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Salicylate Induction of Antibiotic Resistance in *Escherichia coli*: Activation of the *mar* Operon and a *mar*-Independent Pathway

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Since the growth of wild-type *Escherichia coli* in salicylate results in a multiple antibiotic resistance phenotype similar to that of constitutive mutants (Mar) of the chromosomal *mar* locus, the effect of salicylate on the expression of the *marRAB* operon was investigated. The amount of RNA hybridizing with a *mar*-specific DNA probe was 5 to 10 times higher in wild-type cells grown with sodium salicylate (5.0 mM) than in untreated controls. Untreated Mar mutants had three to five times more *mar*-specific RNA than wild-type cells did. When a Mar mutant was treated with salicylate, a 30- to 50-fold increase of *mar*-specific RNA was seen. In wild-type cells bearing a *mar* promoter-*lacZ* fusion on the chromosome, salicylate increased β-galactosidase activity by sixfold. Thus, salicylate induces transcription of the *marRAB* operon. Other inducers of phenotypic multiple antibiotic resistance, e.g., benzoate, salicyl alcohol, and acetaminophen, but not acetate, also increased transcription from the *mar* promoter but to a lesser extent than did salicylate. Both in wild-type and *mar*-deficient strains, growth in salicylate resulted in increased antibiotic resistance, decreased permeation of the outer membrane to cephaloridine, increased *micF* transcription, and decreased amounts of OmpF. However, the magnitude of these changes was generally greater in wild-type (*mar*-containing) cells. Thus, salicylate and other compounds can induce transcription of the *mar* operon and, presumably, give rise to multiple antibiotic resistance via this pathway. However, salicylate can also activate an unidentified, *mar*-independent pathway(s) which engenders multiple antibiotic resistance.

Multiple-antibiotic-resistant (Mar) mutants of *Escherichia coli* can be selected on agar containing low levels of tetracycline, chloramphenicol, or other antibiotics (4, 11). These mutants are resistant to a variety of other structurally unrelated antimicrobial agents, including penicillins, cephalosporins, rifampin, puromycin, nalidixic acid (4, 11), and fluoroquinolones (3). Among other changes, Mar mutants have decreased amounts of OmpF (4), a major porin that allows permeation of certain antibiotics through the outer membrane. This change is linked to increased expression of *micF* RNA in the mutants, which leads to instability of the *ompF* mRNA (4).

The *mar* locus at 34 min (1,636 kbp) on the *E. coli* chromosome map has recently been cloned and sequenced, and its regulation has been studied (2, 14). A regulatory region called *marO* contains a promoter for rightward transcription of the *marRAB* operon (Fig. 1). The putative proteins specified by these genes are MarR, a repressor (2); MarA, a protein whose overexpression leads to multidrug resistance (10a, 12, 27); and a small protein designated MarB which is required for the full resistance phenotype but whose function is yet unknown (2, 27). The *marRAB* operon appears to mediate intrinsic susceptibility of *E. coli* to multiple antibiotics (27), and its transcription is inducible by tetracycline and chloramphenicol (2, 14). Mar mutants have increased transcription of mRNA from the *marRAB* operon as a result of mutations either in the regulatory region *marO* or in *marR* (2). A second promoter present in the *marO* region is responsible for leftward transcription of sequences potentially encoding proteins of 64 and 157 amino acids of unknown function (Fig. 1).

Phenotypic antibiotic resistance (PAR) can be induced in *E. coli* by salicylates and other, related compounds (22). Resistance to chloramphenicol, tetracycline, ampicillin, cephalosporins, and nalidixic acid is expressed when 1 to 5 mM salicylate is present in the growth media. *E. coli* grown in salicylates shows decreased β-lactam antibiotic permeation across the outer membrane (9) associated with increased expression of *micF* and reduced amounts of OmpF porin (24, 26). The resemblance between this reversible resistance phenotype and the fixed resistance phenotype of Mar mutants led us to investigate the possibility of a link between the two antibiotic resistance phenotypes.

In this report, we show that salicylate stimulates expression of the *marRAB* operon, which suggests a role for the *mar* locus in PAR. However, since salicylate leads to increased drug resistance even in *mar*-inactivated strains, salicylate must also be able to induce drug resistance through a *mar*-independent pathway.

MATERIALS AND METHODS

Bacterial strains, plasmids, and bacteriophages. *E. coli* strains used in this study are listed in Table 1. Plasmid pMLB1109 and λRZ5 were obtained from M. Berman and R. Zagursky via A. Wright and C. Turnbough, respectively. Plasmids pHP7.8WT and pHP7.8Mar, containing wild-type and mutant chromosomal *mar* regions, have been described elsewhere (14). To make strains N7603 and N7606, pmicB21 (20a) was isolated from strain SB221 (obtained from Steven Forst) and used to transform AG100 and CH164 to ampicillin resistance.

