

## Roles of TolC-Dependent Multidrug Transporters of *Escherichia coli* in Resistance to $\beta$ -Lactams

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**AcrAB exports some  $\beta$ -lactam antibiotics in the periplasm out of cells via an outer-membrane channel, TolC. It has been reported that eight drug transporters in *Escherichia coli* cooperate with TolC. In this study, the roles of the drug exporters of *E. coli* in  $\beta$ -lactam resistance were examined. We found that five of five resistance-nodulation-cell division-type drug exporters confer  $\beta$ -lactam antibiotic resistance, while no other drug exporters confer any  $\beta$ -lactam resistance even when they cooperate with TolC.**

The emergence of bacterial multidrug resistance has become an increasing problem in the treatment of infectious diseases. Multidrug resistance often results from the overexpression of multidrug efflux transporters (4, 6, 21, 23, 24). Multidrug transporters in bacteria are classified into five families on the basis of sequence similarity. These comprise the major facilitator (MFS), resistance-nodulation-cell division (RND), small multidrug resistance (SMR), multidrug and toxic compound extrusion (MATE), and ATP-binding cassette (ABC) families (1, 25, 26). Recent genome sequence analysis revealed that bacteria have a lot of intrinsic drug transporter genes. In a previous study, Nishino and Yamaguchi cloned 37 known and putative drug transporter genes of *Escherichia coli* and revealed that 20 of them encode exporters of known drugs and/or toxic compounds (21). Among them, RND family transporters in particular play a major role in producing both intrinsic and elevated levels of resistance to a very wide range of noxious compounds in gram-negative bacteria (12, 17, 18). However, not much data are available on the contribution of these transporters (other than AcrAB) to  $\beta$ -lactam resistance (11, 13, 26).

RND transporters usually need two other proteins for their function, a membrane fusion protein (MFP) located in the bacterial periplasm and an outer-membrane channel. For example, AcrB functions through association with periplasmic AcrA (32) and the outer-membrane channel TolC (3a, 8, 30). The AcrAB-TolC system pumps out some  $\beta$ -lactams with multiple charged groups, which have been experimentally shown not to traverse the cytoplasmic membrane (13). Recently, examination of the crystal structure of AcrB revealed the possibility that RND transporters can pump out substrates from the periplasm (15). These data indicate that the AcrAB-TolC system can transport  $\beta$ -lactams from the periplasm into the external medium through the outer-membrane protein TolC (10, 19, 20).

All of the other four RND drug transporter systems (AcrD,

AcrEF, MdtEF [YhiU and YhiV have been renamed MdtE and MdtF following the systematic nomenclature indicated at <http://bmb.med.miami.edu/ecogene/ecoweb/> {27}], and MdtABC) in *E. coli* also need TolC for their functions (3, 5, 16, 21, 22). In addition, two MFS drug transporter systems (EmrAB and EmrKY) and one ABC drug transporter system (MacAB) also need TolC for their functions (7, 9, 22). However, almost none of their capabilities for  $\beta$ -lactam transport have been examined. To elucidate whether or not the capability of  $\beta$ -lactam export from the periplasm is determined by the outer-membrane channel TolC, we cloned all of TolC-dependent drug transporter genes with native promoters into pHSG multicopy plasmids that have a chloramphenicol resistance marker and then investigated their  $\beta$ -lactam resistance phenotypes using as a host an *E. coli* mutant lacking the major multidrug efflux transporter AcrB. We also cloned the *mdfA* (2), *emrE* (31), and *mdtK* (*ydhE* has been renamed *mdtK* following the systematic nomenclature indicated at <http://bmb.med.miami.edu/ecogene/ecoweb/> [27]) (14) genes, which encode TolC-independent multidrug transporters belonging to the MFS, SMR, and MATE families, respectively.

The drug transporter genes were amplified with native promoters from *E. coli* chromosomal DNA by means of PCR using primers containing the restriction enzyme site that exists in the multicloning sites of pHSG398 and pHSG399 (Table 1). The DNA fragments digested with restriction enzymes were ligated into the multicloning sites of pHSG vectors. The fragment sizes and multicloning sites used are shown in Table 2. All drug transporter open reading frames (ORFs) were arranged so as to be in the same orientation as the lactose promoter of the pHSG vectors. Thus, drug transporter genes were expected to be expressed from the lactose promoter. Plasmid DNA was extracted from five independent colonies of every recombinant, followed by transformation of *E. coli* KAM3 (an *acrB*-deficient strain) cells with these plasmids.

