JOURNAL OF BACTERIOLOGY, Oct. 1997, p. 6122–6126 Vol. 179, No. 19
0021-9193/97/$04.00+0

Role of the *acrAB* Locus in Organic Solvent Tolerance Mediated by Expression of *marA*, *soxS*, or *robA* in *Escherichia coli*

DAVID G. WHITE,[1,2] JOHN D. GOLDMAN,[1]† BRUCE DEMPLE,[3] AND STUART B. LEVY[1]*

Center for Adaptation Genetics and Drug Resistance and Department of Molecular Biology and Microbiology, Tufts University School of Medicine, Boston, Massachusetts 02111[1]; Department of Veterinary and Microbiological Sciences, North Dakota State University, Fargo, North Dakota 58105[2]; and Department of Molecular and Cellular Toxicology, Harvard School of Public Health, Boston, Massachusetts 02115[3]

Received 13 May 1997/Accepted 19 July 1997

***Escherichia coli* K-12 strains are normally tolerant to *n*-hexane and susceptible to cyclohexane. Constitutive expression of *marA* of the multiple antibiotic resistance (*mar*) locus or of the *soxS* or *robA* gene product produced tolerance to cyclohexane. Inactivation of the *mar* locus or the *robA* locus, but not the *soxRS* locus, increased organic solvent susceptibility in the wild type and Mar mutants (to both *n*-hexane and cyclohexane). The organic solvent hypersusceptibility is a newly described phenotype for a *robA*-inactivated strain. Multicopy expression of *mar*, *soxS*, or *robA* induced cyclohexane tolerance in strains with a deleted or inactivated chromosomal *mar*, *soxRS*, or *robA* locus; thus, each transcriptional activator acts independently of the others. However, in a strain with 39 kb of chromosomal DNA, including the *mar* locus, deleted, only the multicopy complete *mar* locus, consisting of its two operons, produced cyclohexane tolerance. Deletion of *acrAB* from either wild-type *E. coli* K-12 or a Mar mutant resulted in loss of tolerance to both *n*-hexane and cyclohexane. Organic solvent tolerance mediated by *mar*, *soxS*, or *robA* was not restored in strains with *acrAB* deleted. These findings strongly suggest that active efflux specified by the *acrAB* locus is linked to intrinsic organic solvent tolerance and to tolerance mediated by the *marA*, *soxS*, or *robA* gene product in *E. coli*.**

Organic solvents can be toxic to microorganisms, depending on both the inherent toxicity of the solvent and the intrinsic tolerance of the bacterial species and strain (3, 4, 28). Tolerance to the solvent correlates with the logarithm of its partition coefficient with *n*-octanol and water (log P_{ow}) (12, 24–26). Organic solvents with lower log P_{ow}s are more toxic to microorganisms than are solvents with relatively higher log P_{ow}s. The lowest log P_{ow} in which a strain will grow is known as the index value for that strain, and the solvent with that log P_{ow} is known as the index solvent (5).

The mechanism(s) of organic solvent toxicity is not well understood. However, it has been believed that the solvents cause cell death because they accumulate and break down microbial membranes (13, 27, 30, 48). Therefore, it is of interest that mutants with increased organic solvent tolerance can be isolated from *Escherichia coli* and other species (3, 4, 8, 19, 28, 39, 40).

The index solvent of *E. coli* K-12 is *n*-hexane (log P_{ow}, 3.9). Strains grow in the presence of *n*-hexane but not cyclohexane (log P_{ow}, 3.4) (3–5). Recently, mutants of *E. coli* K-12 strains were derived that were tolerant to both *n*-hexane and cyclohexane (5, 39, 40). These mutants were also found to be resistant to structurally unrelated antibiotics in a pattern that resembled the multiple antibiotic resistance (Mar) phenotype (5, 39, 40). We reported earlier that overexpression of *marA* increased the organic solvent tolerance of *E. coli* strains (19). More recently, other workers have demonstrated that overexpression of *marA* can cause cyclohexane tolerance (8).

The chromosomal *mar* locus, located at 34 min on the *E. coli* chromosomal map, is involved in the regulation of intrinsic susceptibility to structurally unrelated antibiotics (9–11, 18), the expression of antioxidant genes (6, 20), and internal pH homeostasis (43). The *mar* locus consists of two transcription units, *marC* (TU1) and *marRAB* (TU2), which are divergently transcribed from a central putative operator-promoter region (*marO*) (9, 18). *marR* is the repressor of the *marORAB* operon (9, 36, 44). Overexpression of *marA* alone produces the multiple antibiotic resistance phenotype (9, 16, 50). *marB* has no effect of its own; however, when it is present on the same construct with *marA*, it produces a small increase in antibiotic resistance (47). The function of *marC* is unknown; however, it also appears to enhance the multiple antibiotic resistance phenotype when cloned on the same DNA fragment with the *marRAB* operon (18, 47).

