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Toxic Waste Disposal in *Escherichia coli*

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About 10% of the nalidixic acid-resistant (Nal^r) mutants in a transposition-induced library exhibited a growth factor requirement as the result of *cysH*, *icdA*, *metE*, or *purB* mutation. Resistance in all of these mutants required a functional AcrAB-TolC efflux pump, but the EmrAB-TolC pump played no obvious role. Transcription of *acrAB* was increased in each type of Nal^r mutant. In the *icdA* and *purB* mutants, each of the known signaling pathways appeared to be used in activating the AcrAB-TolC pump. The metabolites that accumulate upstream of the blocks caused by the mutations are hypothesized to increase the levels of the AcrAB-TolC pump, thereby removing nalidixic acid from the organism.

Quinolone antibiotics constitute one of the most widely used classes of antibacterial agent. Although the original quinolone, nalidixic acid (Nal) (Fig. 1), was effective primarily against gram-negative bacteria, fluoroquinolone derivatives are potent against a wide variety of gram-negative and gram-positive bacteria. The primary targets of the quinolones are DNA topoisomerase II (DNA gyrase) and topoisomerase IV (10, 19, 37).

Because of the therapeutic importance of these antibiotics, the appearance of bacterial mutants resistant to quinolones is of considerable concern. Many such mutants in a variety of bacteria have been characterized. These fall into two broad classes: those altered in the target topoisomerases and those failing to accumulate the drug to levels seen in the wild type. No mutant resistance stemming from the breakdown or modification of quinolones has been described.

Resistant mutants of *Escherichia coli* and of a wide variety of other gram-negative and gram-positive bacteria selected in the laboratory or isolated from clinical settings have been shown to be altered in one or more of the genes encoding components of the two topoisomerases (*gyrA*, *gyrB*, *parC*, and *parE* in *E. coli*) (34). These mutations produce resistance of various levels, and further mutations often increase the resistance (18).

Quinolone-resistant mutants containing mutations in genes other than those for the topoisomerases are also obtained frequently when only a low level of drug is used in selection or when a secondary mutation increases resistance beyond that of the primary mutation. Where the basis for such mutation has been determined, it has usually resulted from the activation of an efflux pump (31, 32) that removes the quinolone during or after entry so it does not reach a toxic level. In *E. coli*, activation of either the AcrAB-TolC pump (4, 9, 28) or the EmrAB-TolC pump (26) provides resistance. AcrAB-TolC appears to be the main pump providing intrinsic resistance to low levels of many toxic compounds in nature (38). Three signaling pathways controlling the expression of the *acrAB* genes and the formation of the AcrAB-TolC pump are known; these involve the MarAB, SoxRS, and RobA proteins (5, 11, 15, 33, 39). Thus, there are several potential ways of generating low-level resistance to quinolones through the activation of efflux pumps.

Screening mutants of *E. coli* and other bacteria selected on broth plates containing a low level of nalidixic acid revealed that resistance results from mutation at any one of many loci (16, 22). Unexpectedly, resistance mutations sometimes cause blocks in central biosynthetic pathways and corresponding growth factor requirements. The mutations include those in *purB* (adenylosuccinate lyase, adenine requirement) (16) and *icdA* (isocitrate dehydrogenase, glutamate requirement) (17) and others causing requirements for methionine or cysteine (17). In addition, Nal^r mutants stemming from double mutations removing the dual aconitases (*acnA acnB*) (14) have been found (16, 22).

Why should a block in a biosynthetic pathway confer antibiotic resistance on the cell? Results from analysis of the *icdA* mutants provide a clue. These mutants accumulate a large quantity of citrate and isocitrate, the intermediate compounds before the block (Fig. 1). Double mutants lacking citrate synthase as well (*gltA icdA*) neither form these intermediates nor show the resistance to nalidixic acid of the *icdA* mutant (23, 24). Thus, one can hypothesize that the accumulation of the charged intermediate compounds triggers the resistance (23). *purB* mutants also accumulate intermediate compounds formed before the block (13); these compounds (SAICAR and adenylosuccinate; Fig. 1) resemble citrate and isocitrate in part. Thus, the resistance in *purB* mutants may also result from metabolite accumulation.