Media and chemicals. LB broth contained the following per liter: 5 g of tryptone (Difco Laboratories, Detroit, Mich.), 5 g of yeast extract (Difco), and 5 g of NaCl (pH 7.4). LB agar

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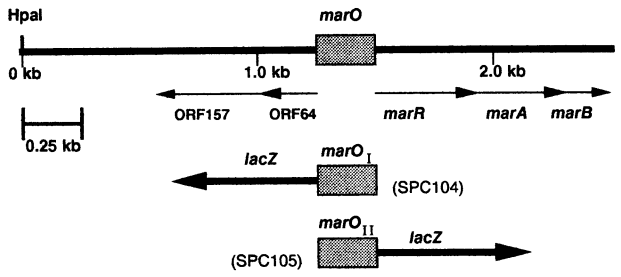

FIG. 1. Schematic of the *mar* locus demonstrating *marO* and transcriptional units from promoter I (ORF64 and ORF157) and promoter II (*marRAB*). The direction of transcription from the two promoters to the *lacZ* fusions in *marO* is shown.

contained 15 g of Bacto Agar (Difco) per liter of LB broth, and LB top agar contained 0.8% agar. In some studies, LB contained 10 g of tryptone per ml (designated LB-10). All antibiotics and salicylates were obtained from Sigma Chemical Co. (St. Louis, Mo.) and used as described previously (22). TMG buffer contained 10 mM Tris, 10 mM $MgSO_4$, and 0.01% gelatin (pH 7.4). Restriction endonucleases and T4 DNA ligase were obtained from New England Biolabs (Beverly, Mass.) and used according to the manufacturer's recommendations.

Measurement of antibiotic susceptibility. Antibiotic susceptibility was measured by several techniques. Gradient plates (3, 14) were used to estimate the MICs of various antibiotics for several strains in the presence or absence of 5 mM sodium salicylate. Agar double-diffusion tests were carried out as described previously (1, 9). The efficiency of plating of various strains on antibiotics, with or without salicylate or salicyl alcohol, was determined as follows. Cultures grown overnight (30°C) in LB-10 broth were diluted in TMG buffer and plated in LB-10 top agar on appropriately supplemented plates. The plates were incubated at 30°C for 2 to 4 days, and the efficiency of plating was calculated by dividing the number of colonies appearing on test plates by the number appearing on the unsupplemented control plates. The dose that inhibited 50% of colony formation was calculated from these data; the mean of several experiments had a variation of ±20%.

Outer membrane analysis and permeability studies. Outer membrane proteins were prepared and analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) as described previously (24). The permeation of the β-lactam cephaloridine through the outer membrane was performed by the method of Zimmerman and Rosselet (28) as modified by Nikaido et al. (21).

Construction of *marO-lacZ* chromosomal transcriptional fusions. A 405-bp *Tha*I fragment from the *marO* region (2) which contains potential divergent promoters was ligated into the *Sma*I site of pMLB1109, a *lacZ* transcriptional fusion plasmid. Two of the resulting plasmids, pMLB1109-I and pMLB1109-II, had *lacZ* gene expression under the presumed control of the leftward (I) or the rightward (II) promoter within the regulatory region. The fusion was introduced into the chromosome by first infecting *E. coli* SPC103 (which lacks the *mar* region), bearing one of the fusion plasmids, with λRZ5. Recombination between the *marO-lacZ* region of the high-copy-number fusion plasmids and λRZ5 resulted in a lysate bearing λRZ5(*marO-lacZ*). This was used to infect the plasmidless SPC103, and Amp^r Lac^+ lysogens were selected on LB agar containing ampicillin (50 μg/ml) and 5-bromo-4-chloro-3-indolyl-β-D-galactoside (40 μg/ml). Lysates from these purified lysogens were then used to infect *E. coli* MC4100, and Amp^r Lac^+ lysogens were again isolated. The resulting strains, SPC104 and SPC105, were confirmed to have a single copy of the fusion region located in the same site on the chromosome (likely the *att*λ site) by Southern hybridization of *Pst*I-digested chromosomal DNA from the strains with a 405-bp *Eco*RI-*Bam*HI fragment isolated from pMLB1109-I. DNA from the lysogens of MC4100 migrated in two bands which hybridized with the probe. One was a 9-kb fragment representing the naturally occurring *marO-marRAB* sequence. The other was a larger fragment (>15 kb) representing the integrated *marO-lacZ* fusion phage and contiguous chromosomal sequences.

DNA manipulations and analyses were performed as described by Maniatis et al. (18). The assay for β-galactosidase (20) was performed on cells grown at 30°C to mid-logarithmic phase in LB broth containing various chemicals or antibiotics, added 1 h prior to the assay.