The MarA protein is homologous to both SoxS, the effector of the *soxRS* regulon (14, 32), and RobA, a small protein that binds to the *E. coli* replication origin and some stress gene promoters (7, 9, 31, 45). The *soxRS* regulon mediates the cell's response to oxidative stress (2, 41, 49). Overexpression of either *soxS* or *robA* in *E. coli* produces both increased organic solvent tolerance and low-level resistance to multiple antimicrobial agents (7, 39, 40).

Overexpression of *marA* causes increased efflux of antibiotics, including fluoroquinolones, tetracycline, and chloramphenicol (10, 17, 37). Transcription of the *acrAB* operon, a multicomponent, multidrug efflux pump whose expression is modulated by global stress signals (33, 34), was shown to be elevated in strains containing *marR* mutations and displaying the Mar phenotype (42). Moreover, inactivation of *acrAB* led to increased antibiotic susceptibility in the wild type and in Mar mutants (42).

We investigated the role of the *acr* and *mar* loci in organic solvent tolerance and the effect of the *mar* locus on organic solvent tolerance mediated by expression of either *robA* or *soxS*.

* Corresponding author. Mailing address: Center for Adaptation Genetics and Drug Resistance, Tufts University School of Medicine, 136 Harrison Ave., Boston, MA 02111. Phone: (617) 636-6764. Fax: (617) 636-0458.

† Present address: Department of Internal Medicine, Polyclinic Medical Health, Harrisburg, PA 17110.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Source or reference(s)
E. coli strains		
AG100	Wild-type *E. coli* K-12	17
AG100-A	AG100; Δ*acrAB*	42
AG100-B	AG100; *acrR* mutant	42
AG102	*marR1* mutant of AG100 selected on tetracycline	9, 17
AG102-A	AG102; Δ*acrAB*	42
MCH164	AG100 with 39-kb deletion from 33.6–34.3 min including *mar* locus; *zdd-230*::Tn*9* from which Tn*9* was spontaneously lost	37
AG100K	Derivative of AG100 in which kanamycin resistance cassette replaced most of *mar* locus	35
GC4468	Wild-type *E. coli* K-12	21, 46
DJ901	GC4468 from which *soxRS901* was deleted; *soxRSΔ901*::Tn*10*Km^r	21
RA4468	GC4468 in which kanamycin resistance cassette was inserted into *rob* locus; *robA*::*kan*	7
JHC1096	GC4468 into which *mar* locus deletion (as in MCH164) was introduced; Tn*9*Km^r	6, 21
JHC1098	GC4468 into which *mar* and *soxRS* deletions of DJ901 and JHC1096 were introduced; Tn*10*Km^r	7, 21
JHC1069	GC4468 bearing *cfxB1*; MarR mutant	7, 21
JTG1078	GC4468; *soxR105* Tn*10*Km^r	20
Plasmids		
pMAK705	Temperature-sensitive, low-copy-number cloning vector; Cml^r	23
pMAK-TU1	pMAK705 with 1-kb chromosomal insertion containing *marO* and *marC* (nt[a] 569–1577 of *mar* locus [9])	18
pMAK-TU2	pMAK705 with 1.28-kb chromosomal insertion (nt 1311–2592 of *mar* locus [9]) containing *marO* and *marRAB* (derived from pHHM193 [9]), which contains *marR5* mutation and constitutively expresses *marRAB* operon	18
pMAK-TU1&TU2	pMAK705 with 2.4-kb chromosomal insertion (nt 163–2592 of *mar* locus [8]) containing *marC* and *marRAB* (derived from pHHM193 [9]), which contains *marR5* mutation and constitutively expresses *marRAB* operon	18
pSE380	*trc* promoter expression vector; high copy number, IPTG inducible; Amp^r	2
pSRob	926-bp *Sal*I-*Sac*I fragment containing entire *rob* gene from pBt35-13 inserted into pSE380	7
pSXS	423-bp *Eco*RI-*Hin*dIII PCR fragment containing entire *soxS* gene inserted into pSE380	2
pSMarAB	699-bp *Eco*RI-*Pst*I PCR fragment (nt 1893–2592 of *mar* locus [9]) containing *marAB* inserted into pSE380	This study

[a] nt, nucleotides.

(Part of this work was previously presented at the 96th General Meeting of the American Society for Microbiology [19].)

MATERIALS AND METHODS

Bacterial strains and plasmids. The *E. coli* strains and plasmids used in this study and their relevant properties are listed in Table 1. Unless otherwise noted, bacteria were grown and maintained at 30°C in Luria-Bertani (LB) broth or LB agar (Difco, Detroit, Mich.) plates with or without the appropriate antibiotics for selection. *E. coli* AG100-A, AG100-B, and AG102-A were kindly provided by H. Nikaido (42).

Chemicals. Organic solvents were obtained from Aldrich Chemical Co. (Milwaukee, Wis.). Antibiotics were obtained from Sigma Chemical Co. (St. Louis, Mo.). Isopropyl-β-D-thiogalactopyranoside (IPTG) was obtained from Alexis Corporation (San Diego, Calif.).

