ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Dec. 1997, p. 2770–2772 Vol. 41, No. 12
0066-4804/97/$04.00+0

Selection of Multiple-Antibiotic-Resistant (Mar) Mutants of *Escherichia coli* by Using the Disinfectant Pine Oil: Roles of the *mar* and *acrAB* Loci

MERRI C. MOKEN,[1] LAURA M. McMURRY,[2]* AND STUART B. LEVY[2,3]

Morristown, New Jersey 07960,[1] and the Center for Adaptation Genetics and Drug Resistance and the Departments of Molecular Biology and Microbiology[2] and of Medicine,[3] Tufts University School of Medicine, Boston, Massachusetts 02111

Received 7 May 1997/Returned for modification 31 July 1997/Accepted 6 October 1997

Mutants of *Escherichia coli* selected for resistance to the disinfectant pine oil or to a household product containing pine oil also showed resistance to multiple antibiotics (tetracycline, ampicillin, chloramphenicol, and nalidixic acid) and overexpressed the *marA* gene. Likewise, antibiotic-selected Mar mutants, which also overexpress *marA*, were resistant to pine oil. Deletion of the *mar* or *acrAB* locus, the latter encoding a multidrug efflux pump positively regulated in part by MarA, increased the susceptibility of wild-type and mutant strains to pine oil.

Pine oil is a disinfectant used in products designed for household use. The possibility that such products might select for antibiotic resistance was investigated. In initial studies of a formulation whose active ingredient was pine oil (Pine-Sol; Chlorox Co., Oakland, Calif.), mutant colonies of *Escherichia coli* sometimes grew within the zone of inhibition surrounding a 6-mm absorbent paper disc impregnated with the product and placed upon a bacterial lawn. These and other pine oil-resistant mutants were tested for resistance to multiple antibiotics, and the genetic basis for the observed phenotype was examined.

Selection of mutants resistant to pine oil formulation or pine oil. Mutants resistant to pine oil formulation or pine oil were obtained from stationary-phase Luria-Bertani broth cultures of *E. coli* WEC (wild-type strain 15-5068 from Carolina Biological Supply Co., Burlington, N.C.) and AG100 (9) at 30°C on nutrient agar (NP3.5GP) or Luria-Bertani agar with 2 to 3 days of incubation in a variety of ways: by the 6-mm-disc method or by plating cells on plates or gradient plates (8) containing pine oil formulation (obtained in supermarkets; contains pine oil [the active ingredient], isopropanol, and surfactants) or pine oil itself (obtained from the White Cap Co., Lester, Pa.). All mutants were single-step isolates and occurred at a frequency of about 10^{-8}.

Resistance to antibiotics of mutants selected on pine oil formulation or pine oil. Antibiotic susceptibility was measured at 30°C by using antibiotic susceptibility discs (Carolina Biological), gradient plates with the drug in the top agar (8), or agar dilution plates (concentration steps of 1.5-fold; inoculum of 10^5 cells/5-μl spot). Mutant NP3.5GP, selected on pine oil formulation from strain WEC, was more resistant than the parent strain to tetracycline, ampicillin, and chloramphenicol (Table 1). Nalidixic acid was included in further tests of 11 independent mutants from strain AG100. While there was a variety of resistance phenotypes, all pine oil formulation-pine oil-selected mutants were also multidrug resistant. For genetic studies, we chose three mutants of AG100 which were resistant to all four antibiotics (Table 1): AP1 and AP5 (selected on pine oil) and APS3 (selected on pine oil formulation).