RNA analyses. Freshly grown cells were incubated for 60 min to mid-logarithmic phase at 30°C in LB broth with or without salicylate (5 mM). After collection by centrifugation, RNA was obtained as previously described (14). Briefly, total cellular RNA was prepared by a hot phenol-acetate method. The RNA was subjected to electrophoresis in 1% agarose containing 8.3% formaldehyde and blotted to nylon membranes (GeneScreen Plus; New England Nuclear, Boston,

TABLE 1. *E. coli* K-12 strains

Strain	Genotype or description	Reference or source
AG100	*argE3 thi-3 rpsL xyl mtl supE44* Δ(*gal-uvr*B)	11
AG102	Mar mutant of AG100 (selected on tetracycline [5 μg/ml])	11
AG1025	AG102 *marA*::Tn*5*	12
AG100-Tc2.5-1	Mar mutant of AG100 (selected on tetracycline [2.5 μg/ml])	14
AG100-Tc2.5-1-kan	*mar*A::Tn*5* transduced by P1 from AG1025 to AG100-Tc2.5-1	14
PLK1738	*trpR trpA his-29 ilv pro-2 arg-427 thyA deo tsx rac gyrA zdd-230*::Tn*9* Δ*mar* (deletion of approximately 39 kbp from 33.6–34.3 min including the *mar* locus)	15
CH164	Same as AG100 but Δ*mar*(33.6–34.3) *zdd-230*::Tn*9*	14
MC4100	F⁻ Δ*lacU169 araD rpsL relA thi flbB*	A. Wright
SM3001	MC4100 Δ*micF1*	19
SPC103	MC4100 Δ*mar zdd-230*::Tn*9*	This study
SPC104	MC4100 $marO_I$-*lacZ* promoter I	This study
SPC105	MC4100 $marO_{II}$-*lacZ* promoter II	This study
N7603	AG100/pmicB21	This study
N7606	CH164/pmicB21	This study

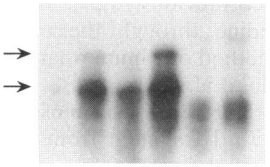

FIG. 2. Effect of salicylate on RNA which hybridized to the *marRAB* probe. RNA was extracted from AG100 (lanes 1 and 2), AG100-Tc2.5-1 (lanes 3 and 4), and AG100-Tc2.5-1-Kan (lanes 5 and 6), grown at 30°C in LB broth for 60 min in the absence (lanes 1, 3, and 5) or presence (lanes 2, 4, and 6) of salicylate (5 mM). The RNA was hybridized by the Northern (RNA) blot technique to a ^{32}P-labeled probe for *marRAB*. The arrows designate the 1.0- and 1.4-kb hybridizing bands.

Mass.). Following transfer and fixation, the RNA blot was probed with a 1.2-kb polymerase chain reaction-generated fragment containing the *marRAB* DNA sequence (2), labeled by the random priming method with [^{32}P]dCTP. Following 2 h of prehybridization in hybridization solution (1% SDS, 1 M sodium chloride, and 10% dextran) at 60°C, denatured ^{32}P-labeled probe was added (10^6 dpm/ml) and allowed to hybridize overnight at 60°C. The membranes were washed as described previously (14) and subjected to autoradiography with Kodak XAR-5 film. The relative intensity of the signal was judged by adjusting the time of exposure to the film or the amount of RNA subjected to electrophoresis.

RESULTS

Effect of salicylate on transcription of the *marRAB* operon. We examined RNA from wild-type, Mar mutant, and *marA*::Tn*5*-inactivated strains grown for 60 min at 30°C in the presence or absence of salicylate (5.0 mM), using the *marRAB* sequence as a probe (Fig. 2). In the absence of salicylate, a prominent 1.0-kb band and a less abundant 1.4-kb RNA which hybridized with the *mar*-specific probe were three- to fivefold increased in the Mar mutant compared with the wild-type as previously found (14). A truncated RNA species was seen in the Tn*5*-inactivated Mar strain. When salicylate was added, the 1.0- and 1.4-kb transcripts were induced 5- to 10-fold in the wild-type strain AG100 and about 30- to 50-fold in the Mar mutant AG100-Tc2.5-1. The transcripts were the same sizes as those observed in Mar mutants exposed to tetracycline or chloramphenicol (14). Similarly, the truncated RNA was also increased by salicylate in the Tn*5*-inactivated strain (Fig. 2).

The induction of *mar*-specific RNA in AG100 by salicylate suggests that PAR induced by exposure of cells to salicylate could occur via transcriptional activation of the *marRAB* operon. The greatly increased level of *mar*-specific RNA in AG100-Tc2.5-1 compared with AG100 when both are grown in the presence of salicylate mimics the increase in *marRAB* transcription caused by other inducers, e.g., tetracycline and chloramphenicol (14).