Using  $10^4$  exponential-phase cells grown in  $2\times$  YT broth (28) supplemented with 20  $\mu$ g of chloramphenicol/ml as the inoculum (Table 3), MICs were determined by serial twofold dilution in YT agar (28). As can be seen, *E. coli* KAM3, which is an *acrB* deletion derivative of TG1, showed hypersensitivity to some

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TABLE 1. Primer pairs used to amplify the drug exporter genes

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
<i>acrAB</i>	CGCGGATCCATGTTTCGTGAATTTACAGGCG	CGCGCATGCAACGCGTCCCCTTCTTAGC
<i>acrD</i>	ACGTTAGTGAGTATAAGCTTTCGCTCGGAGGG	CGCAGCAATCGAGTAAATGAGCTCCACCACGGTATATTG
<i>acrEF</i>	CGCGCATGCGCGAAGGTTAATCTATCACCT	TATTGGTGTGAACCAGTCGACCCTGATCGTCAATAAACCGG
<i>mdtABC</i>	CGCGCATGCTCCTTATTTATGGCCCTTCC	CGCGAATTCCTACTCGGTTACCGTTTGTTT
<i>mdtEF (yhiUV)</i>	CGTTGCCCGAATGCCTGCAGGAAGTGTGGCAGAACGGC	CTCGGCATGTTGGGATCCTTAAGCTGACAATTCATACG
<i>emrAB</i>	GGCGCGGTAGGTATTTTGGTGCACACCAATTGGCATCGCC	CGTGCTGAAAGTGCAGGATCCGAATCAGATCCCGGAAGT
<i>emrKY</i>	CGCGCATGCCCTGGGATAATGTGCAACACA	CGCGGATCCTTACCCGCTATAACCCCTCC
<i>mdfA</i>	GGTTAAACTGGGTACCGCGGCATCGTCTTATTTCC	ATGTGGCTCCTGCGCCAGCTCAAGCTTACCGCCCGC
<i>macAB</i>	CGCCAGCTGCAGCCCGGGGTATTCAAATATAAGC	CGCCAGTGTTATTGTGCCGTCGACGTAGAAGCGGC
<i>emrE</i>	CGCCCCGGTCTTTTCATTGCTGAAAGTGG	CGCGGATCCGAATGCACTTAATCCTAAATC
<i>mdtK (ydhE)</i>	CATTCTGTTACAGCAACTGCAGGTGAAAACGCC	GCCAGACGAAGACGGGATCCGCGCAACGCTCCAGCC

β-lactams (oxacillin [OXA], cloxacillin [MCIPC], nafcillin [NAF], cefuroxime [CXM], cefamandole [FAM], and faropenem [FRPM]) and other toxic compounds (deoxycholate, sodium dodecyl sulfate, novobiocin, erythromycin, and tetraphenylphosphonium

bromide). The pHSGacrAB plasmid (used for overproduction of the AcrAB pump) caused almost full recovery of the intrinsic resistance. These results are in good agreement with previous observations by Mazzariol and coworkers (13).