Role of *mar*, *soxRS*, and *robA* loci. Mutations in the repressor gene *marR* or in its operator *marO* in the *E. coli marRAB* operon (2, 17, 28) lead to enhanced *marA* expression and multiple antibiotic resistance. In host strain HH180, with the entire *mar* region deleted, plasmids pHHM188, pHHM191, and pHHM193 each contain a cloned 9-kb fragment including the entire *mar* locus. In pHHM188, the *mar* locus was wild type, while in the latter two plasmids, *marR* was mutant, resulting in a Mar phenotype (6). These Mar mutants, as well as AG102 (bearing a chromosomal *marR* mutation [6]), were resistant to pine oil formulation (Table 1) and to 100% pine oil (assayed by discs on MacConkey agar [data not shown]) compared to the respective wild-type strains. The *marCORAB* locus was deleted in the pine oil formulation-pine oil mutants and in AG102 by P1 transduction (26) with AG100/Kan (17) as the donor strain and selection on kanamycin. The deletion caused a 60 to 70% reduction in the resistance of all mutants to pine oil formulation (Table 2), down to approximately a wild-type level. The same was true for mutant NP3.5GP (data not shown).

Northern blot analysis for expression of *marA* mRNA in the absence and presence of the inducer salicylate (7) revealed that, like Mar mutant AG102, mutants AP5 and NP3.5GP showed an overexpression of *marA* that was enhanced by salicylate (Fig. 1). Overexpression was also seen in mutant APS3 (data not shown). The wild-type AG100 and the pine oil mutant AP1 showed no detectable signal (Fig. 1). We concluded that AP5, NP3.5GP, and APS3, but not AP1, were probably Mar mutants.

Overexpression of *soxS* and *robA*, two other regulatory genes with homology to *marA*, can also lead to multiple antibiotic resistance (2, 3, 11, 18, 19). We inactivated the *soxRS* and *robA* loci in the pine oil formulation-pine oil and Mar mutants via P1 transduction, with donor strains DJ901, bearing a deletion in *soxRS* very closely linked to *zjc2204*::Tn*10*Km (3, 12), and RA4468, which has a Kan^r insertion in *robA* (3). The inactivations of *sox* or *rob* caused decreased resistance to pine oil formulation only in the mutant AP1 (Table 2). However, mutant AP1 did not overexpress *soxRS* (data not shown; the probe

* Corresponding author. Mailing address: Department of Molecular Biology and Microbiology, Tufts University School of Medicine, 136 Harrison Ave., Boston, MA 02111. Phone: (617) 636-6764. Fax: (617) 636-0458. E-mail: LMcMurry_tet@opal.tufts.edu.

TABLE 1. Susceptibility of pine oil formulation-pine oil and Mar mutants to pine oil formulation and to antibiotics[a]

Strain (reference)	Characteristic(s)	Susceptibility							
		By discs (diam of clearing [mm])			By gradient plates (MIC)[b]				
						μg/ml			
		AP	CM	TC	PS (% by vol)	AP	CM	NAL	TC
WEC derivatives									
WEC	Wild type	22	27	21					
NP3.5GP	Mutant of WEC selected on PS gradient (0–1.5%)	12	11	14					
AG100 derivatives									
AG100	Wild type				0.9	<1.2	2.6	1.7	1.8
AP1	Mutant of AG100 selected by pine oil on disc				>3.6	3.0	7.8	9.7	2.4
AP5	Mutant of AG100 selected as for AP1				>2.9	7.2	21	7.5	4.5
APS3	Mutant of AG100 selected on PS gradient (0–1.5%)				1.8	7.7	>35	8.6	5.3
AG102 (9)	Mar mutant of AG100, selected on TC (two steps)				>4.1	8.5	>35	14.0	>12.8
HH180 (6)	Deletion of 39 kb including *mar* locus; has *zdd-230*::Tn*9* (Cm^r); in host strain MM294				0.3	<0.6	ND[c]	<1.8	<0.6
HH188 (6)	HH180 containing pHHM183 (mar^+)				0.9	<1.0	ND[c]	3.7	1.2
HH191 (6)	HH180 containing pHHM191 (*marR2*)				2.3	5.4	ND[c]	9.1	8.2
HH193 (6)	HH180 containing pHHM193 (*marR5*) 28[d]				3.2	5.9	ND[c]	10.9	>11.4

[a] Abbreviations: PS, pine oil formulation; AP, ampicillin; CM, chloramphenicol; NAL, nalidixic acid; TC, tetracycline.
[b] Gradient plate values were the averages of two to four experiments, except in the case of chloramphenicol, which involved a single determination.
[c] Host strain is Cm^r due to Tn*9*, so values were not determined (ND).
[d] Reference.

was a 432-bp *Eco*RI-*Hin*dIII fragment from pSXS [1], and the constitutive overexpressing strain JTG1078 [11] was a positive control).