Effect of salicylates and other compounds on $marO_{II}$-*lacZ* transcriptional fusions. The nucleotide sequence of the *mar* region has revealed a regulatory region (*marO*) with potential divergent promoters (I and II) for initiating the leftward transcription of ORF64 and ORF157 and the rightward transcription of the *marRAB* operon (2) (Fig. 1). Transcriptional fusions of each of these wild-type promoters to *lacZ* were constructed and introduced into the chromosome so that β-galactosidase activity could be used to monitor the effects of salicylate on transcription (Materials and Methods). Both fusions showed about 100-fold more expression of β-galactosidase activity than did the parent cells without either fusion. Fusions in which expression of the *lacZ* gene was under the control of promoter I (SPC104) showed no significant change from baseline activity when treated with salicylate (data not shown). In contrast, transcription of the *marRAB* operon initiated from promoter II in SPC105 was activated by salicylate more than sixfold (Table 2).

TABLE 2. Effects of salicylates and other compounds on induction of β-galactosidase in SPC105[a]

Compound	Concn (mM)	Induction ratio[b]
None (control)		1.0
Sodium salicylate	2.5	6.3
	5.0	7.1
	10.0	5.7
Acetyl salicylate	2.5	2.4
	5.0	4.4
	10.0	3.1
Sodium benzoate	2.5	1.6
	5.0	2.3
	10.0	3.2
Salicyl alcohol	10	1.7
	15	2.2
	20	2.5
Sodium acetate	25	0.9
	50	0.9
	100	0.9
Cinnamate[c]	2.5	2.1
	5.0	2.9
Acetaminophen[c]	2.5	1.7
	5.0	3.2
	10.0	4.9
2,4-Dinitrophenol	.25	6.3
	.5	9.1
	1.0	5.0

[a] Cells grown logarithmically in LB-10 broth at 30°C were treated with the indicated compounds for 1 h prior to assay for β-galactosidase activity.

[b] The amount of activity with compound was compared to that without compound (237 Miller units), and the fold change was recorded. Except for cinnamate, each compound was tested at least three times, and the standard deviation ranged from 2 to 39%, with a median of 8% and an average of 13%.

[c] Tested in ethanol (≤1%). Induction ratio was corrected for slight induction by ethanol (1.2 at 1%).

The threshold level of salicylate inducibility of the *marRAB* promoter was examined by incubation of SPC105 with different amounts of salicylate. Concentrations of salicylate from 0.01 to 0.1 mM showed little, if any, induction of the *mar* promoter; concentrations of 0.5 to 5 mM gave marked induction (Fig. 3). The finding that concentrations of 0.5 mM or greater are required for salicylate induction of the *marRAB* operon correlates well with the previous findings that salicylate concentrations below 0.5 mM failed to induce substantial PAR (22). Sodium benzoate, acetaminophen, and acetyl salicylate also significantly induced β-galactosidase in SPC105 (Table 2). All of these compounds induce PAR (9, 22, 25). However, on the basis of concentration, sodium salicylate was the best inducer among these related compounds (Table 2).

Other compounds, including salicyl alcohol (saligenin) (Table 2; see Fig. 5) and sodium acetate (22), have been associated with induction of PAR. However, salicyl alcohol at 15 or 20 mM (the concentration needed to produce PAR) showed only a twofold induction of LacZ activity in SPC105. Sodium acetate at 100 mM showed no induction (Table 2). This latter compound is presumably expressing PAR via another pathway. Cinnamate, a precursor of salicylate in plants and a strong

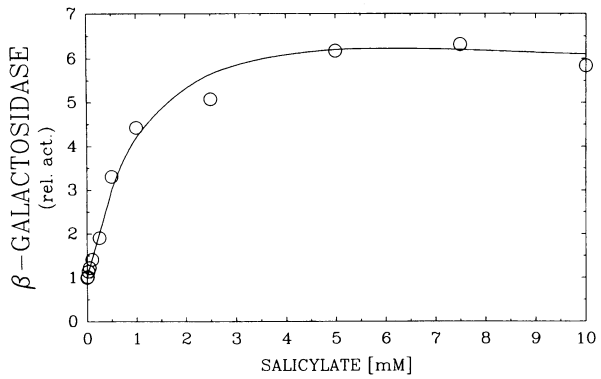

FIG. 3. Effects of different concentrations of salicylate on β-galactosidase activity from the $marO_{11}$-*lacZ* fusion in SPC105.

inducer of cadmium toxicity (23), also showed induction to about the same extent as benzoate. Because of the depolarizing effect of some of the compounds, dinitrophenol was tried. At relatively lower amounts, it proved to be a strong inducer.

Effect of salicylates on OmpF expression and function in *mar*-deleted or *mar*-inactivated strains. The outer membranes of salicylate-treated cells have decreased amounts of OmpF porin (24, 26) and decreased permeability to β-lactam drugs (24). In both salicylate-treated cells (24) and *mar* constitutive mutants (4), these events are mediated, at least in part, by increased transcription of *micF*, which interferes with OmpF translation.

