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Genetic Relationship between *soxRS* and *mar* Loci in Promoting Multiple Antibiotic Resistance in *Escherichia coli*

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Multiple antibiotic resistance in *Escherichia coli* has typically been associated with mutations at the *mar* locus, located at 34 min on the *E. coli* chromosome. A new mutant, *marC*, isolated on the basis of a Mar phenotype but which maps to the *soxRS* (encoding the regulators of the superoxide stress response) locus located at 92 min, is described here. This mutant shares several features with a known constitutive allele of the *soxRS* gene, prompting the conclusion that it is a highly active allele of this gene. The *marC* mutation has thus been given the designation *soxR201*. This new mutant was used to examine the relationship between the *mar* and *sox* loci in promoting antibiotic resistance. The results of these studies indicate that full antibiotic resistance resulting from the *soxR201* mutation is partially dependent on an intact *mar* locus and is associated with an increase in the steady-state level of *mar*-specific mRNA. In addition, paraquat treatment of wild-type cells is shown to increase the level of antibiotic resistance in a dose-dependent manner that requires an intact *soxRS* locus. Conversely, overexpression of MarA from a multicopy plasmid results in weak activation of a superoxide stress response target gene. These findings are consistent with a model in which the regulatory factors encoded by the *marA* and *soxS* genes control the expression of overlapping sets of target genes, with MarA preferentially acting on targets involved with antibiotic resistance and SoxS directed primarily towards components of the superoxide stress response. Furthermore, compounds frequently used to induce the superoxide stress response, including paraquat, menadione, and phenazine methosulfate, differ with respect to the amount of protection provided against them by the antibiotic resistance response.

In the gram-negative bacterium *Escherichia coli*, the intrinsic level of resistance to a variety of antibiotics appears to be affected by at least two unlinked regulatory loci. These loci, which encode the *sox* and *mar* genes, have been identified on the basis of constitutive mutations that increase the level of resistance of this organism to a variety of noxious agents (11, 13, 26). However, most reports describing the roles of individual genes encoded at these loci have focused primarily on the specific substrates for which these systems were originally described: namely, superoxide-generating compounds for the *sox* system and antibiotics for *mar*. Genetic and molecular characterization of these loci has resulted in the identification of putative transcriptional regulatory genes, *soxS* and *marA*, which are thought to modulate the expression of unlinked target genes. The *soxS* and *marA* gene products have significant amino acid sequence similarity, particularly in their candidate DNA recognition domains (1, 5, 9, 27).

Greenberg et al. have provided a bridge between the *sox* and *mar* systems (12). Specifically, these investigators have isolated a mutant, *soxQ1*, that maps to the *mar* locus but confers a phenotype similar to that of a constitutive *soxR* [*soxR*(Con)] allele: i.e., increased resistance to superoxide-generating compounds and elevated expression of target genes involved in the superoxide stress response. Importantly, the activation of the superoxide stress response by *soxQ1* does not require the *soxRS* genes. This mutant also shows increased antibiotic resistance (12). The *soxQ1* mutation has recently been shown to be an allele of the *marR* gene, thought to encode a repressor of the *marRAB* operon (2, 5). *soxR*(Con) mutants also show increased resistance to antibiotics as well as decreased expression of *ompF*, similar to *mar* mutants (3, 13).

While these studies have demonstrated a genetic relatedness between the *soxQ/mar* and *soxRS* loci with respect to the superoxide stress response, the nature of this relationship with respect to antibiotic resistance is not clear. We describe such studies here. This work was prompted by our initial isolation of a *mar*-type mutation that confers multiple antibiotic resistance but which maps to the *soxRS* locus. The relationship between the *sox* and *mar* genes with respect to antibiotic resistance was found to differ from that determined for the superoxide stress response. Our data indicate that antibiotic resistance conferred by the new *soxR*(Con) mutant is partially dependent on an intact *mar* locus and that the steady-state level of *marA* mRNA is elevated in this strain. Comparisons of the new *soxR*(Con) strain with a strain expressing high levels of the *marA* gene product indicate that the *soxRS* system is more effective at activating superoxide stress response target genes, while *marA* is more effective at stimulating antibiotic resistance. In addition, since the *soxRS* system can induce both the superoxide stress response and antibiotic resistance, we investigated the importance of each of these responses in protecting the cell from different superoxide-generating agents.