The association of metabolite accumulation and nalidixic acid resistance in *icdA* and *purB* mutants suggests a further hypothesis relating the two phenomena: the metabolites activate the formation of an efflux pump that removes nalidixic acid from the cell and thus prevents toxicity. In order to test this hypothesis, we isolated Nal^r mutants of *E. coli* induced by transposon insertion, characterized the mutants, and tested the roles of the AcrAB-TolC and EmrAB-TolC pumps in the resistance.

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MATERIALS AND METHODS

Strains, media, and culture conditions. The bacterial strains used in this work are listed in Table 1. Nal^r mutants were selected on HOPS medium, which contains 5 g of NaCl/liter, 16 g of agar/liter, 25 g of tryptone/liter, and 2.5 g of

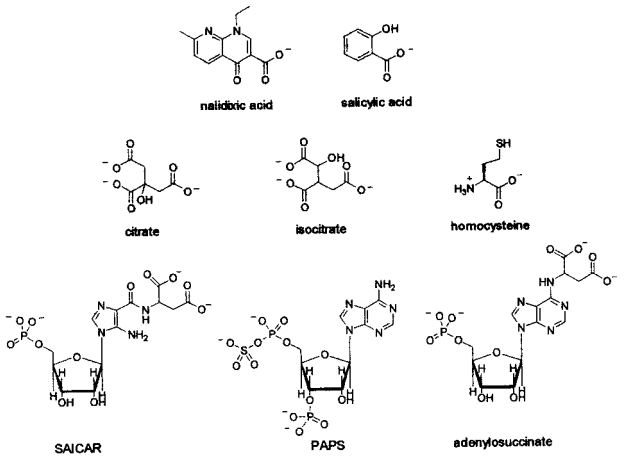

FIG. 1. Structures of nalidixic acid and possible *acrAB* inducers.

yeast extract/liter. After the basic medium was autoclaved, 2.5 ml of 1 M $CaCl_2$, 1 ml of 20% glucose, and 5 ml of supplements (2 mg of adenine, 5 mg of glutamate, 5 mg of tryptophan, and antibiotics as appropriate) were added. Tryptone agar supplemented with adenine (*purB*) or antibiotics as necessary was used for subsequent work. When used, chloramphenicol was added to medium at 5 mg/liter (*acrAB* or *tolC* strains) or at 40 mg/liter (other strains). The same concentrations of kanamycin were used when selecting for kanamycin resistance. Correspondingly, tetracycline was used at 5 or 12 mg/liter.

Construction of a library of Nal^r mutants. Mutants of *E. coli* K-12 strain RBH1211 were induced by transposition from λNK1324 (λ Cam^r; obtained from U. Jacob) essentially as described previously (20). Selection was on HOPS plates containing 4 mg of Nal/liter and 40 mg of chloramphenicol/liter. After incubation overnight at 37°C, colonies were streaked onto similar medium but with 2 mg of Nal/liter, and plates were incubated overnight at 30°C. Single-colony isolates were made for further testing.

Characterization of mutants. Mutants were streaked onto minimal glucose medium to identify growth factor requirements. Auxotrophic mutants were used as donors in transduction to RBH1854 with selection for chloramphenicol resistance. Transductants were tested for nalidixic acid resistance and growth factor requirements. Mutants in which those characters cotransduced with chloramphenicol resistance were assumed to be induced by the transposition. Transductants picked for further analysis were shown to be nonlysogenic for the viruses P1 and lambda.

The locations of the transposon insertions were determined by PCR with an rTth DNA polymerase XL kit (Applied Biosystems) according to the manufacturer's instructions. Twenty-five-nucleotide primers corresponded to the beginning and end of each open reading frame (ORF), and additional upstream primers corresponding to sequences approximately 100 nucleotides upstream of the ORF were used for insertions that did not map within the ORFs.