Role of the *acrAB* locus. The *acrAB* locus, positively regulated by MarA (16) and SoxS and RobA (15), specfies a proton-motive-force-dependent multidrug efflux pump for a wide variety of mostly lipophilic substances (16, 20, 21, 24). Mar mutants and wild-type strains with this locus deleted become equally hypersusceptible to antibiotics (22), suggesting that the *acrAB* pump confers an intrinsic resistance level which is then enhanced in Mar mutants. We mutated the *acrAB* locus in wild-type and mutant strains by P1 transduction, with strain JZM120 (bearing a Kan cassette replacing most of *acrA* and half of *acrB* [16, 22]) as the donor. Deletion of the *acrAB* locus produced a dramatic increase in the susceptibility to pine oil formulation in all strains (Table 2). Since AP1 was affected by inactivation at *mar*, *sox*, *rob*, or *acr*, yet did not overexpress *sox* or *mar*, this mutant may have a mutation in *rob*; alternatively, it may have a mutation in *acrR* (leading to overexpression of *acrAB* [22]) that requires that the wild-type *mar*, *sox*, and *rob* loci be intact for the full resistance phenotype.

Extracted from pine tree wood, pine oil is a clear fragrant liquid relatively insoluble in water; its major ingredients are alpha-terpineol (65%), methyl chavicol (10%), borneol (9%), fenchyl alcohol (8%), and menthanols (4%) (5). All but chavicol are uncharged nonaromatic cyclic hydrocarbons. Our re-

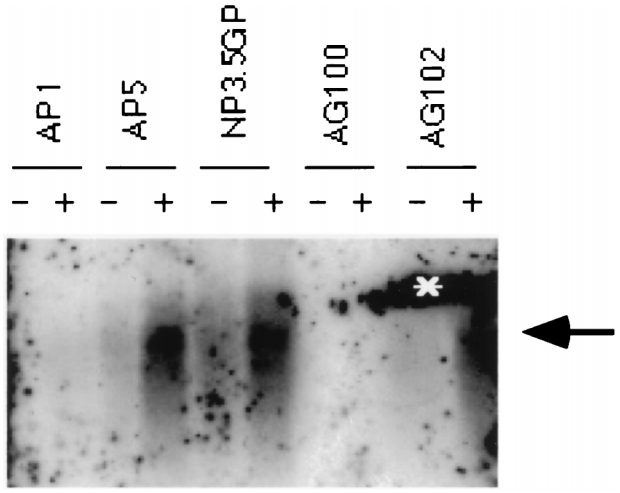

FIG. 1. Northern blot analysis of *marRAB* mRNA. Strains, grown at 30°C in the absence (−) or presence (+) of 5 mM sodium salicylate, were lysed in 3.4% sodium dodecyl sulfate–50 mM Tris-HCl–50 mM Na EDTA, pH 8. RNA was prepared by a CsCl method as described elsewhere (4), except that no guanidine was used and an acid phenol-chloroform step was added prior to the alcohol precipitation. After gel electrophoresis on 1.5% agarose and blotting (10) onto a Nytran Plus membrane (Schleicher and Schuell), the blot was incubated with an [α-^{32}P]dCTP-labeled 387-bp PCR *marA* probe. The hybridization signal was visualized by a PhosphorImager and processed by a linear contrast setting with ImageQuant (both from Molecular Dynamics). AP1, AP5, and NP3.5GP are pine oil formulation-pine oil mutants. AG100 is wild type. AG102 is a Mar mutant. The arrow indicates the *marRAB* transcript. A white asterisk marks an artifactual spot covering both AG102 lanes.