MATERIALS AND METHODS

Strains and media. The strains used in this study are listed in Table 1. Cells were grown in liquid culture in Luria-Bertani medium at 37°C with shaking. Agar was added to 1.5% to solidify the medium, where needed. The following antibiotics were added to selective media at the given concentrations: ampicillin, 50 μg/ml; chloramphenicol, 20 μg/ml; kanamycin, 50 μg/ml; and tetracycline, 15 μg/ml. The above agents were

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TABLE 1. Bacterial strains, plasmids, and phages

Strain, plasmid, or phage	Relevant genotype or phenotype	Source or reference
E. coli strains		
MC4100	F⁻ *araD139 ΔlacU169 rpsL relA thi*	T. Silhavy
PK1738	*trpR trpA9605am his-29am ilv pro-2 arg-427 thyA deoB* or *deoC tsx gyrA* (Nal^r) Rac⁻ *zdd-230*::Tn*9* Cm^r del1738 (Δ*mar*) *sad*	P. Kuempel (17)
B63	MC4100 *marC*	Nitrosoguanidine mutant
B66	MC4100 *marC* zjc::Tn*10kan*	This study
CGSC6661	*uvrA*::Tn*10*; Tet^r UV^s	B. Bachmann
BW802	MC4100 (λIT1); Ap^r Km^r *nfo'-'lacZ*	B. Weiss (26)
BW999	Δ(*argF-lac*)169 *rpsL sup*(Am) (λJW2); Ap^r Km^r *soxS'-'lacZ*	B. Weiss (28)
B222	B66 (λJW2)	λJW2→B66; lysogen
B223	B66 (λIT1)	λIT1→B66; lysogen
BW847	Δ(*argF-lac*)169 *rpsL sup*(Am) Δ*soxR4*::*cat*; *soxR*(Con) Cm^r	B. Weiss (26)
B244	BW802 Δ*soxR4*::*cat*	P1, BW847 × BW802
B247	MC4100 (λJW2)	λJW2→MC4100; lysogen
B248	B247 Δ*soxR4*::*cat*	P1, BW847 × B247
B246	B244(pIT15); Tet^r	This study
B250	B248(pIT15); Tet^r	This study
B227	B222(pIT15); Tet^r	This study
B229	B223(pIT15); Tet^r	This study
B177	MC4100 *zdd-230*::Tn*9* Cm^r Δ*mar*	9
B185	B177 *marC*; Km^r	P1, B66 × B177
BW829	Δ(*argF-lac*)169 *rpsL sup*(Am) Δ*sox-8*::*cat*; Δ(*soxRS*) Cm^r	B. Weiss (26)
B160	MC4100 Δ*sox-8*::*cat*; Δ(*soxRS*)	P1, BW829 × MC4100
MH621	MH20ϕ(*ompF'-'lacZ*)*hyb 16-21*	T. Silhavy (15)
B194	MH621 *zdd-230*::Tn*9* Cm^r Δ*mar*	9
B108	B66 *uvrA*::Tn*10*; Tet^r UV^s Km^s	P1, CGSC6661 × B66
B205	MH621 *uvrA*::Tn*10 marC*; Tet^r UV^s	P1, B108 × MH621
B209	B205 *zdd-230*::Tn*9* Cm^r Δ*mar*	P1, PK1738 × B205
Plasmids		
p9	Ap^r; pBR322/*marA*; Mar	9
pBR328	Ap^r Tet^r Cm^r	25
p40	Ap^r Cm^r pBR328/*marA*; Mar	This study
pIT15	Tet^r; pBR322/*soxRS*	26
pWB34	Ap^r; pUC18/*soxS*	28
pUC18	Ap^r	29
Phages		
λNK1316	Delivery vehicle for mini-Tn*10kan*	N. Kleckner (18)
λIT1	ϕ(*nfo'-'lacZ*); Ap^r Km^r	26
λJW2	ϕ(*soxS'-'lacZ*); Ap^r Km^r	28

from Sigma, while enoxacin (21) and PD079292 were from in-house syntheses. Stocks of PD079292 (molecular weight = 435.9) were prepared by dissolving the compound in 30% dimethylacetamide–70% water at a final concentration of 100 mM.

Genetic and biochemical procedures. Standard procedures for Hfr mating and P1 transduction were used (20, 23). Strains deleted for the *mar* locus were constructed as described previously (9). In some cases, lambda lysogens were constructed by spotting the supernatant from an overnight culture of a lysogen onto a soft agar overlay containing the recipient strain. Centers of turbid plaques were then picked and purified by streaking on selective media. Plasmid transformations were typically performed by the TSS method (4). Nitrosoguanidine mutagenesis was performed as described previously (20) with a level of mutagen treatment that resulted in ~30% survival. Isolation of a Tn*10kan* insertion that was linked to the antibiotic resistance mutation in strain B63 was by the method of Kleckner et al. (18), with the mini-Tn*10* derivative 103 on the λ1316 phage delivery vehicle. Briefly, a collection of mini-Tn*10kan* insertions was generated on strain B63, and a P1 lysate was prepared on the pooled isolates and used to transduce MC4100 to kanamycin resistance. Transductants were then screened for the multiple antibiotic resistance phenotype. A total of 3 of 400 transductants examined had also acquired the resistance phenotype, and one of these, B66, was selected for further study since it exhibited the greatest linkage between the Tn*10kan* element and the antibiotic resistance marker (~40%).