In order to determine the roles of the well-known AcrAB-TolC and EmrAB-TolC efflux pumps in the development of the Nal resistance, the pumps were knocked out in selected Nal^r mutants. Transposon insertions were transduced from the RBH1854 derivatives into RBH1855 (*acrAB*, but otherwise isogenic with RBH1854) by using selection for chloramphenicol. The mutant genes *tolC*::Tn*10* and *emrB*::*kan* were added to mutant derivatives of RBH1854 by transduction by using selection for tetracycline or kanamycin. In the same way, mutations of the signaling pathways (*marA*::miniTn*10kan*, *rob*::*kan*, and *soxS3*::Tn*10*) were transduced into derivatives of RBH1854 with selection for kanamycin or tetracycline.

TABLE 1. Strains used

Strain	Relevant characteristic	Source
RBH996	*marA*::miniTn*10kan*	B159 from P. Miller
RBH1211	Wild type, standard W3110 strain	J. R. Guest collection
RBH1852	*soxS3*::Tn*10*	BW831 from B. Weiss (40)
RBH1854	As RBH1211, but from another source	E. Olson collection (25)
RBH1855	As RBH1854, but Δ*acrAB*	B414 from E. Olson (25)
RBH1864	*rob*::*kan*	RA4468 (5) from M. Bennik and B. Demple
RBH1868	*emrB*::*kan*	OLS103 from J. Tomashek and K. Lewis
RBH1869	*tolC*::Tn*10*	J. Fralick (9)
RBH2086	*lacZ*::Tn*5-131* (Kn^s Tc^s)	CE78 (6) from R. Bender
pNN602S	*acrAB*::*lacZ* fusion plasmid	H. Nikaido (27)

Nalidixic acid resistance. Strains were streaked onto tryptone agar with 0, 1, or 2.5 mg of nalidixic acid/liter and grown overnight at 37°C. The selected mutants grew on all three media, the wild type grew on media with 0 and 1 mg of Nal/liter, and *acrAB* and *tolC* strains grew only on medium lacking the antibiotic. For quantitative assays of resistance, diluted samples of strains grown overnight in tryptone broth at 37°C were spread on tryptone agar plates containing various amounts of Nal and plated at 37°C. Plates were counted after 24 to 40 h.

***acrAB* expression.** Relative transcription rates from the *acrAB* promoter were determined from a p*acrAB-lacZ* fusion in the low-copy-number plasmid pNN602S (27). This construct includes the *acrAB* regulator gene *acrR*. In order to prevent the appearance of LacZ (β-galactosidase) activity stemming from chromosomal *lacZ* transcription, the chromosomal *lacZ*$^+$ gene was replaced with *lacZ*::Tn*5*-131 (Kn^s Tc^r) (6) by using P1-mediated transduction with selection for tetracycline resistance.

In the expression experiments, 0.15 ml of an overnight culture in L broth (30°C) was subcultured in 20 ml of L broth at 30°C, and growth was continued with shaking at 30°C for approximately 2 h until harvest and LacZ assay essentially as described previously (27). Supplements (ethanol to 4%, lactose to 0.2%, nalidixic acid to 2 or 5 mg/liter) were added as appropriate 40 min after subculturing.

RESULTS

Isolation and characterization of Nal^r mutants induced by transposon insertion. From two experiments, a total of 260 Nal^r mutants were recovered after plating organisms infected with λCam^r on medium containing nalidixic acid (to select the mutants) plus chloramphenicol (to select organisms containing the Cam^r transposon) as described in Materials and Methods. Separate selection on plates containing chloramphenicol but not nalidixic acid showed that this corresponded to approximately 46,000 total organisms with transposon insertion, or a Nal^r mutant per insertion frequency of 0.58%, assuming a single transposition per organism.

Single-colony isolates of each mutant were tested for growth on minimal agar medium containing glucose as the sole carbon and energy source. Of the total, 10 required glutamate for growth (*icdA* mutants), 13 required adenine (*purB*), 1 required cysteine, and 1 had an unidentified growth factor requirement satisfied by Casamino Acids. None required methionine, although methionine-requiring mutants were isolated in other screens. Thus, a total of 25 mutants exhibited a growth factor requirement (10%), consistent with a previous report of 2 to 40% auxotrophs among spontaneous mutants (16). None of the mutants showed high-level resistance to nalidixic acid (20 mg/liter).