The requirement of the *mar* locus for these effects of salicylate was examined in *mar*-deficient or *mar*-deleted strains. As was previously shown (24), growth in salicylate substantially reduced the amount of OmpF in the outer membranes of wild-type strains (Fig. 4). Similarly, growth in salicylate severely reduced the amount of OmpF present in the Δ39-kb *mar* deletion strain, CH164, and in the *marA*::Tn*5* strain, AG100-Tc2.5-1-kan (data not shown). Thus, the effect of salicylate on OmpF did not require the *mar* locus. As expected (4), OmpF was undetectable in the *marR* constitutive strain, AG102, with or without salicylate (data not shown).

Likewise, the *mar* locus was not essential for salicylate-dependent reduction of outer membrane permeation of β-lactams (Table 3). Salicylate induced an eightfold decrease in permeation of cephaloridine in the wild-type *E. coli* AG100.

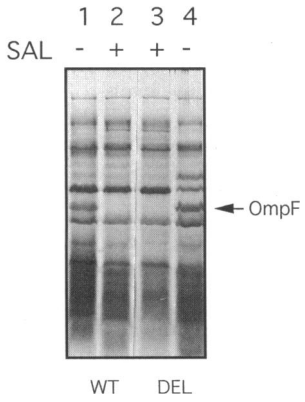

FIG. 4. SDS-PAGE analysis of outer membrane protein preparations from wild-type (WT) and *mar*-deleted (DEL) strains. The position of OmpF is designated. SAL, salicylate.

TABLE 3. Effect of salicylate on cephaloridine permeation of the outer membrane

Strain[a]	Permeation (cm s^{-1}, 10^{-4})[b]		Ratio, −salicylate/+salicylate
	−Salicylate	+Salicylate	
AG100 (WT)	2.03 (0.06)	0.25 (0.01)	8.1
AG100-Tc2.5-1 (Mar)	0.36 (0.02)	0.18 (0.01)	2.0
AG100-Tc2.5-1-kan (*marA*::Tn*5*)	2.02 (0.03)	0.37 (0.01)	5.4
CH164 (Δ*mar*)	2.28 (0.06)	1.03 (0.05)	2.2

[a] All strains carry plasmid pBR322. WT, wild type; Mar, Mar mutant; *marA*::Tn*5*, Tn*5* inactivation of *marAB*; Δ*mar*, 39-kb deletion including all of the *mar* locus.

[b] Measured on cells grown in LB-10 at 30°C as described in Materials and Methods. Each value is the average of five determinations; the standard deviation is indicated in parentheses.

The Mar mutant AG100-Tc2.5-1 (which does not produce OmpF) showed sevenfold-reduced β-lactam permeation compared with AG100. Salicylate induced a further twofold reduction in β-lactam permeation in this strain (Table 3). In the *marA*::Tn*5* strain, permeation was like that in AG100 and was decreased fivefold by salicylate to levels which were a third less than the salicylate effect on the wild-type strain. In the *mar*-deleted strain, CH164, β-lactam permeation was about the same as in wild-type AG100. Although OmpF was diminished by growth in salicylate (Fig. 3), this strain showed only a twofold reduced permeation to cephaloridine, in contrast to the eightfold decrease in the wild-type cells grown in salicylate. This latter finding, along with the data from the *marA*::Tn*5* strain, suggest that permeation is affected by *mar* and by genes located within the 39-kb deletion near *mar* which are independent of OmpF. These data further suggest that decreased β-lactam permeation in Mar mutants reflects changes in addition to reduction in OmpF. The latter conclusion was also made in terms of quinolone resistance in Mar mutants, in which only 25% of resistance could be attributed to the OmpF loss (3).

Increased *micF* transcription has been correlated with decreased translation of OmpF in Mar mutants (4) and in wild-type cells treated with salicylate (24). The role of *mar* in the effect of salicylate on *micF* transcription was examined by comparing isogenic wild-type (N7603) and *mar*-deleted strains (N7606). Baseline *micF* expression was about the same in both strains. Salicylate increased the activity of β-galactosidase from the *micF* promoter in the wild type by sevenfold; however, in the *mar*-deleted strain, the increase was reproducibly only two-thirds that of the wild-type strain (Table 4). Thus, some of

TABLE 4. Expression of β-galactosidase activity in wild-type and *mar*-deleted *micF-lacZ* containing strains[a]

Strain	β-Galactosidase activity (Miller units)[b]		
	No supplement	5 mM salicylate	20 mM salicyl alcohol
N7603 (WT)[c]	53 (1.0)	369 (7.0)	230 (4.3)
N7606 (Δ*mar*)	60 (1.0)	278 (4.6)	227 (3.8)

[a] Cells were grown overnight at 30°C in LB-10 medium with the indicated supplement, diluted and grown for four to five generations in the same medium, harvested, and assayed for β-galactosidase. Results of a representative experiment are shown.