TABLE 2. Effect of inactivation of *mar*, *sox*, *rob*, or *acr* locus upon susceptibility to pine oil formulation

Strain	Relative MIC of pine oil formulation[a]			
	mar[b]	*sox*[b]	*rob*[b]	*acr*[b]
AG100	1.0	0.9	0.8	**<0.06**
AP1	**0.5**	**<0.6**	**0.5**	**<0.02**
AP5	**0.4**	0.9	0.8	**<0.02**
APS3	**0.4**	1.0	1.0	**<0.04**
AG102	**0.4**	1.0	1.0	**<0.03**

[a] Relative MIC is the MIC for the inactivated strain divided by the MIC for the strain before inactivation. Values in boldface indicate notable increases in susceptibility. Values obtained from both gradient plate and agar dilution experiments were averaged.
[b] Inactivated locus.

sults suggest that the AcrAB pump can export one or more of these pine oil constituents.

Pine oil formulation-pine oil and Mar mutants showed no resistance to household disinfectants containing hydrogen peroxide, hypochlorite, alkyl dimethyl benzyl ammonium chloride (a quaternary amine), or chloroxylenol (a phenol) as their active ingredients (data not shown). However, deletion of *acrAB* (but not of *mar*) caused a more than 10-fold increase in the susceptibility of strains to the products containing the quaternary amine or chloroxylenol (data not shown), suggesting that AcrAB was also involved in efflux of those two disinfectants.

Concluding remarks. Plasmid-mediated resistance to antiseptic and disinfectant compounds is common among strains of *Staphylococcus aureus* (14, 29) and can be mediated by transporters such as QacA (23, 27) or Smr-QacC (13) which cause efflux of a variety of lipophilic cations. Antibiotic resistance determinants are also carried on these plasmids (14). In gram-negative bacteria, plasmid-borne integrons carry the *qacE* gene, which specifies a pattern of resistance to different disinfectants that is similar to that of *smr* (25). The integrons also contain antibiotic resistance cassettes (25).

To our knowledge, the selection of chromosomal antibiotic resistance, albeit low level, by a disinfectant has not previously been reported for gram-negative bacteria. Whether pine oil in products meant for household use could lead to a significant problem of antibiotic resistance is not known. However, given the catholic capabilities of pumps such as AcrAB, it seems possible that additional disinfectants might be capable of selecting for resistance to antibiotics and vice versa.

This work was supported in part by U.S. Public Health Service grant no. GM51661.

We thank Hiroshi Nikaido for strains AG100A, AG100B, and JZM120; Bruce Demple for strains DJ901 and RA4468 and plasmid pSXS; and the White Cap Co. for pure pine oil. We are very grateful to Margret Oethinger for performing the mRNA experiments. For counsel and material assistance, M.C.M. thanks Karen J. Shaw (Schering-Plough Research Institute) and Matthew Toth (Novartis [formerly Ciba-Geigy]).