To test the effects of *marA* overexpression on *nfo-lacZ* fusion expression, it was necessary to transfer *marA* to a plasmid vector containing a selectable marker other than ampicillin resistance, since the phage bearing the *nfo-lacZ* fusion carries the *bla* gene (26). Plasmid p40 was thus constructed by subcloning the *Hin*dIII-*Sph*I fragment from p9 (9) into the same sites in the chloramphenicol-resistant cloning vector pBR328 (25). These sites are located in the flanking vector sequences of p9, resulting in the transfer of *marA* sequences from the pBR322 backbone of p9 to pBR328. p9 and p40 are indistinguishable in their ability to confer a Mar phenotype on recipient cells. p40 was then introduced into strains containing the *nfo-lacZ* fusion by selection on 20 μg of chloramphenicol per ml.

RNA was purified from logarithmic-phase cultures with an

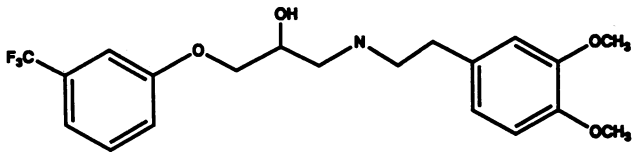

FIG. 1. Chemical structure of PD079292.

RNA Isolation Kit (Stratagene, La Jolla, Calif.), with the protocol modified as follows. A 5-ml culture was poured into a centrifuge tube containing an equal volume of a frozen solution containing 400 μg of chloramphenicol per ml and 100 mM sodium azide and mixed until the frozen material had just melted. The culture was then centrifuged, and the pellet was resuspended in 180 μl of buffer containing 100 mM Tris-HCl (pH 7.5), 100 mM KCl, and 5 mM $MgCl_2$. Twenty microliters of a fresh 3-mg/ml solution of lysozyme was then added, and the cell suspension was then frozen at −20°C, thawed in a 37°C water bath, and mixed with 2 ml of buffer D from the RNA Isolation Kit. RNA purification was completed from this point according to the instructions provided by the manufacturer.

Denaturing agarose gel electrophoresis of RNA samples and Northern (RNA) transfer to Nytran membranes were performed according to protocols from the supplier (Schleicher & Schuell, Keene, N.H.). Samples were loaded in duplicate on gels and transferred to the Nytran membrane, and then the membrane was divided in half. One half of the blot was stained with methylene blue (19) to reveal molecular weight markers and rRNA bands, while the other half was probed for *mar* sequences. The *mar* hybridization probe was the 705-bp *SalI-PvuII* fragment from p9 (9) that contains the 3′ end of *marR*, all of *marA*, and the 5′ end of *marB* (5). Labeling of the fragment, hybridization, and processing of the blot were performed with the Genius I Kit and the LumiPhos 530 chemiluminescent detection reagent, following the manufacturer's instructions (Boehringer Mannheim, Indianapolis, Ind.).

β-Galactosidase assays were performed as described by Miller (20), by the chloroform-sodium dodecyl sulfate method, and with cultures grown to mid-logarithmic phase.

Antibiotic susceptibility testing. Antibiotic gradient plates were prepared and inoculated as described previously (9). MICs were calculated from gradient plate measurements. The validity of this method was confirmed by comparing gradient plate results with measurements from agar dilution tests, performed by diluting saturated overnight cultures of test strains to ~3,000 CFU/ml and spotting 100-μl aliquots of the diluted cultures on agar plates containing different concentrations of antibiotic. The lowest amount of drug that resulted in >90% reduction in colony numbers (compared with no drug controls) was considered the MIC. The MICs differed by <20% between these two methods, and fold differences in susceptibility among strains were the same for both methods.

RESULTS

Isolation of a multiply antibiotic-resistant mutant containing a constitutive allele of *soxR*. This research project evolved from genetic efforts to obtain mutants of *E. coli* that were resistant to an in-house compound with moderate antibacterial activity, PD079292 (Fig. 1). Our standard laboratory strain, MC4100, is susceptible to this agent at concentrations of approximately 300 μM (= MIC of 130 μg/ml). MC4100 cells were mutagenized with nitrosoguanidine, and the survivors were screened for growth on Luria-Bertani agar plates containing 1 mM (= 435 μg/ml) PD079292. A single resistant mutant was isolated. Further testing revealed four- to eightfold increases in MICs for chloramphenicol, tetracycline, ampicillin, and enoxacin, a fluoroquinolone. This mutation did not revert to the parental antibiotic-sensitive phenotype after growth on nonselective media and exhibited a higher level of resistance than typical single-step *mar* mutants (10). It thus seemed possible that this mutation was present in a gene, which we tentatively named *marC*, that was located outside of the *mar* locus. We then chose to use the other antibiotics as test agents in genetic experiments and no longer focused on PD079292.