In order to determine whether the nalidixic acid resistance and growth alterations were caused by a separate mutation rather than by the transposon insertion, we transduced Cam^r from some original isolates to a wild-type recipient by using selection on tryptone agar containing chloramphenicol. After single-colony isolation, we scored the transductants for Nal^r and for growth properties.

In summary, 10 of 10 *icdA* mutations cotransduced with the Cam^r insertion and with Nal^r, and 4 of 6 *purB* mutations co-

transduced with Cam^r and with Nal^r. (In the other two cases, neither *purB* nor Nal^r cotransduced with Cam^r.) Nal^r cotransduced with Cam^r in 10 of 26 original isolates that showed no growth factor requirement. Thus, most of the Nal^r auxotrophs were caused by transposon insertion, but many of the original Nal^r isolates showing normal growth were caused by mutation other than the insertion of the transposon. The locations of the transposon insertions were verified by PCR for two *icdA* and two *purB* mutants. For each type of mutant (*icdA* and *purB*), one insertion was within the ORF and one was immediately upstream.

The cysteine-requiring mutant responded to cysteine, sulfide, or sulfite, and so it was blocked in the reduction of sulfate to sulfite (21, 30). PCR showed that the transposon insertion was within the *cysH* gene encoding 3′-phosphoadenosine 5′-phosphosulfate (PAPS) sulfotransferase. A methionine-requiring mutant isolated earlier when testing the procedure responded to vitamin B_{12}, as well, but not to homocysteine-thiolactone. This pinpointed the mutational defect to the last step in methionine synthesis, the formation of methionine by homocysteine methyltransferase, which is the product of the gene *metE*. PCR verified that the transposon insertion was within *metE*.

Characterizing the role of the AcrAB-TolC and EmrAB-TolC efflux pumps in resistance. In order to see if the resistance of our mutants to Nal was associated with its efflux through one of these pumps, we modified the Nal^r mutants so that they also contained *acrAB*, *tolC*, or *emrB* mutations (see Materials and Methods). Streaking onto tryptone agar containing nalidixic acid showed that *emrB*::*kan* had no obvious effect on the Nal resistance of *icdA* (five tested), *purB* (two tested), *metE*, or *cysH* mutants. However, each of the same mutants became Nal sensitive when the organism was deficient in the AcrAB-TolC pump because of defective AcrAB (Δ*acrAB*) or TolC (*tolC*::Tn*10*) (as shown for *icdA* derivatives by quantitative assay in Fig. 2). We conclude that in each of the mutants, the AcrAB-TolC pump must be functional in order to express Nal resistance but that EmrAB-TolC plays no obvious role.

Three signaling pathways for activating the AcrAB-TolC pump have been characterized (see the introduction). In order to determine which of the three, if any, were responsible for triggering drug resistance in our mutants, we inserted mutations inactivating each signal pathway (*soxS3*::Tn*10*, *rob*::*kan*, or *marA*::*kan*) separately into *icdA* and *purB* mutants. To our surprise, streak tests showed no diminution in resistance when any one signaling pathway was knocked out in these mutants. Quantitative assays supported the results from the streak tests (Fig. 2). These results suggested that either an unknown activation pathway was used or more than one of the known pathways triggered resistance in the mutants.

In order to determine which possibility was correct, we deleted pairs of the signaling pathways from the same *icdA* and *purB* mutants to see if resistance was lost. Streaking onto Nal-containing medium showed that for each Nal^r mutant, the loss of either MarA and SoxS or Rob and SoxS prevented the development of resistance. Quantitative assays with an *icdA* derivative show the results clearly (Fig. 2). Thus, it appears that each signaling pathway (MarAB, Rob, and SoxRS) has a role in the development of resistance to nalidixic acid in the mutants tested. When a single path is inactivated, a small diminution in resistance may be seen via the quantitative assay, but it is too small to detect by streaking.

