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# Analysis of the Genetic Requirements for Inducible Multiple-Antibiotic Resistance Associated with the *mar* Locus in *Escherichia coli*

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**A series of novel genetic constructs derived from the *marRAB* operon was used to determine the role of this gene cluster in salicylate-inducible multiple-antibiotic resistance in *Escherichia coli*. Our findings indicate that regulated antibiotic resistance associated with this locus requires only the products of *marR* and *marA*, without any neighboring genes.**

In the gram-negative bacterium *Escherichia coli*, exposure to the weak acid salicylate (SAL) induces a condition of phenotypic resistance to a number of chemically unrelated antibiotics, including tetracycline, chloramphenicol, β-lactams, and quinolones (17). Mutants that exhibit increased resistance to these same agents have been isolated and found to contain mutations in either the *mar* (multiple-antibiotic resistance) or *soxRS* (superoxide stress response) locus, both of which encode related regulatory genes (1, 7–10, 16). Recent experiments have demonstrated that the treatment of cells with SAL results in the induction of *mar* gene expression, providing the beginnings of a mechanistic explanation for the effects of this agent (6).

While recent efforts have begun to unravel the genetic organization of the *mar* locus, some features of its structure remain unclear. For instance, initial cloning experiments suggested that the *mar* locus encompasses nearly 8 kb of sequence (11). However, known alleles that affect *mar*-related antibiotic resistance phenotypes have been found in only two linked genes, *marR* and *marA* (1, 5, 7), which are part of a SAL-inducible operon (6). This 1-kb operon, which contains the *marRAB* genes, appears to be regulatory in nature. *marA* encodes a positive regulator of antibiotic resistance that affects unlinked genes (7), while *marR* has been proposed to encode a repressor of the *marRAB* operon (1, 5). The purpose of this study was to clarify the role of the *marRAB* operon in *mar*-mediated antibiotic resistance relative to other adjacent sequences.

**Construction and characterization of *mar-lacZ* reporter strains and *marRA*(*B*) plasmids.** To facilitate studies of the regulation of *mar* gene expression, we constructed protein fusions between *marR* and *marA* and *lacZ* under the control of the normal *mar* regulatory sequences. Specific constructions involved the cloning of PCR products that contained the *mar* operator-promoter region (as defined in reference 5) and extended into *marR* or *marA* into the *lacZ* fusion plasmid pRS552 and recombining these onto the *ind*$^-$ phage λRS88 to generate single-copy elements integrated at the λ attachment site (20). (The structures of the *marRAB* operon and the corresponding fusions are shown in Fig. 1.) The initial constructions resulted in in-frame translational fusions between codon 75 of *marR* (*marR75*) or codon 25 of *marA* (*marA25*) and the eighth codon of *lacZ*. It is important to note that the *marA* fusion construct contains an intact copy of *marR*, while *marR* fusions do not. The expression of these fusions in either a wild-type background or a strain containing a deletion of the *mar* region of the chromosome was determined by assaying β-galactosidase levels (15) in cultures grown in the absence or presence of SAL, a known inducer of *mar* transcription (6).

As shown in Table 1, *marR75* and *marA25* fusions expressed different basal levels of fusion enzyme activity in the wild-type background. This may reflect differences in the levels of expression, protein stability, specific activity, or any combination of these. However, fusions were similarly induced (15- to 30-fold) in the wild-type background following treatment with 2.5 mM SAL. To determine if genes located in the *mar* region played a role in this induction scheme, a 39-kbp deletion that eliminates the *mar* operon, Δ1738 (11, 12), was introduced by P1 transduction (19) into fusion strains. In the Δ1738 background, the *marA* fusion continued to show a wild-type pattern of SAL-mediated induction, indicating that the genetic information defined by the Δ1738 deletion that is involved in SAL-inducible *mar* gene expression is present on the λ*marA25* element. In contrast, expression of the *marR* fusion became elevated in the Δ1738 background, even in the absence of SAL treatment. Since the major difference between *marR* and *marA* fusions is the presence of *marR* on the *marA* fusion construct, this derepression of the *marR* fusion is consistent with the proposed role of MarR as a repressor of *mar* gene expression (5).

