several recent studies of *E. coli* isolates that reveal the role of *acrR* mutations in high-level fluoroquinolone resistance in the absence of *mar* mutations (12, 20, 21).

Nucleotide sequence accession numbers. The nucleotide sequences of the *E. aerogenes* BW16627 *acrRAB* and *tolC* and the *E. aerogenes* EA27 *tolC* loci have been assigned EMBL accession nos. AJ306389, AJ306390, and AJ421426, respectively.

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