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Resistance to Methotrexate Due to AcrAB-Dependent Export from *Escherichia coli*

STEPHAN J. KOPYTEK,† JOHN C. D. DYER, GWENDOWLYN S. KNAPP, AND JAMES C. HU*

Department of Biochemistry and Biophysics and Center for Advanced Biomolecular Research, Texas A&M University, College Station, Texas 77843-2128

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Many laboratory strains of *Escherichia coli* are resistant to methotrexate (MTX), a folate analogue that binds dihydrofolate reductase (DHFR). Mutations that inactivate either *tolC* or *acrA* confer MTX sensitivity. Further, overexpression of a fusion protein with DHFR activity reverses this sensitivity by titrating out intracellular MTX. These results suggest that MTX accumulates in cells where mutations in *acrA* or *tolC* have inactivated the TolC-dependent AcrAB multidrug resistance efflux pump.

Methotrexate (MTX) is a folate analogue that inhibits the activity of dihydrofolate reductase (DHFR) (16), which catalyzes the NADPH-dependent reduction of dihydrofolate to tetrahydrofolate. Reduced folates are substrates in a number of one-carbon transfers in purine, pyrimidine, and amino acid biosynthesis (3). Inhibition of DHFR activity initially results in the depletion of N^5,N^{10}-methylene tetrahydrofolate, followed by inhibition of DNA synthesis and ultimately cell death (8). DHFR is thus a well-studied target of antibiotic and antineoplastic therapy.

Although MTX binds both human and *Escherichia coli* DHFR very tightly, with K_i values of 3.4 and 1.0 pM, respectively (2), all of the *E. coli* isolates we tested (genotypes of the strains used in this study are listed in Table 1), which included both common laboratory strains (MG1655, MC4100, AG1688, and ZK126) and clinical isolates (O157:H7, RM74A, STM1, LL, RM52B, DD, and RM33B), were resistant to MTX added to solid medium at concentrations of up to 1 mM, the highest concentration we tested (data not shown).

Antibiotic resistance can occur by a variety of mechanisms, including failure of the drug to bind its target, overexpression of the drug target, modification or degradation of the drug, creation of permeability barriers, or active export of the drug. It is increasingly recognized that active efflux plays a major role in the resistance of many organisms to a plethora of agents (11, 20). A wide variety of antibiotics are exported from *E. coli* by one of several active efflux systems (11, 12, 19, 20). At least two of these systems, the AcrAB and EmrAB efflux pumps, have been shown to depend on the outer membrane protein TolC (1, 7, 12, 19, 20).

To determine whether the MTX resistance was due to a TolC-dependent efflux pump, we examined the effect of a *tolC*::Tn*10* mutation. LBB1175, in which *tolC* had been inactivated by the Tn*10* insertion, was sensitive to 1 mM MTX, while W4573, the isogenic TolC$^+$ control, was resistant. Similar results were obtained using the common laboratory strain MG1655, which is the reference wild-type *E. coli* K-12 strain used for the genome sequence (4), and AG1688 (see below). Strains carrying Tn*10* at a different chromosomal location remained resistant to MTX. These results suggest that MTX resistance is mediated by a TolC-containing multidrug resistance efflux pump (MDR).

tolC mutants are pleiotropic (17, 26) and are hypersensitive to many hydrophobic agents (18). Thus, the loss of MTX resistance in the *tolC* mutant might not be due to the loss of function of an MDR. To address this possibility, we tested the effects of mutations that inactivate specific TolC-dependent MDRs. The AcrAB pump belongs to the RND (for resistance, nodulation, and division) family, and its substrates include sodium dodecyl sulfate, basic dyes, novobiocin, and tetracycline (19, 20); the EmrAB pump belongs to the MF (major facilitator) family, and its substrates include carbonyl cyanide *m*-chlorophenylhydrazone, nalidixic acid, and phenyl mercury acetate (19, 20). A strain containing the *acrA1* mutation (N43) was sensitive to 1 mM MTX, while its isogenic parent (W4573) was resistant. In contrast, both the *emrB* mutant (OLS103) and its isogenic parent (AMS6) were MTX resistant. These results show that the MTX sensitivity of the *tolC* strains is at least partly due to inactivation of the AcrAB MDR, while the EmrAB pump does not have a major role in MTX export.