TABLE 2. Comparison of *marC* with a constitutive allele of *soxR*

Allele	Plasmid	β-Galactosidase (U)[a]		% Growth (MIC)[b]
		nfo-lacZ	*soxS-lacZ*	
Wild type	None	410	180	13 (0.05)
marC	None	6,280	7,370	>90 (0.45)
marC	pIT15	290	1,650	71 (0.28)
soxR4	None	1,320	2,470	25 (0.1)
soxR4	pIT15	480	120	19 (0.08)

[a] Enzyme activity was determined for mid-logarithmic phase cultures and is expressed in Miller units as described previously (16).

[b] Value indicates extent of growth across a linear gradient of 0.0 to 0.4 μg of enoxacin per ml. The number in parentheses indicates the MIC in micrograms per milliliter. (See Materials and Methods.)

Although the resistance phenotypes of the mutant were significantly different from the wild-type parent, difficulties were encountered in directly selecting for the resistance marker in transduction experiments. We were, however, successful in isolating a mini-Tn*10kan* insertion that was linked (~40%) to the *marC* mutation. It thus became possible to construct *marC* strains by transducing recipients to kanamycin resistance and then screening for *marC*-associated chloramphenicol resistance. Backcrosses of the resistance mutation into the MC4100 parent consistently resulted in the same frequency of cotransduction of the resistance marker with the Tn*10kan* element, as well as the same levels of antibiotic resistance as the original isolate, indicating that a single mutation, or very tightly clustered mutations, was involved. Using standard genetic methods (24), we were able to map the mini-Tn*10* element, and consequently the antibiotic resistance mutation, to the 92-min region of the *E. coli* chromosome. More specifically, we observed that the antibiotic resistance phenotype was tightly linked to and downstream from the *uvrA* gene (~90% linkage). This linkage and positioning with respect to *uvrA* were nearly identical to those reported for the *soxR* locus (13, 26), which encodes two genes that govern the stress response induced by superoxide radicals. Importantly, increased chloramphenicol resistance associated with constitutive alleles of *soxR* has been reported (13). We therefore attempted to obtain additional evidence to determine if the *marC* mutation represented a constitutive allele of *soxR*.

Constitutive alleles of *soxR* [*soxR*(Con)] result in increased expression of superoxide-inducible genes in the absence of inducing conditions (13, 26). In addition, this phenotype can be partially suppressed by a multicopy plasmid expressing the wild-type *soxR* gene (26). To determine if the *marC* mutant also displayed these properties, we transferred the mutation into strains containing either superoxide-inducible *nfo-lacZ* or *soxS-lacZ* fusions and measured β-galactosidase levels (Table 2). The *marC* mutation was able to promote an increase in the expression of both fusions, albeit to higher levels, similar to that observed for the *soxR4* allele, a known *soxR*(Con) muta-

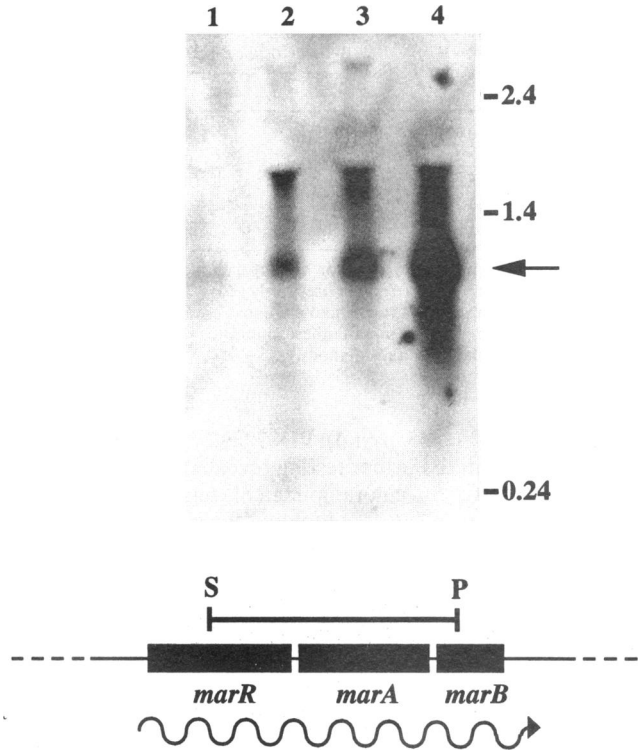

FIG. 2. Northern blot hybridization of *mar*-specific mRNA in wild-type and *sox* strains. An autoradiogram of hybridization signals from wild-type (lane 1), *soxR201* (lane 2), multicopy *soxS* (lane 3), and salicylate-treated wild-type (lane 4) strains is shown at top. Positions and sizes (in kilobases) of RNA markers are shown to the right, as well as the position of the 1.0-kb *mar* transcript (arrow). The structure of the *marRAB* operon is shown below (5), along with the *SalI-PvuII* fragment that was used as a probe (bar).

tion (26). This effect correlated well with the effects on enoxacin resistance. In addition, both the increase in fusion enzyme expression and the increased level of resistance to enoxacin were suppressed by the multicopy *soxRS* plasmid pIT15, consistent with previous observations of *soxR*(Con) mutations (26). On the basis of the identity between our mapping data for *marC* and that of known *soxR* alleles with respect to *uvrA*, and the similar results in the genetic tests shown in Fig. 2, we conclude that the antibiotic resistance mutation isolated here almost certainly represents a highly active, constitutive allele of *soxR*. We have named this allele *soxR201*. It is worth noting that PD079292 does not induce expression of either the *nfo-lacZ* or the *soxS-lacZ* fusion, indicating that it is not an inducer of superoxide radicals.

