JOURNAL OF BACTERIOLOGY, Jan. 1996, p. 306–308 Vol. 178, No. 1
0021-9193/96/$04.00+0

AcrAB Efflux Pump Plays a Major Role in the Antibiotic Resistance Phenotype of *Escherichia coli* Multiple-Antibiotic-Resistance (Mar) Mutants

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Multiple-antibiotic-resistance (Mar) mutants of *Escherichia coli* are resistant to a wide variety of antibiotics, and increased active efflux is known to be responsible for the resistance to some drugs. The identity of the efflux system, however, has remained unknown. By constructing an isogenic set of *E. coli* K-12 strains, we showed that the *marR1* mutation was incapable of increasing the resistance level in the absence of the AcrAB efflux system. This experiment identified the AcrAB system as the major pump responsible for making the Mar mutants resistant to many agents, including tetracycline, chloramphenicol, ampicillin, nalidixic acid, and rifampin.

Multiple-antibiotic-resistance (Mar) mutants of *Escherichia coli* express elevated levels of resistance to a wide range of structurally unrelated antibiotics (5, 6, 8), and the resistance to some agents, such as tetracycline and fluoroquinolones, has been shown to result from increased levels of active efflux (2, 5). The alleles that affect the Mar phenotype are located in the *marRAB* operon (1, 23). The *marA* gene encodes a positive regulator of antibiotic resistance whose sequence shows strong similarity to those of such transcriptional regulators as SoxS, XylS, and AraC (1, 7, 8), while *marR* encodes a repressor of the *marRAB* operon (1). The function of *marB* is not known.

Although MarA appears to be a global regulator that affects distant chromosomal genes (7, 8), only a handful of genes responsible for Mar-induced multiple-antibiotic resistance have been identified. Elevated levels of MarA have been found to increase *micF* transcription, thereby causing a decrease in OmpF expression (3), but this effect alone is not sufficient to explain the antibiotic resistance of Mar mutants (1, 2). Random Tn*phoA* and Tn*lacZ* fusions were used recently to identify other genes regulated by the *marRAB* operon (22). However, the precise functions of these genes have not yet been defined.

We previously cloned a chromosomal fragment of *E. coli* containing the *acrAB* operon and the *acrR* gene and demonstrated that AcrAB is a drug efflux pump that is homologous to other efflux systems (14) and that its expression is regulated to some extent by the repressor AcrR (13). AcrB is an efflux transporter belonging to the resistance-nodulation-cell division family (21), and AcrA belongs to the membrane fusion protein family (4) that is thought to connect the transporter protein physically to an outer membrane channel so that the drugs can be exported directly into the external medium, bypassing the outer membrane barrier (16, 19).

While investigating the regulation of the expression level of *acrAB*, we observed that the transcription of *acrAB* was also elevated in several *marR* mutants displaying a Mar phenotype (15). These results, however, did not indicate how important a role the AcrAB pump plays in the drug resistance phenotype of Mar mutants.

In order to determine the role of AcrAB, we constructed a set of isogenic *E. coli* strains. This was done by transducing various mutations with P1*clr100* (17) into a pair of isogenic strains, AG100 (K-12 *argE3 thi-1 rpsL xyl mtl* Δ(*gal-uvrB*) *supE44*) and AG102 (AG100 *marR1*) (5), kindly furnished by S. B. Levy. The *marR1* mutation (earlier called *marA1*) changes an arginine residue in MarR into leucine and causes increased expression of the MarA regulator protein (1). The transduced mutations were Δ*acrAB*::Tn*903* Kan^r (from strain KZM120 [15]) and *acrR*::Tn*903* Kan^r (from strain WZM124 [13]; this mutation contains a 1.3-kb Tn*903* Kan^r cartridge at the sole *Bgl*II site of the *acrR* gene). As a control, we also transduced a mutant copy (*emrB*::Tn*kan* from a mutant strain kindly given to us by O. Lomovskaya) of *emrB*, which codes for a transporter component of another efflux system (12). Kanamycin resistance, which is not affected by either the AcrAB or the EmrAB efflux system, was used as the selective marker in transduction. Strains derived from AG100 are here called AG100A to -C, and those derived from AG102 (and therefore containing the *marR1* mutation) are called AG102A to -C.

The antibiotic resistances of these strains were compared by measuring the MICs of various antibiotics for these strains by inoculating 10^3 cells of an overnight culture into 0.5 ml of serial twofold dilutions in LB broth (17). Growth was scored after a 20-h incubation at 37°C. The experiments were repeated three times and gave consistent MICs.

The MIC data for six antibiotics are shown in Table 1. As demonstrated previously (2, 5), the *marR1* mutation increased the MIC two- to fourfold (Table 1; compare AG102 and AG100). The increase in the MIC for *acrR* strain AG100B was smaller than that for AG102, consistent with the limited repressor function of AcrR (13). The Δ*acrAB* mutant, AG100A, was hypersusceptible to all compounds, suggesting that the AcrAB drug efflux pump plays an important role in determining the intrinsic level of resistance in *E. coli*. Most importantly, when the two mutations *marR1* and Δ*acrAB* coexisted in a single strain (AG102A), the MICs of various antibiotics for that strain were exactly the same as those for the *marR*$^+$ Δ*acrAB* strain, AG100A, except in the case of tetracycline. This outcome suggested that the AcrAB pump plays a predominant role in making *marR* mutants resistant to all these com-

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TABLE 1. Comparison of the MICs of various antibiotics