DNA manipulations. Plasmid DNA was prepared by using the Wizard Prep Kit (Promega, Madison, Wis.). Restriction endonucleases and T4 DNA ligase were purchased from New England Biolabs (Beverly, Mass.) and used under conditions suggested by the supplier. PCR amplification was carried out with a Perkin Elmer-Cetus DNA thermal cycler 480. *Taq* polymerase and reagents were provided by Perkin Elmer-Cetus and used as directed by the supplier. Primers were created which flanked the coding sequence and allowed amplification of *marAB* (1,893 to 2,592 bp of the published sequence; reference 9). This PCR amplicon was approximately 699 bp long. Restriction endonuclease sites for *Eco*RI and *Pst*I were incorporated into the ends of the PCR primers to ensure that insertion of fragments was in the correct orientation when they were cloned into pSE380, a high-copy-number expression vector (Invitrogen, Carlsbad, Calif.). The resulting plasmid construct was called pSMarAB.

DNA fragments from low-melting-point agarose gels, as well as PCR products, were purified by using the Qiagen gel extraction kit (Qiagen Inc., Chatsworth, Calif.). DNA transformation was performed by using the $CaCl_2$ procedure as previously described (9), and pSE380 clones were selected by using LB agar plates containing ampicillin (100 μg/ml), IPTG (0.1 mM), and 5-bromo-4-chloro-3-β-D-galactopyranoside (X-Gal; 40 μg/ml).

Organic solvent tolerance assays. Organic solvent tolerance of bacterial strains grown to late logarithmic phase was measured in cultures diluted to a concentration of approximately 10^7 cells/ml. A 5-μl aliquot of the bacterial suspension was plated on LB agar and allowed to dry. An organic solvent was overlaid to a depth of 2 to 3 mm. The plate was sealed and incubated overnight at 30°C (5). Cyclohexane, *n*-hexane, and *n*-pentane were used as the organic solvents. IPTG was added to the agar plates at a concentration of 0.5 mM when induction of plasmid genes was required. For efficiency-of-plating (EOP) experiments, logarithmic-phase cultures were diluted to an optical density at 530 nm of 0.2 and 100-μl aliquots of cells from serial dilutions were spread onto LB agar plates. As mentioned above, an organic solvent was then overlaid to a depth of 2 to 3 mm and the plate was sealed and incubated overnight at 30°C. Platings were done in duplicate, and colonies were counted. Growth was recorded as confluent growth (++), visible growth (≤100 colonies; +), or no growth (−).

RESULTS

The organic solvent tolerance of *E. coli* K-12 strain AG100 was compared to that of an isogenic strain that constitutively expresses the *mar* operon due to a mutation in *marR* (AG102) (9). AG100 grew in the presence of *n*-hexane only; AG102 grew in the presence of *n*-hexane, cyclohexane (Table 2), and *n*-pentane (data not shown). Thus, constitutive expression of the *mar* locus changed the index solvent from *n*-hexane (log P_{ow}, 3.9) to *n*-pentane (log P_{ow}, 3.3).

In the wild-type *E. coli* AG100 background, overexpression of *marA* (on plasmid pSMarAB or pMAK-TU2), *soxS* (on pSXS), or *robA* (on pSRob) resulted in cyclohexane tolerance (Table 2). *marC* by itself (pMAK-TU1) had no effect on cyclohexane tolerance; however, introduction of *marCORAB* on low-copy plasmid pMAK705 (pMAK-TU1&TU2) resulted in cyclohexane tolerance (Table 2).

When the *mar* locus was inactivated by replacement with a kanamycin resistance cassette (AG100K) (35), the strain became hypersusceptible to *n*-hexane compared to the wild-type strain (Table 2). MCH164 (a derivative of AG100 from which 39 kb of chromosomal DNA, including the entire *mar* locus, had been deleted [18, 37]) was, as expected, also hypersusceptible to organic solvents (Table 2). Expression in *trans* of *marA*, *soxS*, or *robA* in AG100K restored *n*-hexane tolerance and increased cyclohexane tolerance in the cell (Table 2). Expression in *trans* in AG100K of *marA*, specified from plasmid

TABLE 2. Organic solvent tolerance of wild-type and *mar* mutant strains bearing *mar*, *soxS*, and *robA* plasmids