SoxS is the proximal activator of antibiotic resistance. The *soxR* locus consists of two genes, *soxR* and *soxS*, that form an atypical two-component signaling system (22, 28). The SoxR protein is thought to act as a sensor that is activated by superoxide radicals or some related agent. Activated SoxR then promotes the transcription of the adjacent and divergently transcribed *soxS* gene. The SoxS protein is also a transcriptional activator and likely effects the increased expression of the superoxide-inducible genes (22, 28). It is thus possible that the antibiotic resistance phenotype associated with *soxR*(Con) mutations is due either to direct action of the SoxR protein on target genes or indirectly to increased levels of SoxS. To distinguish between these two possibilities, we examined the effect of overexpressing *soxS* from the multicopy plasmid pWB34 (28) on antibiotic resistance. The results obtained were virtually identical to those described for the *soxR201* mutant: the *soxS* plasmid transformant grew completely across gradients of 0 to 0.4 μg of enoxacin per ml or 0 to 10 μg of chloramphenicol per ml (data not shown). Thus, SoxS is probably the direct effector of antibiotic resistance in *soxR*(Con) strains.

TABLE 3. Dependence of *soxR201* on the *mar* locus

Genotype	*ompF-lacZ* expression[a]	Growth (% of gradient)[b]	
		Enoxacin	Tetracycline
Wild type	18,500	21 (0.05)	13 (1.0)
soxR201	4,700	>95 (0.45)	48 (3.6)
soxR201 Δ*mar*	2,450	62 (0.16)	21 (1.6)

[a] Enzyme activity was determined for mid-logarithmic phase cultures and is expressed in Miller units as described previously (20). The strain background for fusion enzyme experiments was MH621. The actual strains used were (from top to bottom) MH621, B205, and B209 (Table 1).

[b] Values represent the extent of growth across a linear gradient of 0.0 to 0.25 μg of enoxacin per ml or 0.0 to 7.5 μg of tetracycline per ml. The number in parentheses indicates the MIC in micrograms per milliliter. The strain background for antibiotic resistance measurements was MC4100. The actual strains used were (from top to bottom) MC4100, B66, and B185 (Table 1).

Activation of the *mar* locus by the *soxRS* regulators. The levels and spectrum of antibiotic resistance mediated by the *soxR201* allele are reminiscent of those observed for certain *mar* mutants, e.g., strains that overexpress the MarA positive regulator from a multicopy plasmid (9). To determine if the Mar phenotype resulting from the *soxR201* mutation requires an intact *mar* locus, a *soxR201* strain containing a deletion of the *mar* region of the chromosome was constructed and compared with the original mutant. As is shown in Table 3, the levels of antibiotic resistance conferred by the *soxR201* allele were considerably lower in strains containing a deletion of the *mar* locus. A similar effect was observed for *soxS* plasmid transformants (data not shown).

On the bases of these data and the fact that *marA* expression is inducible and associated with antibiotic resistance (5, 6, 9, 14), Northern blot hybridization was used to determine if the *soxR201* mutation results in increased levels of *mar*-specific mRNA. RNA samples were prepared from *soxR201* and *soxS* plasmid-containing strains and hybridized with a probe derived from the *marRAB* operon. As is shown in Fig. 2, the steady-state level of an ~1.0-kb *mar* transcript was significantly increased in strains containing either the *soxR201* mutation or the *soxS* plasmid compared with the wild-type strain, although not to the same level observed for sodium salicylate treatment, a known inducer of the *mar* locus (6). Thus, constitutive activation of *soxR*, and consequently increased expression of *soxS*, results in an increased accumulation of *marA* transcripts.

On the basis of these results, it would be predicted that an agent that induces the superoxide stress response because of superoxide radical production would also induce multiple antibiotic resistance in a manner that is completely dependent on *soxRS* and partially dependent on *mar*. To test this prediction, isogenic strains differing only at their *soxRS* or *mar* locus were inoculated onto enoxacin gradient plates containing different concentrations of paraquat (a generator of superoxide radicals) uniformly distributed throughout the plates. The wild-type strain (MC4100) exhibited increasing levels of enoxacin resistance as a function of increasing paraquat concentration (Table 4). A similar effect was observed when tetracycline was used as the challenge antibiotic (data not shown). This resistance pattern depended on the presence of an intact *soxRS*