[b] The activity of the supplemented culture divided by that of the untreated culture is shown in parentheses.

[c] In the absence of the *micF-lacZ*-bearing plasmid, the expression of the wild-type (WT) *lacZ* gene was 4 to 6 U and was not significantly affected by 5 mM salicylate or 20 mM salicyl alcohol.

TABLE 5. MICs in the absence and presence of salicylate

Strain	MIC (μg/ml)[a]								
	Tetracycline			Chloramphenicol			Ampicillin		
	−Sal	+Sal	Ratio, +Sal/−Sal	−Sal	+Sal	Ratio, +Sal/−Sal	−Sal	+Sal	Ratio, +Sal/−Sal
AG100 (WT)	3.1	7.9	2.5	5.3	18.1	3.4	2.2	4.1	1.9
AG100-Tc2.5-1 (Mar)	8.3	11.9	1.4	25	31	1.2	10.7	9.3	0.9
AG100-Tc2.5-kan (*marA*::Tn*5*)	1.6	3.6	2.3	2.9	9.6	3.3	1.9	3.6	1.9
CH164 (Δ*mar*)	1.7	4.0	2.4	NT[b]	NT		1.1	2.2	2.0

[a] Determined on LB agar gradient plates with or without the addition of 5 mM sodium salicylate (Sal). Fresh 30°C LB-grown mid-log-phase cultures, diluted to an optical density at 530 of 0.2 with phosphate-buffered saline, were applied to the plates with a cotton swab. Results are averages of four replicas.
[b] NT, not tested since the strain contains Tn*9*, rendering it highly Cm^r.

the effect of salicylate on *micF* is attributable to the *mar* locus or the region near it. Nevertheless, the *mar* locus is not absolutely required for increased *micF* transcription in response to salicylates. Salicyl alcohol, at the concentration needed to induce PAR, increased β-galactosidase activity in the wild-type strain and *mar*-deleted strain to about the same extent: by 4.3- and 3.8-fold. Thus, very little, if any, effect of salicyl alcohol on *micF* transcription depends on the *mar* locus.

Effects of salicylate on antibiotic susceptibility of wild-type, Mar, and *mar*-deficient strains. To examine more closely the role of *mar* in PAR, we measured susceptibility to tetracycline, chloramphenicol, and ampicillin by a gradient plate method. The strain deleted for all of the *mar* region was considerably more susceptible to tetracycline and ampicillin (chloramphenicol could not be tested in CH164) (Table 5). The strain bearing Tn*5* in *marA* was also more susceptible to tetracycline and to chloramphenicol. However, there was less change in ampicillin susceptibility. The latter finding implies that other genes within the 39-kb deletion decrease ampicillin susceptibility.

In the presence of salicylate, the wild-type, Δ*mar*, and Tn*5*-inactivated strains all showed an increase in resistance (decreased susceptibility) to the antibiotics (chloramphenicol could not be tested in CH164). Of interest, the fold increase caused by salicylate was similar for the wild-type and *mar*-deficient strains, although the level of resistance attained was lower in the *mar*-deficient strains. In contrast, salicylate had only a minimal effect on the Mar strain.

While salicylate and salicyl alcohol are closely related compounds, a much greater concentration of the latter (15 to 20 mM) was needed to induce PAR (data not shown) and *marRAB* than of the former (5 mM or less) (Table 2). Furthermore, the effect of salicyl alcohol on *micF* transcription appeared to be independent of *mar* (Table 4). We therefore examined the effects of both compounds on susceptibility to other antibiotics, namely, the quinolones nalidixic acid (Fig. 5A) and norfloxacin (Fig. 5B), in the wild-type, Mar mutant, and *mar*-deficient strains. Both salicyl alcohol and salicylate decreased susceptibility to nalidixic acid, even in the *mar*-deficient strains. However, the extent of the effects with salicyl alcohol was not as great as that seen with salicylate. Resistance to norfloxacin was also increased by salicylate, and to a lesser extent by salicyl alcohol, even in the *mar*-deficient strains. In contrast to the findings with ampicillin, tetracycline, and chloramphenicol (Table 5), salicylate also increased resistance to the quinolone antibiotics in Mar mutants. While the *mar* locus provided higher levels of salicylate-induced resistance to both drugs in the Mar strain, there was little difference in susceptibility to norfloxacin between induced wild-type and *marA*::Tn*5* strains (Fig. 5). In the *mar*-deletion strain, however, intrinsic and induced resistance was much lower than in the wild-type strain, suggesting that, as with ampicillin susceptibility, other genes within the 39-kb deletion affect cellular susceptibility to quinolones. Thus, it is clear that in the absence of a functional *mar* operon, both salicylate and salicyl alcohol can induce resistance to multiple antibiotics, in particular the quinolone group of antimicrobial agents.