Strain	Plasmid[b]	Growth in presence of organic solvent[a]	
		n-Hexane (3.9)[c]	Cyclohexane (3.4)[c]
AG102 (*marR* mutation)		++	++
AG100 (wild type)		++	−
AG100	pMAK-TU1	++	−
AG100	pMAK-TU2	++	+
AG100	pMAK-TU1&TU2	++	++
AG100	pSMarAB	++	+
AG100	pSXS	++	++
AG100	pSRob	++	++
AG100K (*marCORAB*::*kan*)		+	−
AG100K	pMAK-TU1	+	−
AG100K	pMAK-TU2	++	+
AG100K	pMAK-TU1&TU2	++	++
AG100K	pSMarAB	++	+
AG100K	pSXS	++	++
AG100K	pSRob	++	++
MCH164 (Δ*mar*)		+	−
MCH164	pMAK-TU1	+	−
MCH164	pMAK-TU2	++	−
MCH164	pMAK-TU1&TU2	++	++
MCH164	pSMarAB	++	−
MCH164	pSXS	++	−
MCH164	pSRob	++	−
AG100-B (*acrR* mutant)		++	+
AG100-A (Δ*acrAB*)		−	−
AG102-A (*marR1* Δ*acrAB*)		−	−
AG102-A	pSMarAB	−	−
AG102-A	pSXS	−	−
AG102-A	pSRob	−	−

[a] ++, confluent growth; +, visible growth (≤100 colonies); −, no growth.
[b] IPTG was added to plates at a concentration of 0.5 mM when induction of plasmid genes was required (pSE380 derivatives).
[c] Value in parentheses is log P_{ow}.

pMAK-TU1&TU2, restored *n*-hexane tolerance and produced higher cyclohexane tolerance (Table 2). The better effect of the latter plasmid than those containing only *marA* could be linked to greater production of MarA protein from this plasmid (as visualized by antibody to MarA [1]). While introduction of either *marA*, *soxS*, or *robA* restored *n*-hexane tolerance in MCH164, only pMAK-TU1&TU2 produced cyclohexane tolerance in this larger deletion mutant (Table 2).

Since *acrAB* deletion dramatically decreased multiple drug resistance in Mar mutants (42), we examined its possible role in organic solvent tolerance. Overexpression of *acrAB*, because of a mutation in *acrR* in AG100-B, enabled the strain to grow in the presence of cyclohexane (Table 2). Deletion of *acrAB* from wild-type AG100 (AG100-A) resulted in *n*-hexane sensitivity (Table 2). Deletion of *acrAB* from the Mar mutant AG102 (AG102-A) resulted in sensitivity to both *n*-hexane and cyclohexane. Expression of *marA*, *soxS*, or *robA* in AG102-A failed to restore organic solvent tolerance, further demonstrating the critical role of *acrAB* (Table 2).

We next investigated a series of isogenic strains in which *sox*, *mar*, and *robA* were either overexpressed, deleted, or inactivated (Table 3). *E. coli* strains overexpressing MarA (JHC1069; *cfxB1*/MarR mutation) or SoxS (JTG1078; *soxR*105 mutation) grew in the presence of both *n*-hexane and cyclohexane, whereas wild-type GC4468 grew only in the presence of *n*-hexane (Table 3). Much like the situation in AG100, introduction of either pSMarAB, pMAK-TU2, pMAK-TU1&TU2, pSXS, or pSRob into GC4468 produced cyclohexane tolerance. Inactivation of *robA* by insertion of a kanamycin resistance cassette (RA4468) caused *n*-hexane susceptibility (Table 3). Introduction of either *marA* (on pMAK-TU1&TU2, pMAK-TU2, or pSMarAB), *soxS* (on pSXS), or *robA* (on pSRob) into the *robA*-inactivated strain increased tolerance to both *n*-hexane and cyclohexane (Table 3). Deletion of *soxRS* (DJ901) had little effect on *n*-hexane tolerance (Table 3). Introduction of *marA*, *soxS*, or *robA* into the Δ*soxRS* strain produced cyclohexane tolerance (Table 3). In all of these complementations, the best effect of *marA* was obtained with plasmid pMAK-TU1&TU2.

Since Mar mutant strain AG102 grew confluently in the presence of cyclohexane and the *acrR* mutant form of strain AG100 (AG100-B) grew less well, we tried a different method, namely, an EOP assay to compare growth in the presence of cyclohexane (Table 4). AG102 had a greater EOP (73%) in cyclohexane than did AG100-B (EOP, 13%). The sixfold difference confirmed, although perhaps less dramatically, the spot test results.

DISCUSSION

The *mar* locus affects intrinsic multidrug susceptibility or resistance in *E. coli* and when activated provides the cell with

TABLE 3. Organic solvent tolerance of wild-type, Δ*soxRS*, and *robA*::*kan* strains bearing *mar*, *soxS*, and *robA* plasmids

Strain	Plasmid[b]	Growth in presence of organic solvent[a]	
		n-Hexane (3.9)[c]	Cyclohexane (3.4)[c]
GC4468 (wild type)		++	−
JHC1069 (*cfxB1*)		++	++
JTG1078 (*soxR105*)		++	++
GC4468	pMAK-TU1	++	−
GC4468	pMAK-TU2	++	+
GC4468	pMAK-TU1&TU2	++	++
GC4468	pSMarAB	++	+
GC4468	pSXS	++	++
GC4468	pSRob	++	++
RA4468 (*robA*::*kan*)		+	−
RA4468	pMAK-TU1	+	−
RA4468	pMAK-TU2	++	+
RA4468	pMAK-TU1&TU2	++	++
RA4468	pSMarAB	++	+
RA4468	pSXS	++	++
RA4468	pSRob	++	++
DJ901 (Δ*soxRS*)		++	−
DJ901	pMAK-TU1	++	−
DJ901	pMAK-TU2	++	+
DJ901	pMAK-TU1&TU2	++	++
DJ901	pSMarAB	++	+
DJ901	pSXS	++	++
DJ901	pSRob	++	++

[a] ++, confluent growth; +, visible growth (≤100 colonies); −, no growth.
[b] IPTG was added to plates at a concentration of 0.5 mM when induction of plasmid genes was required (pSE380 derivatives).
[c] Value in parentheses is log P_{ow}.