TABLE 4. Induction of antibiotic resistance by paraquat

Strain	Growth (% of gradient)[a] in presence of paraquat at μg/ml:			
	0	5	25	50[b]
MC4100	21 (0.08)	35 (0.14)	60 (0.24)	71 (0.28)
MC4100Δ*soxRS*	14 (0.06)	16 (0.06)	13 (0.05)	ND[c]
MC4100Δ*mar*	13 (0.05)	37 (0.15)	59 (0.24)	67 (0.27)

[a] Values represent the extent of growth across a linear gradient of 0.0 to 0.4 μg of enoxacin per ml, with paraquat uniformly distributed throughout the plate at the indicated concentrations. Values in parentheses indicate the MIC in micrograms per milliliter. The actual strains used were (from top to bottom) MC4100, B160, and B177 (Table 1).

[b] Growth of all strains was partially inhibited at this concentration of paraquat.

[c] ND, growth was not detectable.

TABLE 5. Role of antibiotic resistance in providing protection from superoxide-generating compounds

Genotype	Growth (% of gradient)[a]			
	ENX	PQ	MEN	PMS
Wild type	19 (0.08)	71 (142)	74 (93)	48 (17)
Wild type; p9	71 (0.28)	22 (44)	48 (60)	58 (20)
soxR201	90 (0.36)	65 (130)	61 (76)	55 (19)
Δ*soxRS*	13 (0.05)	36 (72)	45 (56)	<5 (≤2)
Δ*soxRS*; p9	77 (0.31)	29 (58)	39 (49)	36 (13)

[a] Values indicate extent of growth across a linear gradient of 0.0 to 0.4 μg of enoxacin (ENX) per ml, 0 to 200 μg of paraquat (PQ) per ml, 0 to 125 μg of menadione (MEN) per ml, or 0 to 35 μg of phenazine methosulfate (PMS) per ml. Values in parentheses indicate MIC in micrograms per milliliter.

locus, as a strain containing a deletion of these genes failed to respond to paraquat exposure and showed hypersensitivity to higher concentrations of this agent. Surprisingly, paraquat-induced enoxacin resistance showed no dependence on the *mar* locus. However, the highest level of antibiotic resistance that was induced by paraquat treatment was less than that conferred by the *soxR201* mutation and comparable to that of the *soxR201* Δ*mar* double mutant (Table 3). Higher concentrations of paraquat than those shown in Table 4 were toxic to wild-type strains.

Dependence on the *mar* locus for the downregulation of *ompF* expression by *soxR*(Con) alleles. Chou et al. have recently shown that strains containing constitutive alleles of *soxR* have repressed levels of OmpF porin and that this repression is mediated by *micF* (3). Multiple antibiotic resistance resulting from the activation of the *mar* locus is also associated with a *micF*-dependent reduction in *ompF* translation (7, 9). To determine if the *mar* locus plays a role in the repression of *ompF* expression by *soxR*, the *soxR201* mutation was transduced into a Δ*mar* strain containing an *ompF-lacZ* translational fusion and β-galactosidase levels were measured. (The deletion of the *mar* locus has no effect on *ompF-lacZ* expression [reference 9 and data not shown].) As is shown in Table 3, the *soxR201* mutation caused a downregulation of *ompF-lacZ* protein fusion expression independent of the allelic state of the *mar* locus. Thus, the inhibition of *ompF* expression and the increased antibiotic resistance that result from constitutive activation of the *soxRS* locus differ in their dependence on *mar*. Moreover, these observations are consistent with previous findings indicating that *ompF* downregulation plays little role in the antibiotic resistance mechanism (7, 9).

Overexpression of *marA* can activate a superoxide-inducible gene. The amino acid sequences of the regulators encoded by *marA* and *soxS* are very similar, particularly in their DNA binding regions (5, 9). Since it is now evident that the elevated expression of either gene can increase antibiotic resistance, it was of interest to determine if *marA* could, conversely, stimulate the expression of a *soxS* target gene. One such gene is *nfo*, which encodes the superoxide-inducible repair enzyme endonuclease IV (8). To test this, a plasmid that overproduces the *marA* gene product was introduced into a strain containing an *nfo-lacZ* fusion, and β-galactosidase levels were determined. These experiments used plasmid p40 as a source of *marA*. p40 is identical to the *marA* plasmid p9 except that the parent vector for p40 is pBR328 (see Materials and Methods). The *marA* plasmid p40 produced a twofold increase in *nfo-lacZ* enzyme expression (720 ± 46 U for the p40 transformant compared with 345 ± 23 U for the control strain containing the pBR328 vector). This increase is significant ($P = 0.001$) but is much less than that observed with the *soxR201* mutant (Table 2). It is not known at this time whether this effect is dependent on *soxRS*. This result is consistent with recent experiments demonstrating that mutations in *marR* that confer a Mar phenotype result in increased expression of superoxide stress response target genes (2).