Strain	Genotype	MIC (mg/liter)					
		Tetracycline	Ampicillin	Puromycin	Nalidixic acid	Rifampin	Chloramphenicol
AG100	Wild type	1.25	2.5	100	5	12.5	5
AG102	*marR* mutant	5	5	400	10	25	10
AG100A	Δ*acrAB*	0.3	0.6	3	0.6	5	0.6
AG102A	*marR* mutant Δ*acrAB*	0.6	0.6	3	0.6	5	0.6
AG100B	*acrR* mutant	2.5	2.5	200	10	12.5	10
AG102B	*marR* mutant *acrR* mutant	10	10	800	20	12.5	>160
AG100C	*emrB* mutant	2.5	2.5	200	5	12.5	5
AG102C	*marR* mutant *emrB* mutant	5	5	400	10	25	10

pounds, because the *marR1* mutation had no effect on the MIC in the absence of this pump. The small effect of *marR1* on the MIC of tetracycline for AG102A is probably due to the MarA-induced increases in the expression of some other pump(s) that may prefer tetracycline as the substrate. The *E. coli* genome is known to contain several pumps that are homologs of either AcrB or EmrB (9, 16).

Because the nearly total absence of any effect of the *marR1* mutation on the MICs for the Δ*acrAB* strain was unexpected, we made certain that the MarA level was elevated in the *marR1* Δ*acrAB* AG102A strain. (i) Production of MarA is known to increase the level of glucose 6-phosphate dehydrogenase (7). Indeed, in two independent experiments, the specific activities of this enzyme in AG102A were 2.7- and 3.6-fold higher than that in AG100 and were identical (with a <5% difference) to that found in AG102. The presence of Δ*acrAB* alone (in AG100A) had no detectable effect on the activity of this enzyme. (ii) MarA is also known to elevate the transcription of *micF* antisense RNA (3). The expression of *micF* was measured by the introduction of *micF-lacZ* fusion reporter plasmid pmicB21 (plasmid IV in reference 18; a gift of N. Delihas and M. Inouye). Again, in two experiments, the expression levels in AG102A were 2.1- and 1.9-fold higher than that in the *marR*$^+$ Δ*acrAB* AG100A strain. Although the extent of the increase in expression for AG102A was slightly less than that for the *acrAB*$^+$ strains, in which 2.5- and 2.7-fold inductions were caused by the presence of a *marR* mutation in AG102 (compared with AG100), these results clearly indicate that MarA expression is significantly elevated in AG102A.

Since efflux pumps such as AcrAB are thought to produce a multiprotein complex traversing the cytoplasmic as well as the outer membrane (16, 19), it may be argued that the absence of one or two of the component proteins (which was expected, for example, for Δ*acrAB* strains) might result in the assembly of a defective complex, which could make the cells hypersusceptible by allowing the leakage of drugs into the cells. If so, the low MICs for AG102A might not be the consequence of the loss of the efflux process per se. However, this possibility is contradicted by the following data. (i) The entry of [^{3}H]tetracycline during the first 30 s into cells poisoned with 50 μM carbonyl cyanide *m*-chlorophenylhydrozone (to eliminate efflux) occurred at exactly the same rates for AG100 and AG100A (data not shown). (ii) A null mutation of *emrB*, whose product is a transporter component of a three-protein efflux complex of a structure similar to that containing AcrA and AcrB (9, 12), was introduced into AG100. The MICs for the resultant strain, AG100C, were very similar to those for AG100 (Table 1). Furthermore, in both the presence and absence of the EmrB transporter, *marR1* increased MICs to exactly the same extent (Table 1; compare AG102 and AG102C), most probably by increasing the expression level of the AcrAB pump. Thus, at least in this case, the presence of a defective pump protein did not cause a hypersusceptible phenotype or the influx of antibiotics through leakage.

Additional evidence for the major role of the AcrAB pump was obtained by combining *marR1* and *acrR*::Tn*903*. Null mutations in *acrR* raise the baseline expression levels of *acrAB* about twofold, but even under these conditions, the *acrAB* operon still responds strongly to other global stress regulators, including MarA and its homologs (13, 15, 16). Thus, in the absence of AcrR, MarA is expected to increase the level of the transcription of *acrAB* further. Indeed, strain AG102B was more resistant than AG102 to almost all agents (with the exception of rifampin).

In summary, deletion of *acrAB* made *marR1* totally ineffective in increasing the MICs of various antibiotics, and a null mutation in the negative regulator of the *acrAB* operon, *acrR*, made *marR1* more effective in increasing MICs. These results indicate that AcrAB plays the major role in the antibiotic resistance phenotype of Mar mutants and most probably in the resistance created by the physiological response to the presence of antibiotics, which is reported to be mediated by the Mar regulatory system (8).

Other aspects of this study merit comment. First, the major function of the AcrAB system of *E. coli* in antibiotic efflux is consistent with the observation that another AcrAB homolog, AcrEF, is not normally expressed in K-12 (16) and that the closest homolog of AcrAB in *Pseudomonas aeruginosa*, MexAB, also plays a major role in making that organism intrinsically resistant to most of the commonly used antibiotics, except aminoglycosides (10, 20). Second, EmrAB, which contains a transporter of another (major facilitator) class (9, 12), has little effect on resistance to clinically important classes of antibiotics, although it is involved in the efflux of proton conductors and an antibiotic of natural origin, thiolactomycin (9). Finally, the MIC of ampicillin decreased strikingly in Δ*acrAB* strains and increased significantly in the *acrAB* overexpression strain (AG102B). This result strongly suggests that the increased resistance of Mar mutants of *E. coli* to β-lactam is largely caused by an increased efflux whose effect is synergistically enhanced by the decreased OmpF porin level. Homologs of AcrAB in *P. aeruginosa* have indeed been shown to pump out β-lactam compounds efficiently (11).

D. Ma thanks J. E. Hearst for support and encouragement.

This study was supported in part by grant AI-09644 from the National Institutes of Health.

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