TABLE 4. Effect of a *marR* or *acrR* mutation on *E. coli* cyclohexane tolerance

Strain	Avg no. of colonies ± SE		EOP[b]
	Control plate[a]	Cyclohexane plate	
AG102 MarR mutant	182 ± 5	133 ± 7	0.73
AG100-B AcrR mutant	196 ± 10	25 ± 4	0.13

[a] Control LB and cyclohexane-layered LB agar plate data are averages of two plates.

[b] EOP experiment comparing growth on cyclohexane-overlaid plates with that on control plates. A 10^{-6} dilution was plated, and colonies were counted.

a response to a variety of environmental stimuli and stresses (6, 11, 18, 20, 38, 43). One such stress could certainly include growth in an environment containing organic solvents. This possibility was suggested in work by Aono et al. (5), who reported that cyclohexane-tolerant mutants of *E. coli* also displayed the Mar phenotype (low-level multidrug resistance).

Various genes are thought to be involved in organic solvent tolerance in *E. coli*, but the mechanisms of tolerance have not been identified. Of those investigated, a gene designated *ostA*, when transduced from an *n*-hexane-tolerant *E. coli* strain into a hexane-sensitive strain, restored hexane tolerance (4). Unexpectedly, a gene encoding alkylhydroperoxide reductase also produced organic solvent resistance (15). A *soxR* mutation, overexpression of the cloned *soxS* gene, or overexpression of *robA* increased the levels of organic solvent tolerance in *E. coli* (39, 40).

We showed earlier that an *E. coli* Mar mutant (AG102) selected for multiple antibiotic resistance was more tolerant to organic solvents than was its wild-type parent strain AG100 (19). Conversely, a mutant of AG100 selected for growth in the presence of cyclohexane was found to be multidrug resistant with overexpression of *marA* (22). Introduction of *marA* on low- or high-copy-number plasmids into AG100 generated cyclohexane tolerance (19) (Table 2). Therefore, the *marA*-mediated increased organic solvent tolerance can be produced in a wild-type background without mutations elsewhere on the chromosome.

Inactivation or deletion of the *mar* locus has been shown to restore wild-type antibiotic susceptibilities to Mar mutants (9, 17) and abolish the protection provided against the rapid bactericidal effects of fluoroquinolones (18). When the wild-type *mar* locus was inactivated by replacement with a kanamycin resistance cassette (AG100K) or by a 39-kb deletion (MCH164), the strains became hypersusceptible to *n*-hexane compared with the wild-type strain (Table 2).

In a hypersusceptible strain background with *mar* deleted (AG100K), overexpression of *soxS*, *robA*, or *marA* restored tolerance to *n*-hexane and produced growth on cyclohexane. However, in a strain missing the *mar* locus and ~35 kb of adjacent DNA (MCH164), overexpression of *soxS*, *robA*, or *marA* could not induce cyclohexane tolerance (Table 2). Only the plasmid containing *marRAB* and *marC*, carried on the large fragment cloned in pMAK-TU1&TU2, produced growth on cyclohexane. These findings suggested that other genes deleted in MCH164 are required for *robA*-, *soxS*-, or *marA*-mediated cyclohexane tolerance. Alternatively, much higher expression of these activators may be needed in this strain background, although it is interesting that *soxS* and *robA* on the same plasmids were able to generate strong cyclohexane tolerance in the other strains tested (Tables 2 and 3).

Expression of TU1 (*marC*) by itself had no effect on organic solvent tolerance. As discussed above, when present with *marAB* (TU2) on the same chromosomal fragment, it enhanced the complementation in *trans* by *marA*. Western blot studies (data not shown) (1) with antibodies to MarA showed that this was at least partially due to increased expression of MarA, compared to TU2 alone, although the presence of *marC* may be additionally involved. It is interesting that the TU1&TU2 fragment was also required for resistance to rapid cell killing by fluoroquinolones in this strain (18).