Role of antibiotic resistance in protecting against superoxide-generating compounds. Since *soxR*(Con) strains are multiply antibiotic resistant and paraquat treatment of wild-type cells can induce the same phenotype, it was of interest to determine the role of the antibiotic resistance response in protecting the cell from various compounds that promote the formation of superoxide radicals (16). To address this, we employed the plasmid p9, which constitutively expresses the *marA* gene, a positive regulator of antibiotic resistance (9). Strains transformed with p9 show patterns and levels of antibiotic resistance that are very similar to those of *soxR201* strains. The ability of p9 to confer resistance to superoxide-generating agents in a strain deleted for the *soxRS* locus was then examined (Table 5). The results depended upon the specific compound in question. While p9 did elicit the expected enoxacin resistance phenotype in the deletion strain, it did not reverse the hypersensitivities to paraquat or menadione associated with the lack of a functional *soxRS* locus. In fact, p9 consistently exacerbated this hypersensitive phenotype, an effect that was also observed in a wild-type background. In contrast, p9 restored a significant level of resistance to phenazine methosulfate in a *soxRS* deletion strain and also promoted a slight increase in resistance in a wild-type background, similar to the *soxR201* strain.

DISCUSSION

The results described in this report suggest that a high-level, *mar*-type antibiotic resistance mutation, isolated in our laboratory, results from an activating mutation in the *soxR* gene. Although the initial selective agent, PD079292, is not a common antibiotic, its antimicrobial activity does not appear to involve the generation of superoxide radicals, since the *soxRS* system is not induced by this compound (20a). Thus, this is the first report, to our knowledge, of the isolation of a *soxR* mutant on the basis of a Mar phenotype. This mutant, which we call *soxR201*, conferred properties which were qualitatively similar to those of a known *soxR*(Con) allele, *soxR4*. However, the *marC* strain showed higher levels of fusion enzyme expression as well as higher levels of antibiotic resistance (Table 2). The *soxR4* mutation was isolated on the basis of *nfo-lacZ* fusion enzyme expression, which apparently can identify less active alleles of *soxR* (26). For example, the *soxR4* mutation would not have been selected under the conditions described here.

Nonetheless, two pieces of evidence strongly support the idea that the two mutations are alleles of the same gene. First, the *soxR201* mutation promotes high-level expression of a *soxS-lacZ* fusion, and only constitutive mutations in *soxR* are known to have this property. Second, both the elevated levels of fusion enzyme expression and the increased resistance to enoxacin conferred by the *soxR201* mutant are suppressed by a multicopy plasmid carrying a wild-type copy of *soxR*, also a feature of *soxR*(Con) mutants. This effect appears to reflect a competition between wild-type SoxR protein and the activated mutant form, either in the formation of a regulatory complex or in the recognition of a target site (26, 28). The partial suppression observed here is consistent with results from a previous study, in which the extent of the suppressing effect appeared to be allele specific (26). We have also shown that the increase in antibiotic resistance associated with *soxR*(Con) mutants results from increased expression of *soxS*. Although we have not characterized the *soxR201* mutation at the molecular level and thus cannot rule out the possibility that mutations in other genes linked to *soxR* are also present in these strains, explanations based solely on the known properties of *soxR* are sufficient to interpret all of our observations.

These results also represent an important extension to experiments previously described regarding the relationship between the *mar* and *soxRS* loci (2, 3, 12). Specifically, Greenberg et al. described a mutant, *soxQ1*, that confers increased resistance to the superoxide-generating compound menadione as well as increased expression of *soxRS* target genes but which maps to the *mar* locus. These investigators showed that the *soxQ/mar* and *soxRS* systems act independently with respect to the induction of genes involved in the superoxide stress response (12). From the results reported here, it seems likely that the genes involved in antibiotic resistance represent a distinct class of target genes, in that the full measure of antibiotic resistance associated with the *soxR201* mutation is partially dependent on *mar*. An exception to this situation is *ompF*, which is repressed by *soxR*(Con) mutants in a *mar*-independent manner. Thus, a picture emerges in which both MarA and SoxS can independently control the expression of genes that function to promote antibiotic resistance, but to different extents. In addition, the *marRAB* operon may also be a target for SoxS, since the high level of antibiotic resistance induced by either the *soxR201* mutation or multiple copies of *soxS* is reduced in strains deleted for the *mar* locus (Table 3), and the steady-state level of *marA*-specific mRNA is increased in these hyperactive *soxRS* strains (Fig. 2). Alternatively, overexpression of *soxS* may somehow perturb the cell in such a way that the *mar* locus is activated indirectly, contributing to antibiotic resistance. The physiological significance of this interaction is not clear, since we were unable to demonstrate a requirement for *mar* when antibiotic resistance was induced by paraquat treatment (Table 4). However, paraquat may be too toxic an agent for these studies, since the maximum level of antibiotic resistance induced by this agent is less than that observed for the *soxR201* strain. Similar arguments could be made regarding the activation of the *nfo-lacZ* fusion by the *marA* plasmid p9, although *mar* mutants have been reported to confer increased resistance to the superoxide-generating compounds menadione and phenazine methosulfate (12) and to stimulate the expression of certain superoxide stress response genes (2). Nonetheless, the overlapping effects of MarA and SoxS overexpression are consistent with the observed sequence similarity in the putative DNA binding domains for these proteins (5, 9). The slight divergence between these sequences may explain why MarA overexpression has a more pronounced effect on antibiotic resistance than on expression of superoxide stress response targets, while the opposite pattern is seen with *soxS* induction (see Results and Table 2) (2). Identification and comparison of operator regions from individual responder genes should provide insights into the mechanism of target gene discrimination and should represent a useful system in which to compare the mechanisms of DNA sequence recognition by these closely related proteins.