TABLE 2. Bacterial strains and plasmids used in this study<sup>a</sup>

Strain or plasmid	Relevant phenotype or genotype <sup>b</sup>	Reference or origin
<i>E. coli</i> strains		
TG1	<i>supE hsd Δ5 thi Δ[lac-proAB] F' [traD36 proAB lacI<sup>q</sup> lacZ ΔM15]</i>	28
KAM3	Derivative of TG1 that lacks <i>acrB</i>	14
TG1Δ <i>tolC</i>	Derivative of TG1 that lacks <i>tolC</i>	16
DH5α	<i>recA endA1 hsdR17 supE4 gyrA96 relA1 Δ(lacZYA-argF)U169 (φ80<i>dlacZ</i>Δ M15)</i>	28
Plasmids		
General		
pHSG398	Vector; CP <sup>r</sup> ; multiple cloning site in <i>lacZ</i>	Takara Bio Inc.
pHSG399	Vector; CP <sup>r</sup> ; multiple cloning site in <i>lacZ</i>	Takara Bio Inc.
Plasmids carrying RND-type transporter ORFs		
pHSGacrAB	5.1-kb <i>Bam</i> HI- <i>Sph</i> I fragment containing <i>acrA/B</i> (MFP/multidrug transporter) genes cloned into pHSG398, CP <sup>r</sup>	This study
pHSGacrD	3.5-kb <i>Hind</i> III- <i>Sac</i> I fragment containing <i>acrD</i> (multidrug transporter) gene cloned into pHSG399, CP <sup>r</sup>	This study
pHSGacrEF	5.0-kb <i>Sph</i> I- <i>Sal</i> I fragment containing <i>acrE/F</i> (MFP/multidrug transporter) genes cloned into pHSG399, CP <sup>r</sup>	This study
pHSGmdtABC	7.7-kb <i>Sph</i> I- <i>Eco</i> RI fragment containing <i>mdtA/B/C</i> (MFP/multidrug transporter/multidrug transporter) genes cloned into pHSG399, CP <sup>r</sup>	This study
pHSGmdtEF	4.7-kb <i>Pst</i> I- <i>Bam</i> HI fragment containing <i>mdtE/F</i> (formerly named <i>yhiU/V</i> ) (MFP/multidrug transporter) genes cloned into pHSG399, CP <sup>r</sup>	This study
Plasmids carrying MFS-type transporter ORFs		
pHSGemrAB	3.9-kb <i>Sal</i> I- <i>Bam</i> HI fragment containing <i>emrR/A/B</i> (regulator/MFP/multidrug transporter) genes cloned into pHSG399, CP <sup>r</sup>	This study
pHSGemrKY	2.8-kb <i>Sph</i> I- <i>Bam</i> HI fragment containing <i>emrK/Y</i> (MFP/bile acid transporter) genes cloned into pHSG399, CP <sup>r</sup>	This study
pHSGmdfA	1.6-kb <i>Kpn</i> I- <i>Hind</i> III fragment containing <i>mdfA</i> (multidrug transporter) gene cloned into pHSG398, CP <sup>r</sup>	This study
Plasmid carrying ABC-type transporter ORFs		
pHSGmacAB	3.7-kb <i>Pst</i> I- <i>Sal</i> I fragment containing <i>macA/B</i> (MFP/macrolide-specific transporter) genes cloned into pHSG399, CP <sup>r</sup>	This study
Plasmid carrying SMR-type transporter ORFs		
pHSGemrE	1.2-kb <i>Sma</i> I- <i>Bam</i> HI fragment containing <i>emrE</i> (multidrug transporter) gene cloned into pHSG398, CP <sup>r</sup>	This study
Plasmid carrying MATE-type transporter ORFs		
pHSGmdtK	1.6-kb <i>Pst</i> I- <i>Bam</i> HI fragment containing <i>mdtK</i> (formerly named <i>ydhE</i> ) (multidrug transporter) gene cloned into pHSG399, CP <sup>r</sup>	This study

<sup>a</sup> Gene fragments were cloned into pHSG vectors in the same orientation as the lactose promoter.

<sup>b</sup> CP, chloramphenicol; MFP, membrane fusion protein.

TABLE 3. Susceptibility of *E. coli* drug transporter overproducing strains to  $\beta$ -lactams and toxic compounds