Inactivation of *robA* by insertion of a kanamycin resistance cassette (RA4468) resulted in increased *n*-hexane susceptibility, evidently the first phenotype described for *robA*-inactivated cells. However, deletion of *soxRS* (DJ901) caused no detectable effect on *n*-hexane tolerance (Table 3). Thus, the *mar* and *rob* loci are more involved with basal organic solvent tolerance than is *soxRS*. Introduction of any of the three transcriptional activators into a *robA*- or *soxRS*-inactivated strain increased *n*-hexane tolerance and increased cyclohexane tolerance. These findings contrast with a previous report that organic solvent tolerance mediated by overexpression of *robA* was dependent on *soxRS* (39). This unexplained disparity may be due to strain differences. Therefore, each of the three loci can mediate organic solvent tolerance independently of the others.

Recent work has identified the AcrAB efflux system as the major mechanism responsible for the multidrug resistance in Mar mutants (42). Deletion of *acrAB* from either wild-type AG100 (AG100-A) or the Mar mutant form of AG102 (AG102-A) resulted in loss of tolerance to the index organic solvent *n*-hexane. Increased organic solvent tolerance was not restored in AG102-A by any of the transcriptional activators (Table 2). Mutation of *acrR* (in AG100-B, in which *acrAB* is overexpressed) permitted the strain to grow on cyclohexane (Table 2). These results indicate that AcrAB plays a major role in the organic solvent tolerance phenotype of *E. coli*. In a comparison between isogenic strains overexpressing *marA* or *acrAB*, the Mar mutant showed greater cyclohexane tolerance (Tables 2 and 4). This finding may reflect greater expression of *acrAB* in Mar mutants or a combination of *acrAB* overexpression and the other changes occurring in a Mar mutant. It is interesting that efflux was associated with toluene resistance in *Pseudomonas putida*, although the genes were not identified (29).

In summary, all three transcriptional activators (*marA*, *soxS*, and *robA*) can independently produce organic solvent tolerance in *E. coli*; all require the *acrAB* locus. For complementation of the large deletion mutant MCH164, however, only the plasmid bearing both transcriptional units of the mutant *mar* locus produced cyclohexane tolerance (Table 2). These results strongly suggest that these transcriptional activators produce organic solvent tolerance by up regulating the *acrAB* locus, resulting in increased efflux of the solvents from the cell.

ACKNOWLEDGMENTS

This work was supported in part by a Johnson and Johnson Focused Giving Award (S.B.L.) and by grants from the National Institutes of Health (T32 AI-07329 [J.D.G.], CA3731 [B.D.], and GM51661 [S.B.L.]).

REFERENCES

1. **Alekshun, M., and S. B. Levy.** Unpublished data.
2. **Amabile-Cuevas, C. F., and B. Demple.** 1991. Molecular characterization of the *soxRS* genes of *Escherichia coli*: two genes control a superoxide stress regulon. Nucleic Acids Res. **19:**4479–4484.
3. **Aono, R., K. Aibe, A. Inoue, and K. Horikoshi.** 1991. Preparation of organic solvent tolerant mutants from *Escherichia coli*. Agric. Biol. Chem. **55:**1935–1938.
4. **Aono, R., T. Negishi, and H. Nakajima.** 1994. Cloning of organic solvent