An intriguing point emerged concerning the role of the antibiotic resistance response in protecting the cell from agents that stimulate the intracellular formation of superoxide radicals. Specifically, activation of the *soxRS* system stimulates the expression of one set of genes whose products help to detoxify superoxide radicals and modulates the expression of a second set of genes that results in the reduced accumulation of antibiotics. Null alleles of *soxRS* are not, by themselves, informative in resolving the specific roles of these two classes of genes in the resistance scheme, since both the superoxide-protecting enzymes and the antibiotic resistance functions are uninducible by paraquat in this genetic background (Table 4) (13, 26). By using a *soxRS* deletion strain transformed with the *marA* plasmid p9, it was possible to examine the protective role of the antibiotic resistance system against challenge by superoxide-generating agents in the absence of a vigorous superoxide stress response. The results of these experiments varied with respect to the agent in question (Table 5) and demonstrated that the spectrum of *mar*-mediated antibiotic resistance does not include paraquat or menadione, indicating that the program of superoxide-detoxifying enzymes induced by *soxRS* is essential for protection against these agents. Moreover, although *mar* mutants have been shown to have increased levels of certain gene products associated with the superoxide stress response (2, 12), this response must differ in some important qualitative way from the induction of these same genes by *soxRS*, since overexpression of *marA* cannot complement a *soxRS* deletion with respect to paraquat resistance (Table 5). In contrast, resistance to phenazine methosulfate was enhanced by p9, suggesting that the accumulation of this agent is reduced in a significant manner by the *mar* system. The lack of an effect of p9 on menadione resistance is apparently at odds with a previous study in which a *mar* mutant was shown to increase resistance to this compound (12). However, strain and/or allele differences may be involved, since these investigators also saw no effect of a *soxR* deletion on menadione sensitivity (12), in contrast to our observations (Table 5) and those of Tsaneva and Weiss (26).

The finding that it is in fact possible to select for a multiply antibiotic-resistant isolate resulting from a constitutive mutation in the *soxR* gene is, by itself, interesting. Prior genetic investigations of Mar mutants have focused on the *mar* locus, a genetic complex whose organization is beginning to be unraveled (5, 9, 11). While it has been shown that constitutive alleles of *soxR* also exhibit a Mar phenotype (13), these mutants were isolated on the basis of superoxide stress response phenotypes. The reasons that *soxR*(Con) mutants were not identified in previous searches for Mar strains are unknown but may include the fact that the *soxR* gene represents a rather small target, and that *soxR*(Con) alleles that confer high levels of antibiotic resistance may be a small subset of those that result in elevated expression of superoxide stress response target genes. Indeed, low-level *mar* mutants arise quite frequently (14), whereas it was necessary to employ a mutagen to isolate the *soxR201* strain described here. Moreover, the *soxR* mutations that confer antibiotic resistance are gain-of-function in nature, whereas typical *mar* mutants result from loss-of-function mutations in *marR*, a negative regulator

of *mar* expression, or its putative operator target site, *marO*, and thus would be expected to occur more frequently (5). It is also possible that since nitrosoguanidine was used as the mutagen, the *soxR201* allele contains more than one mutation in the *soxR* gene, and that such a complex genetic alteration is required to achieve the high levels of antibiotic resistance associated with it. That the antibiotic resistance phenotype promoted by the *soxRS* system has physiological relevance is supported by the demonstration that exposure of cells to paraquat, a *soxRS*-dependent inducer of the superoxide stress response, promotes increased antibiotic resistance in a dose-dependent manner (Table 4).

In summary, we have investigated in greater detail the genetic relationship between the *soxRS* and *mar* loci in promoting multiple antibiotic resistance in *E. coli*. Although similarities exist with respect to the consequences of induction of the *mar* and *soxRS* systems, important functional differences indicating that these are not redundant regulators have been identified. The strong degree of conservation in the DNA binding regions of SoxS and MarA suggests that they interact with a common set of target genes (2, 5, 9). The differences in these binding regions may explain in part why SoxS can stimulate both the superoxide stress response genes and the antibiotic resistance target genes, with a preference for the former, while MarA activation appears to be biased primarily towards the latter group.

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