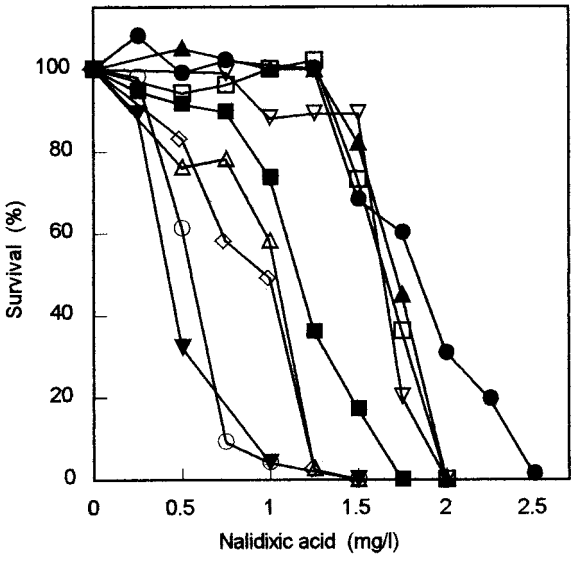

FIG. 2. Role of the AcrAB-TolC efflux pump and its activating pathways in the development of resistance of *icdA* mutants to nalidixic acid. ●, *icdA* RH1854; ■, wild-type (RH1854); ▼, Δ*acrAB* (RBH1855); ○, *icdA* Δ*acrAB* (*icdA* RBH1855). Other strains are derivatives of *icdA* RBH1854. Additional mutations are *marA* (▲), *rob* (▽), *soxS* (□), *mar sox* (◇), and *rob sox* (△).

***acrAB* expression.** In order to see whether the activation of the *acrAB-tolC* pump implied by the above results stemmed from increased transcription, we measured relative transcription rates by using a p*acrAB-lacZ* fusion. The results (Fig. 3) show clearly that transcription from the *acrAB* promoter in each Nal^r mutant was increased to over twice the level in the wild-type organism. Ethanol is known to induce the *acrAB* operon (27) and so served as a positive control. As expected, lactose did not affect transcription of the *lacZ* gene fused to the *acrAB* promoter. Expression was decreased by nalidixic acid. The lack of a functional chromosomal *acrAB* locus resulted in greater transcription from the *acrAB* promoter of the plasmid. The increased *acrAB* transcription in the *icdA* mutant was reduced by *marA* or *robA* mutation but was still greater than that in the wild type, consistent with the results shown in Fig. 2.

DISCUSSION

These results show that in each of the mutants tested (*cysH*, *icdA*, *metE*, and *purB*), the expression of Nal resistance required the AcrAB-TolC efflux pump but not the EmrAB-TolC pump. In each type of mutant, expression from the *acrAB* promoter was increased. Each of the known signaling pathways for activating the expression of the *acrAB* genes played a role in developing the resistance in the two types of mutant examined in more detail (*icdA* and *purB*). When only a single signaling pathway was available, the resistance level was intermediate between that expressed by the wild type and that of the *acrAB* mutant. When any pair of pathways was available, the resistance level was slightly below that of an *icdA* mutant with