Since the *micF* locus is required for reduced OmpF synthesis in the Mar mutants (4), we compared the plating efficiency and colony sizes of strain MC4100 with its Δ*micF* derivative SM3001 for salicylate induction of tetracycline resistance. Both strains were able to grow on tetracycline (5 μg/ml) only when supplemented with salicylate; however, there was a noticeable decrease in colony size for SM3001 relative to its parent strain (data not shown). Thus, although resistance is inducible in the absence of *micF*, the level of resistance is lower. This result is consistent with previous findings that *micF* promotes, but is not required for, the reduced amounts of OmpF in salicylate-treated cells (24).

It was previously reported that salicylate increased the susceptibility of the wild-type *E. coli* to aminoglycosides such as kanamycin (1). This effect was also seen for the salicylate-treated Mar and *mar*-deletion strains (data not shown). Thus, this effect of salicylate appears to be independent of the *marRAB* operon and DNA within the 39-kb deletion.

DISCUSSION

The similarities between the multiple antibiotic resistance phenotypes that result either from particular mutations in the *marRAB* operon or from growth in salicylates prompted us to investigate the possibility that salicylate enhanced the expression of *mar*. Since previous studies showed that *marRAB* expression is inducible by at least two of the antibiotics to which it mediates resistance, i.e., tetracycline and chloramphenicol (14), it was possible that salicylate also induced the operon, thereby generating the PAR. The present studies show that salicylate increases the levels of *marRAB*-hybridizing mRNA in wild-type bacteria (Fig. 2). Several other chemicals that induce PAR (benzoate, acetaminophen, and acetyl salicylate) were also found to induce *marRAB* transcription in a $marO_{11}$-*lacZ* fusion assay (Table 2). Using this assay, we found that the concentrations of salicylate that significantly induced β-galactosidase synthesis (Table 2) were also those that elicit PAR (22). Furthermore, growth of the Mar mutant AG100-Tc2.5-1 in salicylate resulted in a 30- to 50-fold increase in *marRAB* mRNA (Fig. 2), similar to that found in the Mar mutant upon treatment with tetracycline or chloramphenicol. This evidence clearly shows a mechanistic link between salicylate and the *mar* operon.

To explore further the relationship between *mar* and PAR, the effect of salicylate on antibiotic resistance was examined in

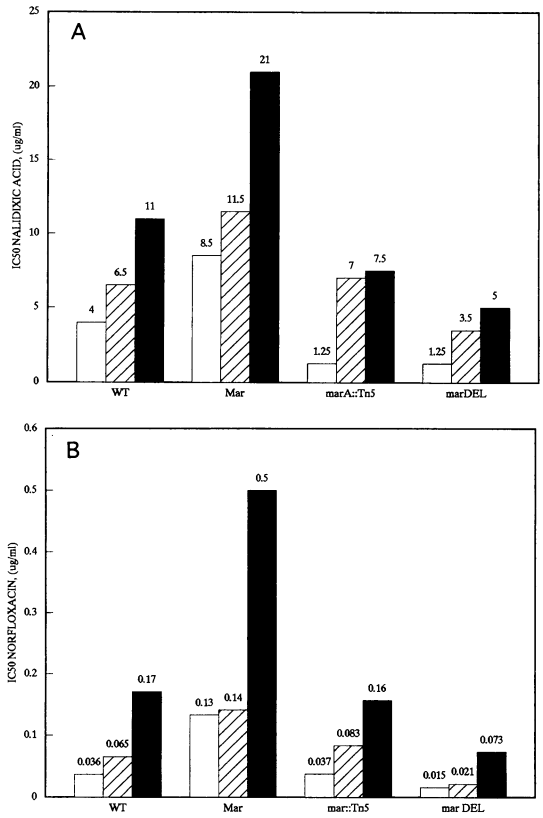

FIG. 5. Effects of 5 mM salicylate or 20 mM salicyl alcohol on the 50% inhibitory concentration (IC_{50}) for nalidixic acid (A) or norfloxacin (B) of wild-type (WT), Mar mutant (AG100-Tc2.5-1), *marA*::Tn*5*, and CH164 (*mar*-deletion [mar DEL]) strains. Open bars, control; cross-hatched bars, salicyl alcohol; solid bars, salicylate.

three *mar* variants: a Mar constitutive mutant; a *mar* mutant in which *marA* is inactivated by a Tn*5* insertion; and a strain in which 39 kb of DNA including the *mar* region has been deleted. If PAR operates exclusively through *mar*, we should not expect induction of PAR in the *mar*-inactivated or *mar*-deleted strains. This was not observed (Table 5; Fig. 5). Thus, salicylate must be able to induce PAR via a *mar*-independent pathway. This pathway, however, has several features in common with the *mar* pathway: *micF* expression is elevated (Table 4), OmpF is severely reduced (Fig. 4), and outer membrane permeation by cephaloridine is reduced (Table 3). Nevertheless, the levels of resistance to some antibiotics induced by salicylate in the absence of functional *mar* (Table 5) are only about half as high as in the presence of *mar*. Moreover, salicylate-induced decreased β-lactam permeation is one-third less in the *mar*-inactivated strain (Table 3).