MICs of MTX were determined for a set of isogenic *E. coli* strains containing combinations of *acrA*, *emrB*, and *tolC* mutations (Table 2). The wild-type strain (W4573) was resistant to 1,024 μM MTX, the highest concentration tested. The *emrB* mutation did not affect the MIC, either alone (compare W4573 to SK636) or in combination with *acrA1* (compare N43 to SK627) or *tolC*::Tn*10* (compare SK642 to SK660). Inactivation of either *acrA* or *tolC* resulted in a decrease of the MTX MIC to 256 or 64 μM, respectively. Since the *acrA1* allele is an IS*2* insertion in the second codon of *acrA* (14), it is unlikely that the remaining MTX resistance in the *acrA1* mutant is due to residual activity of the *acrA* gene product. The *tolC* gene product seems to have more than one role in MTX resistance. It is unclear if this is due to the loss of function of another, unidentified TolC-containing MDR or the highly pleiotropic effects of *tolC* mutations on outer membrane structure (17, 26). Similar alterations have not been found in the outer membrane of *acr* mutants (19, 21, 24).

The additional role of TolC is not related to the EmrAB MDR, since the *acrA1 emrB* double mutation (in SK627) yielded an MIC identical to that yielded by the *acrA1* single

* Corresponding author. Mailing address: Department of Biochemistry and Biophysics, Texas A&M University, College Station, TX 77843-2128. Phone: (979) 862-4054. Fax: (979) 845-4946. E-mail: jimhu@tamu.edu.

† Present address: Columbia University, Department of Chemistry, New York, NY 10027.

TABLE 1. *E. coli* strains used

Strain	Genotype	Reference and/or source
MG1655	K-12 F$^-$ λ$^-$	22; D. Siegele
MC4100	F$^-$ *araD139* Δ(*argF-lac*)*U169 rpsL150 relA1 deoC1 rbsR fthD5301 fruA25* λ$^-$	5; D. Siegele
AG1688	F′128 *lacI*^{q} *lacZ*::Tn*5*/*araD*139 Δ(*ara-leu*)7697 Δ(*lac*)*X74 galE15 galK16 rpsL*(Str^r) *hsdR2 mcrA mcrB1*	MC1061 F′128 *lacI*^{q} *lacZ*::Tn*5* (9)
ZK126	W3110 Δ(*lac*)*U169 tna-2*	6; D. Siegele
O157:H7	*E. coli* isolate from human	10; D. Siegele
RM74A	Group I *E. coli* from human female	15; D. Siegele
STM1	Group I *E. coli* from human male	27; D. Siegele
LL	Group II *E. coli* from human infant	23; D. Siegele
RM52B	Group II *E. coli* from human female	15; D. Siegele
DD	Group III *E. coli* from human infant	27; D. Siegele
RM33B	Group III *E. coli* from human female	15; D. Siegele
W4573	K-12 F$^-$ *lac ara mal xyl mtl gal rpsL*	14; J. A. Fralick
LBB1175	W4573 *tolC*::Tn*10*	J. A. Fralick
N43	W4573 *acrA1*	14; J. A. Fralick
OLS103	AMS6 *emrB*::Km	13; J. A. Fralick
AMS6	K-12 F$^-$ Δ*lacU169*	13; J. A. Fralick
SK627	W4573 *acrA1 emrB*::Km	N43 × P1 *vir* (OLS103)
SK636	W4573 *emrB*::Km	W4573 × P1 *vir* (OLS103)
KH803	MC4100 *tolC*::Tn*10*	R. Young
SK642	W4573 *acrA1 tolC*::Tn*10*	N43 × P1 *vir* (KH803)
SK660	W4573 *acrA1 emrB*::Km *tolC*::Tn*10*	SK627 × P1 *vir* (KH803)
SK037	AG1688 *tolC*::Tn*10*	AG1688 × P1 *vir* (KH803)
SK029	AG1688(pSK029)	pSK029, a pBR322-derived plasmid that expresses a λ cI-*E. coli* DHFR fusion protein from P_{lac}UV5, was introduced by M13-mediated transduction (25) into AG1688
XZ020	AG1688(pXZ020)	pXZ020, a pBR322-derived plasmid that expresses a λ cI-GCN4 leucine zipper fusion protein from P_{lac}UV5, was introduced by M13-mediated transduction into AG1688

mutation. Similar combinations of mutations in a different background (AG1688) yielded identical MICs (data not shown). This further demonstrates that the observed effects are not strain specific.