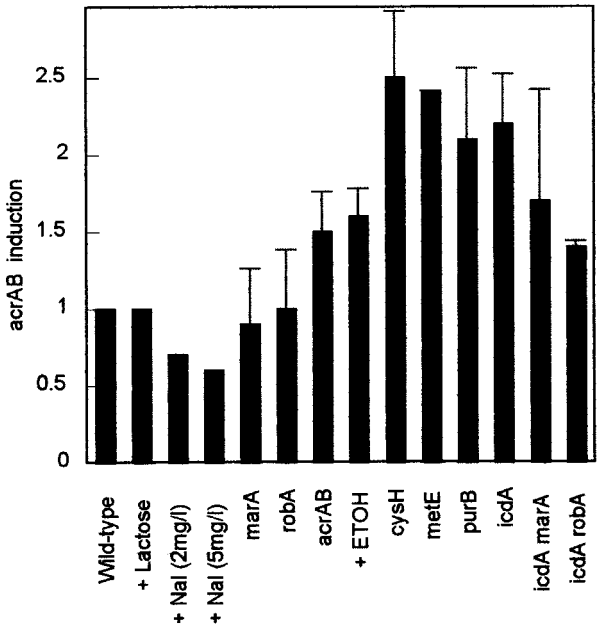

FIG. 3. Increased *acrAB* transcription in Nal^r mutants. β-Galactosidase activity from the *acrAB-lacZ* fusion in different mutants or after different treatments is expressed relative to that in the wild-type organism. Standard error bars are shown, except for the lactose or nalidixic acid additions, which show the results from single experiments.

all three pathways intact (Fig. 2), and *acrAB* transcription was correspondingly affected in the signaling mutants examined (*marA* and *robA*).

Comparison of the structures of the intermediate compounds formed before the metabolic block in *metE* (8, 36), *icdA* (23), and *purB* (13) mutants (Fig. 1) shows that each has at least one carboxylate. The well-studied AcrAB-TolC inducer, salicylate, also has a carboxylate (Fig. 1). Salicylate is known to induce the AcrAB-TolC efflux pump by at least two mechanisms, one of which involves Mar, the other(s) being unknown but not SoxRS (7). The repressor of *marRAB*, MarR, binds at the *marRAB* promoter; binding is antagonized in vitro by salicylate and some other inducers of the Mar pathway but not by inducers such as tetracycline (1, 2, 3, 29).

cysH mutants are thought to accumulate PAPS (21, 30); PAPS does not contain a carboxylate but is highly charged as a consequence of the sulfate and phosphate groups (Fig. 1). It seems likely that the anionic nature of the compounds is important to their inducing activity. However, structural studies with another multidrug-binding protein, QacR, show that a single regulatory protein can bind structurally diverse inducers in different ways (35).

As noted in the introduction, *icdA* mutants that also lack citrate synthase (*gltA*) are Nal^s. In contrast, mutants deficient in both aconitases and also in citrate synthase remain resistant to the drug (14), contrary to what is expected if citrate is not formed. However, recent work suggests that in these triple mutants, some citrate is formed by an alternative enzyme to citrate synthase, 2-methylcitrate synthase, and then trapped without further metabolism because of a lack of aconitase (12). Citrate is also formed in small amounts by an unknown pathway (presumably 2-methylcitrate synthase) in *icdA gltA* double mutants (23). However, in those strains the citrate does not form a pool large enough to induce AcrAB-TolC, probably because it is converted by aconitase to isocitrate, which then drains through the glyoxylate pathway (24). This route for further metabolism of citrate is not available in the *acnA acnB gltA* triple mutants.

Why should the internal metabolites induce an efflux pathway? Is it an accident or the product of long-term natural selection? We suggest that the AcrAB-TolC pump provides a means for damping infrequent short-term accumulations of charged metabolites that are inhibitory in high concentrations. By this reasoning, the charged intermediate compounds accumulating in the mutants are inducers of the pump that removes them from the organism if their concentrations become high as the result of temporary surges. The residual activity of the AcrAB-TolC pump would reflect the titration of the sum of the pools of the internal inducers. If true, the affinity or capacity of the pump for some inducers must be relatively low, because the complete blockage of the pathways in *icdA* and *purB* mutants results in large internal pools of the metabolites. Fortuitously, nalidixic acid is a substrate for the pump, and so activation by metabolite accumulation provides resistance against the antibiotic.

We envisage four roles for efflux pumps. Their best-known role is in the removal of toxins entering from outside. Other pumps remove end products of metabolism, for example, fermentation end products and toxins directed against other organisms. Although the pumps usually require an input of energy, some may conserve or capture potential energy. Finally, as suggested by these studies, pumps may serve to buffer the organism against infrequent surges in pools of potentially toxic normal metabolites. It will be interesting to learn which of these functions is primitive.

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