Strain	MIC ( $\mu\text{g/ml}$ ) <sup>a</sup>																	
	AMP	OXA	MCIPC	NAF	CAR	SBPC	CXM	FAM	CTX	CMZ	ATM	FRPM	DOC <sup>b</sup>	SDS <sup>b</sup>	NOV <sup>b</sup>	ERM <sup>b</sup>	TPP <sup>b</sup>	
TG1	1.56	>100	200	>400	1.56	1.56	1.56	0.39	0.025	0.78	0.05	0.39	>40,000	>400	50	50	200	
KAM3, KAM3/pHSG398 or pHSG399	0.78	0.39	0.78	1.56	1.56	1.56	0.20	0.10	0.012	0.39	0.05	0.10	625	50	0.78	1.56	3.13	
KAM3/pHSGacrAB	1.56	>100	100	200	1.56	1.56	1.56	0.39	0.025	0.78	0.05	0.39	>40,000	>400	25	12.5	200	
KAM3/pHSGacrD	0.78	12.5	12.5	50	25	50	0.39	0.20	0.012	0.78	0.39	0.10	>40,000	>400	6.25	1.56	3.13	
KAM3/pHSGacrEF	0.78	50	100	200	1.56	1.56	0.78	0.39	0.025	0.78	0.05	0.10	>40,000	>400	25	12.5	200	
KAM3/pHSGmdtABC	0.78	3.13	6.25	6.25	6.25	12.5	0.20	0.10	0.012	0.39	0.10	0.10	20,000	200	6.25	1.56	1.56	
KAM3/pHSGmdtEF	0.78	12.5	12.5	25	1.56	1.56	0.20	0.10	0.012	0.39	0.05	0.10	20,000	200	0.78	12.5	25	
KAM3/pHSGemrAB <sup>c</sup>	0.78	0.39	0.78	1.56	1.56	1.56	0.20	0.10	0.012	0.39	0.05	0.10	2,500	100	3.13	1.56	1.56	
KAM3/pHSGemrKY	0.78	0.39	0.78	1.56	1.56	1.56	0.20	0.10	0.012	0.39	0.05	0.10	1,250	50	ND <sup>e</sup>	1.56	ND	
KAM3/pHSGmacAB	0.78	0.39	0.78	1.56	1.56	1.56	0.20	0.10	0.012	0.39	0.05	0.10	625	50	0.78	12.5	1.56	
KAM3/pHSGmdfA	0.78	0.39	0.78	1.56	1.56	1.56	0.20	0.10	0.012	0.39	0.05	0.10	625	50	0.78	1.56	12.5	
KAM3/pHSGemrE <sup>d</sup>	0.78	0.39	0.78	1.56	1.56	1.56	0.20	0.10	0.012	0.39	0.05	0.10	625	50	0.78	1.56	3.13	
KAM3/pHSGmdtK	0.78	0.39	0.78	1.56	1.56	1.56	0.20	0.10	0.012	0.39	0.05	0.10	625	50	0.39	1.56	25	
TG1 $\Delta$ tolC	0.78	0.39	0.78	0.78	0.78	1.56	0.20	0.10	0.012	0.39	0.05	0.10	313	ND	ND	ND	ND	
TG1 $\Delta$ tolC/pHSGacrAB	0.78	0.39	0.78	0.78	0.78	1.56	0.20	0.10	0.012	0.39	0.05	0.10	313	ND	ND	ND	ND	
TG1 $\Delta$ tolC/pHSGacrD	0.78	0.39	0.78	0.78	0.78	1.56	0.20	0.10	0.012	0.39	0.05	0.10	313	ND	ND	ND	ND	
TG1 $\Delta$ tolC/pHSGacrEF	0.78	0.39	0.78	0.78	0.78	1.56	0.20	0.10	0.012	0.39	0.05	0.10	313	ND	ND	ND	ND	
TG1 $\Delta$ tolC/pHSGmdtEF	0.78	0.39	0.78	0.78	0.78	1.56	0.20	0.10	0.012	0.39	0.05	0.10	313	ND	ND	ND	ND	
TG1 $\Delta$ tolC/pHSGmdtABC	0.78	0.39	0.78	0.78	0.78	1.56	0.20	0.10	0.012	0.39	0.05	0.10	313	ND	ND	ND	ND	

<sup>a</sup> MIC determination was repeated at least three times. Values in bold differ from the KAM3/pHSG398 or 399 control strain value by  $\geq 4$ -fold. MIC values of benzylpenicillin (12.5  $\mu\text{g/ml}$  for all strains listed in the table), cephalothin (1.56  $\mu\text{g/ml}$ ), cefsulodin (12.5  $\mu\text{g/ml}$ ), ceftazidime (0.10  $\mu\text{g/ml}$ ), flomoxef (0.025  $\mu\text{g/ml}$ ), and imipenem (0.05  $\mu\text{g/ml}$ ) were not changed by overexpression of any drug exporters tested in this study. Abbreviations: AMP, ampicillin; SBPC, sulbenicillin; CTX, cefotaxime; CMZ, cefmetazole; DOC, deoxycholate; SDS, sodium dodecyl sulfate; NOV, novobiocin; ERM, erythromycin; TPP, tetraphenylphosphonium bromide.

<sup>b</sup> These compounds were used to confirm the overproduction of the pumps. Additionally, CCCP (carbonyl cyanide *m*-chlorophenylhydrazone) and acriflavine were used to confirm the overproduction of EmrAB and EmrE, as described below.

<sup>c</sup> pHSGemrAB conferred CCCP resistance (MIC, 6.25  $\mu\text{g/ml}$  for KAM3 versus 50  $\mu\text{g/ml}$  for KAM3/pHSGemrAB).

<sup>d</sup> pHSGemrE conferred acriflavine resistance (MIC, 12.5  $\mu\text{g/ml}$  for KAM3 versus 200  $\mu\text{g/ml}$  for KAM3/pHSGemrE).

<sup>e</sup> ND, not determined.