tolerance gene *ostA* that determines *n*-hexane tolerance level in *Escherichia coli*. Appl. Environ. Microbiol. **60:**4624–4626.
5. **Aono, R., M. Kobayashi, H. Nakajima, and H. Kobayashi.** 1995. A close correlation between improvement of organic solvent tolerance levels and alteration of resistance toward low levels of multiple antibiotics. Biosci. Biotech. Biochem. **59:**213–218.
6. **Ariza, R. R., S. P. Cohen, N. Bachhawat, S. B. Levy, and B. Demple.** 1994. Repressor mutations in the *marRAB* operon that activate oxidative stress genes and multiple antibiotic resistance in *Escherichia coli*. J. Bacteriol. **176:**143–148.
7. **Ariza, R. R., Z. Li, N. Ringstad, and B. Demple.** 1995. Activation of multiple antibiotic resistance and binding of stress-inducible promoters by *Escherichia coli* Rob protein. J. Bacteriol. **177:**1655–1661.
8. **Asako, H., H. Nakajima, K. Kobayashi, M. Kobayashi, and R. Aono.** 1997. Organic solvent tolerance and antibiotic resistance increased by overexpression of *marA* in *Escherichia coli*. Appl. Environ. Microbiol. **63:**1428–1433.
9. **Cohen, S. P., H. H. Hachler, and S. B. Levy.** 1993. Genetic and functional analysis of the multiple antibiotic resistance (*mar*) locus in *Escherichia coli*. J. Bacteriol. **175:**1484–1492.
10. **Cohen, S. P., L. M. McMurry, D. C. Hooper, J. S. Wolfson, and S. B. Levy.** 1989. Cross-resistance to fluoroquinolones in multiple-antibiotic-resistant (Mar) *Escherichia coli* selected by tetracycline or chloramphenicol: decreased drug accumulation associated with membrane changes in addition to OmpF reduction. Antimicrob. Agents Chemother. **33:**1318–1325.
11. **Cohen, S. P., L. M. McMurry, and S. B. Levy.** 1988. *marA* locus causes decreased expression of OmpF porin in multiple-antibiotic-resistant (Mar) mutants of *Escherichia coli*. J. Bacteriol. **170:**5416–5422.
12. **Corwin, H., and S. M. Anderson.** 1967. The effect of intramolecular hydrophobic bonding on partition coefficients. J. Org. Chem. **32:**2583–2586.
13. **De Smet, M. J., J. Kingman, and B. Witholt.** 1978. The effect of toluene on the structure and permeability of the outer and cytoplasmic membrane of *Escherichia coli*. Biochim. Biophys. Acta **506:**64–80.
14. **Fawcett, W. P., and R. E. Wolf, Jr.** 1994. Purification of a MalE-SoxS fusion protein and identification of the control sites of *Escherichia coli* superoxide-inducible genes. Mol. Microbiol. **14:**669–679.
15. **Ferrante, A. A., J. Augliera, K. Lewis, and A. M. Klibanov.** 1995. Cloning of an organic solvent-resistance gene in *Escherichia coli*: the unexpected role of alkylhydroperoxide reductase. Proc. Natl. Acad. Sci. USA **92:**7617–7621.
16. **Gambino, L., S. J. Gracheck, and P. F. Miller.** 1993. Overexpression of the MarA positive regulator is sufficient to confer multiple antibiotic resistance in *Escherichia coli*. J. Bacteriol. **175:**2888–2894.
17. **George, A. M., and S. B. Levy.** 1983. Amplifiable resistance to tetracycline, chloramphenicol, and other antibiotics in *Escherichia coli*: involvement of a non-plasmid-determined efflux of tetracycline. J. Bacteriol. **155:**531–540.
18. **Goldman, J. D., D. G. White, and S. B. Levy.** 1996. The multiple antibiotic resistance (*mar*) locus protects *Escherichia coli* from rapid cell killing by fluoroquinolones. Antimicrob. Agents Chemother. **40:**1266–1269.
19. **Goldman, J. D., D. G. White, and S. B. Levy.** 1996. A central role for the multiple antibiotic resistance (*mar*) locus in *E. coli* in organic solvent tolerance, abstr. A115, p. 153. *In* Abstracts of the 96th General Meeting of the American Society for Microbiology 1996. American Society for Microbiology, Washington, D.C.
20. **Greenberg, J. T., J. H. Chou, P. Monach, and B. Demple.** 1991. Activation of oxidative stress genes by mutations at the *soxQ*/*cfxB*/*marA* locus of *Escherichia coli*. J. Bacteriol. **173:**4433–4439.
21. **Greenberg, J. T., P. Monach, J. H. Chou, P. D. Josephy, and B. Demple.** 1990. Positive control of global antioxidant defense regulon activated by superoxide-generating agents in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **87:**6181–6185.
22. **Gustafson, J., and S. B. Levy.** Unpublished data.
23. **Hamilton, C. M., M. Aldema, B. K. Washburn, P. Babizke, and S. Kushner.** 1989. New method for generating deletions and gene replacement in *Escherichia coli*. J. Bacteriol. **173:**2888–2894.
24. **Hansch, C., M. R. Muir, T. Fujita, P. P. Maloney, F. Geiger, and M. Streich.** 1963. The correlation of biological activity of plant growth regulators and chloromycetin derivatives with Hammett constants and partition coefficients. J. Am. Chem. Soc. **85:**2817–2824.
25. **Hansch, C., and T. Fujita.** 1964. p-r-π analysis. A method for correlation of biological activity and chemical structure. J. Am. Chem. Soc. **86:**1616–1626.
26. **Harnisch, M., H. Mockel, and G. Schulze.** 1983. Relationship between low P_{ow} shake-flask values and capacity factors derived from reversed-phase high performance liquid chromatography for *n*-alkylbenzeds and some OECD reference substances. J. Chromatogr. **282:**315–332.