To determine whether inhibition of DHFR was sufficient to explain the MTX sensitivity of *tolC* strains, we examined whether the MTX sensitivity of AG1688 *tolC*::Tn*10* (SK037) could be suppressed by overexpression of DHFR activity. In the course of other (unpublished) studies, we had constructed a plasmid, pSK029, which expresses a fusion protein, cI-DHFR, in which the N-terminal DNA binding domain of the bacteriophage λ repressor is fused to *E. coli* DHFR; the fusion protein is expressed under the control of the *lac*UV5 promoter. Neither pSK029 nor pXZ020, a control plasmid expressing cI-GCN4 (a fusion to the leucine zipper of GCN4), affected the MTX resistance of wild-type AG1688 whether or not the fusion proteins were overexpressed (Table 3, lines 3 and 5). AG1688 *tolC*::Tn*10* strains containing either plasmid were sensitive to MTX under conditions in which the fusion proteins were uninduced (Table 3, lines 4 and 6). However, in the presence of isopropyl-β-D-thiogalactopyranoside (IPTG), which induces the overexpression of cI-DHFR, SK029 *tolC*::Tn*10* was resistant to high concentrations of MTX (Table 3, line 4). In contrast, IPTG-induced overexpression of the control protein cI-GCN4 had no protective effect on the *tolC* strain (Table 3, line 6).

These results show that MTX sensitivity in the *tolC* strain is due to the inhibition of endogenous DHFR by the drug. When cI-DHFR is overexpressed, the DHFR activity provided by the DHFR domain in the fusion protein cannot be titrated out, which strongly suggests that the plasmid-coded DHFR acts to sequester MTX that is added to the medium. Increasing the level of DHFR should not relieve sensitivity due to mechanisms that do not involve uptake of MTX.

The results of this study can be summarized as follows. (i) All of the TolC$^+$ AcrA$^+$ strains of *E. coli* we tested were resistant to at least 1 mM MTX when grown on solid medium containing the drug. (ii) MTX resistance is decreased by mutations that disrupt *tolC* or *acrA*, genes that code for integral components of the AcrAB MDR, suggesting that resistance is due to active export of MTX via the AcrAB MDR. (iii) Mutation of the *emrB* gene does not decrease MTX resistance, suggesting that MTX is not a substrate of this MDR. (iv) The difference between the MICs for *tolC*::Tn*10* and *acrA*::IS*2*

TABLE 2. Sensitivities of *acrA*, *tolC*, and *emrB* mutants to MTX

Strain	Relevant genotype[a]	MIC[b] of MTX (μM)
W4573	Wild type	>1,024
SK636	*emrB*	>1,024
N43	*acrA1*	256
SK627	*acrA1 emrB*	256
LBB1175	*tolC*	64
SK642	*acrA1 tolC*	64
SK660	*acrA1 tolC emrB*	64

[a] For a full listing of genotypes, see Table 1.

[b] MICs were determined by examining the growth of 2-ml liquid cultures containing twofold serial dilutions of MTX (2 to 1,024 μM) in Luria-Bertani liquid broth. The inoculum (20 μl) contained approximately 10^5 cells per ml. The MIC was determined as the lowest concentration that prevented visible growth after 8 h on a roller drum at 37°C. All the tested strains grew to saturation in the absence of MTX. The values are the averages of three separate experiments.

TABLE 3. Suppression of the MTX sensitivities of TolC mutants by cI-DHFR

Line	Strain[b]	Fusion protein	*tolC* genotype	Growth[a]			
				−IPTG		+IPTG	
				−MTX	+MTX	−MTX	+MTX
1	AG1688	None	*tolC*$^+$	+	+	+	+
2	SK037	None	*tolC* mutant	+	−	+	−
3	SK029	cI-DHFR	*tolC*$^+$	+	+	+	+
4	SK029 *tolC*::Tn*10*	cI-DHFR	*tolC* mutant	+	−	+	+
5	XZ020	cI-GCN4	*tolC*$^+$	+	+	+	+
6	XZ020 *tolC*::Tn*10*	cI-GCN4	*tolC* mutant	+	−	+	−

[a] Cultures were grown to saturation in Luria-Bertani broth at 37°C overnight and diluted in M9 salts to approximately 5,000 CFU/ml. A total of 10 μl of each diluted culture was pipetted onto Luria-Bertani agar plates containing no IPTG (−IPTG) or 1 mM IPTG (+IPTG) and either no MTX (−MTX) or 1 mM MTX (+MTX) as indicated. The spots were allowed to dry and then the plates were incubated at 37°C overnight. +, growth; −, no growth. The observations are from at least three separate experiments.

[b] *tolC*::Tn*10* was introduced into the indicated strains by P1 *vir* transduction using KH803 as the donor.

strains suggests the possibility of another mechanism for low-level TolC-dependent MTX resistance.

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