If, on the other hand, salicylate's induction of multidrug resistance occurred only via a pathway separate from *mar*, one might have expected a similar increase in resistance in Mar mutants as in the wild type. This was not the case. In fact, there was only a minimal increase in resistance to tetracycline, chloramphenicol, and ampicillin in Mar mutants grown in salicylate. The latter finding suggests that once expressed, the *mar* locus provides all of the resistance to these drugs that could be otherwise induced by salicylate. Salicylates presumably produce resistance to these drugs by activating directly or indirectly some of the same genes which are activated by the *marRAB* operon. This was not true of the quinolone family of antimicrobial agents, resistance to which was induced equally well in wild-type and *marA*::Tn*5*-inactivated strains. Resistance to these drugs was, however, greatly increased in salicylate-induced Mar mutants, suggesting that more than one group of genes may be activated.

The existence of a *mar*-independent route to multidrug resistance was also suggested by studies with salicyl alcohol, which was a less effective inducer of transcription of the *marRAB* operon. This compound caused an increase in *micF* to the same extent in cells with or without the *mar* locus. Thus, the twofold increased expression of the *mar* operon caused by salicyl alcohol (Table 2) had little effect on its induction of *micF* expression. Its principal action, therefore, may be through a *mar*-independent pathway. This pathway is likely to be the one used by salicylate in the absence of *mar*, since salicylate-induced *micF-lacZ* expression in the *mar*-deleted strain was the same as that of the wild-type or deleted strains with salicyl alcohol. This finding suggests that at least a third of the effect of salicylate on *micF* expression and β-lactam permeability (Table 3) can be attributed to *mar*. In view of the lower levels of induced resistance in *mar*-deficient strains, the *mar*-independent pathway must lack, either in quantity or in quality, some element(s) present in the *mar*-responsive pathway.

Previous work has shown that the antibiotic resistance of Mar mutants results from a severalfold increase in *marRAB* RNA (14). When such cells were grown with salicylate, the RNA level increased about 30- to 50-fold (Fig. 2), yet the antibiotic resistance of this strain was increased no more than 2.5-fold (Table 5; Fig. 5). This finding suggests that some other component(s) of the antibiotic resistance mechanism regulated by *mar* has become rate limiting.

Although salicylate is a potent inducer of the *marRAB* operon, the operon does not appear to affect resistance to growth-inhibitory concentrations of salicylate itself; no significant difference in resistance was seen among wild-type, *mar*-deficient, and Mar mutant strains (data not shown). Thus, salicylate appears to be a gratuitous inducer of *marRAB*, as it is for other effects on *E. coli*. Both salicylate and salicyl alcohol potentiate susceptibilities to aminoglycosides (1) and to Cd^{2+} (23), the latter effect involving the *cysB* gene (13). However, in the *Pseudomonas* plasmid NAH7, the induction of naphthalene catabolism by salicylate is not gratuitous. Salicylate alters the binding of the *nahR*-encoded transcriptional activator to promoters that are responsible for the degradation of naphthalene and salicylate (16).

DNA homologous to *marRAB* has been found among many members of the family *Enterobacteriaceae*, including *Klebsiella* species (5). Similarly, induction of PAR by salicylate and acetyl salicylate has been commonly observed among 58 clinical enteric isolates tested (10). In *Klebsiella pneumoniae*, *Serratia marcescens*, and *Pseudomonas cepacia*, salicylate decreased the presence of OmpF-like outer membrane porins (1a, 26). Furthermore, in *Klebsiella* species, salicylates increased resistance to various antibiotics (including β-lactams and tetracycline), decreased resistance to aminoglycosides, and decreased the amounts of capsular polysaccharide (6, 7). Thus, it is reasonable to anticipate that *mar*-like loci may be involved in salicylate induction of PAR in many enterobacteria.

In plants, in which salicylate is widely distributed, it can have important effects on thermogenesis, flowering, and defense against viral (17), bacterial, and fungal pathogens (for a review, see reference 8). The antipyretic, anti-inflammatory, and analgesic properties of salicylate in humans have been known since antiquity. It may be anticipated that the insights gained

by studying the potent bioactivities of salicylates in any one of these systems will shed light on its role in the other systems.

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