27. **Heipieper, H. J., F. J. Weber, J. Sikkema, H. Keweloh, and J. A. M. de Bont.** 1994. Mechanisms of resistance of whole cells to toxic organic solvents. Trends Biotechnol. **12:**409–415.
28. **Inoue, A., and K. Horikoshi.** 1989. A pseudomonas thrives in high concentrations of toluene. Nature (London) **338:**264–265.
29. **Isken, S., and J. A. M. de Bont.** 1996. Active efflux of toluene in a solvent-resistant bacterium. J. Bacteriol. **178:**6056–6058.
30. **Jackson, R. W., and J. A. Demoss.** 1965. Effects of toluene on *Escherichia coli*. J. Bacteriol. **90:**1420–1425.
31. **Jair, K.-W., X. Yu, K. Skarstad, B. Thony, N. Fujita, A. Ishihama, and R. E. Wolf, Jr.** 1996. Transcriptional activation of promoters of the superoxide and multiple antibiotic resistance regulons by Rob, a binding protein of the *Escherichia coli* origin of chromosomal replication. J. Bacteriol. **178:**2507–2513.
32. **Li, Z. Y., and B. Demple.** 1994. SoxS, an activator of superoxide stress genes in *Escherichia coli*: purification and interaction with DNA. J. Biol. Chem. **269:**18371–18377.
33. **Ma, D., D. N. Cook, M. Alberti, N. G. Pon, H. Nikaido, and J. E. Hearst.** 1995. Genes *acrA* and *acrB* encode a stress induced efflux system of *Escherichia coli*. Mol. Microbiol. **16:**45–55.
34. **Ma, D., M. Alberti, C. Lynch, H. Nikaido, and J. E. Hearst.** 1996. The local repressor AcrR plays a modulating role in the regulation of *acrAB* genes of *Escherichia coli* by global stress signals. Mol. Microbiol. **19:**101–112.
35. **Maneewannakul, K., and S. B. Levy.** 1996. Identification of *mar* mutants among quinolone-resistant clinical isolates of *Escherichia coli*. Antimicrob. Agents Chemother. **40:**1695–1698.
36. **Martin, R. G., and J. L. Rosner.** 1995. Binding of purified multiple antibiotic resistance repressor protein (MarR) to *mar* operator sequences. Proc. Natl. Acad. Sci. USA **92:**5456–5460.
37. **McMurry, L. M., A. M. George, and S. B. Levy.** 1994. Active efflux of chloramphenicol in susceptible *Escherichia coli* strains and in multiple-antibiotic-resistant (Mar) mutants. Antimicrob. Agents Chemother. **38:**542–546.
38. **Miller, P. F., L. F. Gambino, M. C. Sulavik, and S. J. Gracheck.** 1994. Genetic relationship between *soxRS* and *mar* loci in promoting multiple antibiotic resistance in *Escherichia coli*. Antimicrob. Agents Chemother. **38:**1773–1779.
39. **Nakajima, H., K. Kobayashi, M. Kobayashi, H. Asako, and R. Aono.** 1995. Overexpression of the *robA* gene increases organic solvent tolerance and multiple antibiotic and heavy metal ion resistance in *Escherichia coli*. Appl. Environ. Microbiol. **61:**2302–2307.
40. **Nakajima, H. K., H. Kobayashi, R. Aono, and K. Horikoshi.** 1995. *soxRS* gene increased the level of organic solvent tolerance in *Escherichia coli*. Biosci. Biotechnol. Biochem. **59:**1323–1325.
41. **Nunoshiba, T., E. Hidalgo, C. F. Amabile-Cuevas, and B. Demple.** 1992. Two-stage control of an oxidative stress regulon: the *Escherichia coli* SoxR protein triggers redox-inducible expression of the *soxS* regulatory gene. J. Bacteriol. **174:**6054–6060.
42. **Okusu, H., D. Ma, and H. Nikaido.** 1996. AcrAB efflux pump plays a major role in the antibiotic resistance phenotype of *Escherichia coli* multiple-antibiotic-resistance (Mar) mutants. J. Bacteriol. **178:**306–308.
43. **Rosner, J. L., and J. L. Slonczewski.** 1994. Dual regulation of *inaA* by the multiple antibiotic resistance (Mar) and superoxide stress response (SoxRS) systems of *Escherichia coli*. J. Bacteriol. **176:**6262–6269.
44. **Seoane, A. S., and S. B. Levy.** 1995. Characterization of MarR, the repressor of the multiple antibiotic resistance (*mar*) operon in *Escherichia coli*. J. Bacteriol. **177:**3414–3419.
45. **Skarstad, K., B. Thony, D. Hwang, and A. Kornberg.** 1993. A novel binding protein of the origin of *Escherichia coli* chromosome. J. Biol. Chem. **268:**5365–5370.
46. **Touati, D.** 1983. Cloning and mapping of the manganese superoxide dismutase gene (*sodA*) of *Escherichia coli* RDEC-1 and DNA sequence of the major structural subunit. Infect. Immun. **58:**1124–1128.
47. **White, D. G., W. Yan, and S. B. Levy.** 1994. Functional characterization of the chromosomal multiple antibiotic resistance (*mar*) locus in *Escherichia coli*, abstr. A-104, p. 20. *In* Abstracts of the 94th General Meeting of the American Society for Microbiology 1994. American Society for Microbiology, Washington, D.C.
48. **Woodringh, C. L.** 1973. Effects of toluene and phenylethyl alcohol on the ultrastructure of *Escherichia coli*. J. Bacteriol. **114:**1359–1361.
49. **Wu, J., and B. Weiss.** 1991. Two divergently transcribed genes, *soxR* and *soxS*, control a superoxide response regulon of *Escherichia coli*. J. Bacteriol. **173:**2864–2871.
50. **Yan, W., S. P. Cohen, and S. B. Levy.** 1992. Three putative proteins in the *mar* operon mediate intrinsic multidrug resistance in *Escherichia coli*, abstr. A-26, p. 5. *In* Abstracts of the 92nd General Meeting of the American Society for Microbiology 1992. American Society for Microbiology, Washington, D.C.