REFERENCES

1. **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.
2. **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.
3. **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.
4. **Ausubel, F. M., R. Brent, R. E. Kingston, et al. (ed.).** 1996. Current protocols in molecular biology, vol. 1, p. 4.2.3–4.2.5. John Wiley & Sons, New York, N.Y.
5. **Claus, E. P., V. E. Tyler, and L. R. Brady.** 1970. Pharmacognosy. Lea and Febiger, Philadelphia, Pa.
6. **Cohen, S. P., H. Hächler, and S. B. Levy.** 1993. Genetic and functional analysis of the multiple antibiotic resistance (*mar*) locus in *Escherichia coli*. J. Bacteriol. **175:**1484–1492.
7. **Cohen, S. P., S. B. Levy, J. Foulds, and J. L. Rosner.** 1993. Salicylate induction of antibiotic resistance in *Escherichia coli*: activation of the *mar* operon and a *mar*-independent pathway. J. Bacteriol. **175:**7856–7862.
8. **Curiale, M. S., and S. B. Levy.** 1982. Two complementation groups mediate tetracycline resistance determined by Tn*10*. J. Bacteriol. **151:**209–215.
9. **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.
10. **Goda, S. K.** 1995. A simple procedure for gel electrophoresis and Northern blotting of RNA. Nucleic Acids Res. **23:**3357–3358.
11. **Greenberg, J. T., J. H. Chou, P. A. Monach, and B. Demple.** 1991. Activation of oxidative stress genes by mutations at the *soxQ1*/*cfxB1*/*marA* locus of *Escherichia coli*. J. Bacteriol. **173:**4433–4439.
12. **Greenberg, J. T., P. Monach, J. Chou, P. D. Josephy, and B. Demple.** 1990. Positive control of a multilevel antioxidant defense regulon activated by superoxide-generating agents in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **87:**6181–6185.
13. **Grinius, L. L., and E. B. Goldberg.** 1994. Bacterial multidrug resistance is due to a single membrane protein which functions as a drug pump. J. Biol. Chem. **47:**29998–30004.
14. **Lyon, B. R., and R. Skurray.** 1987. Antimicrobial resistance of *Staphylococcus aureus*: genetic basis. Microbiol. Rev. **51:**88–134.
15. **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.
16. **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.
17. **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.
18. **Martin, R. G., K.-W. Jair, R. E. Wolf, Jr., and J. L. Rosner.** 1996. Autoactivation of the *marRAB* multiple antibiotic resistance operon by the MarA transcriptional activator in *Escherichia coli*. J. Bacteriol. **178:**2216–2223.
19. **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.
20. **Nikaido, H.** 1994. Prevention of drug access to bacterial targets: permeability barriers and active efflux. Science **264:**382–387.
21. **Nikaido, H.** 1996. Multidrug efflux pumps of gram-negative bacteria. J. Bacteriol. **178:**5853–5859.
22. **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.
23. **Paulsen, I. T., M. H. Brown, T. G. Littlejohn, B. A. Mitchell, and R. A. Skurray.** 1996. Multidrug resistance proteins QacA and QacB from *Staphylococcus aureus*: membrane topology and identification of residues involved in substrate specificity. Proc. Natl. Acad. Sci. USA **93:**3630–3635.
24. **Paulsen, I. T., M. H. Brown, and R. A. Skurray.** 1996. Proton-dependent multidrug efflux systems. Microbiol. Rev. **60:**575–608.
25. **Paulsen, I. T., T. G. Littlejohn, P. Radström, L. Sundström, O. Sköld, G. Swedberg, and R. A. Skurray.** 1993. The 3′ conserved segment of integrons contains a gene associated with multidrug resistance to antiseptics and disinfectants. Antimicrob. Agents Chemother. **37:**761–768.
26. **Provence, D. L., and R. I. Curtiss.** 1994. Gene transfer in gram-negative bacteria, p. 317–347. *In* P. Gerhardt, R. G. E. Murray, W. A. Wood, and N. R. Krieg (ed.), Methods for general and molecular bacteriology. American Society for Microbiology, Washington, D.C.
27. **Rouch, D. A., D. D. Cram, D. DiBerardino, T. G. Littlejohn, and R. A. Skurray.** 1990. Efflux-mediated antiseptic resistance gene *qacA* from *Staphylococcus aureus*: common ancestry with tetracycline- and sugar-transport proteins. Mol. Microbiol. **4:**2051–2062.
28. **Seoane, A. S., and S. B. Levy.** 1995. Characterization of MarR, the repressor of the multiple antibiotic resistance (*mar*) operon of *Escherichia coli*. J. Bacteriol. **177:**3414–3419.
29. **Tennant, J. M., B. L. Lyon, M. T. Gillespie, J. M. May, and R. A. Skurray.** 1985. Cloning and expression of *Staphylococcus aureus* plasmid-mediated quaternary ammonium resistance in *Escherichia coli*. Antimicrob. Agents Chemother. **27:**79–83.