As shown in Table 3, pHSG-acrD, -acrEF, -mdtABC, and -mdtEF also conferred  $\beta$ -lactam resistance to *E. coli* KAM3. Among these, pHSGacrEF conferred the highest level of  $\beta$ -lactam resistance to *E. coli*. Overproduction of AcrEF caused elevated resistance to OXA (128-fold higher than the level seen with KAM3), MCIPC (128-fold), NAF (128-fold), CXM (4-fold), and FAM (4-fold). Except for that seen with FRPM, the drug resistance spectrum of AcrEF against  $\beta$ -lactams is quite similar to that of AcrAB, probably because AcrF exhibits high sequence homology to AcrB (84% similarity and 77% identity in the amino acid sequences). The resistance spectra of AcrEF and AcrAB against other toxic compounds are almost identical (21).

pHSGacrD conferred resistance to OXA (32-fold higher than the level seen with KAM3), MCIPC (16-fold), NAF (32-fold), CAR (carbenicillin) (16-fold), SBPC (sulbenicillin) (32-fold), and ATM (aztreonam) (8-fold). pHSGmdtABC conferred resistance to OXA (8-fold), MCIPC (8-fold), NAF (4-fold), CAR (4-fold), and SBPC (8-fold). pHSGmdtEF conferred resistance to OXA (32-fold), MCIPC (16-fold), and NAF (16-fold). Except for that of ATM, the drug resistance spectrum of AcrD against  $\beta$ -lactams is quite similar to that of MdtABC. These transporters also have similar substrate specificities for other compounds (novobiocin, deoxycholate, and sodium dodecyl sulfate) (Table 3) (21). Recently, Hirakawa et al. and Elkins and Nikaido found that AcrD, like AcrB, needs TolC and AcrA for its function (3, 5). These data indicate that the substrate specificities of these exporters are determined by the inner-membrane pumps.

pHSG-emrAB, -emrKY, -macAB, -mdfA, -emrE, and -mdtK

did not confer any  $\beta$ -lactam resistance, while these plasmids conferred resistance against other compounds (Table 3).

All plasmids that confer  $\beta$ -lactam resistance to *E. coli* encode TolC-dependent transporters. Using a TolC-deficient strain, we investigated whether or not TolC is required for  $\beta$ -lactam resistance. Neither pHSG-acrAB, -acrD, -acrEF, -mdtABC, or -mdtEF conferred any  $\beta$ -lactam resistance in TolC-deficient strain *E. coli* TG1 $\Delta$ tolC (Table 3). Thus, it is concluded that TolC is required for  $\beta$ -lactam export from the periplasm. TolC-independent transporters such as MdfA, EmrE, and MdtK did not confer  $\beta$ -lactam resistance, probably due to the lack of cooperation with the outer-membrane channel.

Although EmrAB (an MFS type), EmrKY (an MFS type), and MacAB (an ABC type) are TolC-dependent exporters but not RND-type transporters, none of them conferred resistance to  $\beta$ -lactams. These observations suggest that only RND-type transporters, which probably have vestibules open at the side of the periplasmic headpiece, can recognize  $\beta$ -lactams (15).

In this study, we showed that all of the five RND-type drug exporters of *E. coli*, i.e., AcrAB, AcrD, AcrEF, MdtABC, and MdtEF, confer resistance to *E. coli* against  $\beta$ -lactams and that none of the other exporters confer  $\beta$ -lactam resistance, even when they cooperate with the outer-membrane channel TolC. Previously, Sulavik et al. (29) constructed *E. coli* strains with deletions of putative drug exporters and outer-membrane channels. They reported that the deletion of *acrAB* increased the drug susceptibility of *E. coli* cells but that strains from which *acrD*, *acrEF*, *mdtABC*, or *mdtEF* genes were deleted showed no change in susceptibility against tested compounds. There are two possibilities to explain why these deletions had

no effect on drug susceptibility. One possibility is that the effect of deletion of these genes might have been masked by AcrAB. The other possibility is these genes might not be expressed under normal conditions. Outer-membrane channel TolC is required for the functions of all of the five RND-type drug exporters. Our data showed that the deletion of *tolC* increased  $\beta$ -lactam susceptibility to the same level as that of the *acrAB* single-deletion mutant. This observation supports the latter of the two above-named possibilities. Thus, expression cloning of an individual gene into an AcrAB-deficient strain may be necessary for the discovery of a potential drug efflux transporter gene. Our results suggest that RND-type drug transporter genes will become a potential source for  $\beta$ -lactam resistance to pathogens.

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