The *marR75-lacZ* fusion in the Δ1738 background was still stimulated an additional two- to threefold by SAL (Table 1). Although *mar*-deleted strains have also been shown to retain a small degree of SAL-inducible antibiotic resistance (6), it was formally possible that the regulation observed in the Δ1738 strain was due to repressor activity associated with the 75-amino-acid portion of MarR present in the fusion. To test this, we constructed an additional fusion in which *lacZ* was fused to the fifth codon of *marR* (*marR5-lacZ*). Although the absolute levels of fusion enzyme expression were much lower for the new fusion, it behaved identically to the *marR75* construct in regulation experiments. Specifically, the *marR5* fusion was stimulated approximately 50-fold by 2.5 mM SAL in the wild-type background, and introduction of the Δ1738 allele

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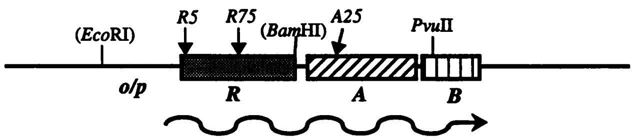


FIG. 1. Structure of the *marRAB* operon. Arrows designated *R5*, *R75*, and *A25* indicate the positions of junctions in the *marR5*-, *marR75*-, and *marA25-lacZ* fusions described in the text. The *Eco*RI site in parentheses was introduced by PCR 135 bp upstream from the GTG initiation codon of *marR* and represents the 5′ cloning junction for all the plasmid and fusion constructs described. The 3′ cloning junction for all *lacZ* fusions was a *Bam*HI site created by PCR and used to insert the *mar* PCR products in frame with *'lacZ* in plasmid pRS552 (20), as described in the text. For plasmids used in complementation experiments, the 3′ cloning junction was either a *Pvu*II site present after the 10th codon of *marB* (*marRAB'*; p43 and p49) or a *Bam*HI site (indicated in parentheses) introduced by PCR immediately after the *marR* stop codon (*marR*; p50 and p51). The wavy arrow represents *marRAB* mRNA and shows the direction of transcription. *o/p*, operator-promoter region.

resulted in approximately 30-fold induction in the absence of SAL. As was observed for the *marR75* fusion, expression of the *marR5* construct in the Δ1738 background was induced an additional threefold by SAL. Thus, the additional stimulation of *marR-lacZ* fusions in a *mar* deletion background does not involve factors encoded at the *mar* locus.

The *mar* deletion strain containing the *marR75-lacZ* fusion phage generated in the experiments described above was used to determine the minimal amount of cloned *mar* sequence needed to restore wild-type patterns of both *marR75-lacZ* fusion regulation and SAL-inducible antibiotic resistance. Cloned sequences included the *mar* operator-promoter region and extended either to the end of the *marR* coding region or through *marA* to a *Pvu*II site after the 10th codon of *marB* (Fig. 1). These segments were introduced into both the high-copy-number plasmid pBR322 (2) and low-copy-number plasmid pGB2 (4). As shown in Tables 1 and 2, plasmids that contained *marR*, *marA*, and part of *marB* under the control of the *mar* promoter (p43 and p49) were able to reestablish both of these activities. Moreover, antibiotic susceptibility patterns more closely resembled those of the wild-type strain when cloned sequences were present on the low-copy-number plasmid pGB2 rather than on the higher-copy-number pBR322 vector (compare p49 and p43 [Table 2]). Recombinant plasmids that contained only *marR* under the control of the adjacent *mar* operator-promoter region restored low basal levels of *marR-lacZ* fusion enzyme expression to cells cultured in the absence of SAL (Table 1). In addition, the exposure of this strain to SAL resulted in stimulation of fusion enzyme expression that was similar to that of the wild-type strain (Table 1). However, plasmids that contained only *marR* did not complement the defect in inducible antibiotic resistance, consistent with the requirement of *marA* for this function (Table 2). Taken together, these results indicate that the only sequences necessary for SAL-inducible *mar* gene expression and antibiotic resistance located in the region defined by the Δ1738 deletion are the *marRAB* operon.

TABLE 1. Expression of *marR-lacZ* and *marA-lacZ* fusions

| Strain background[a] | Fusion with *lacZ* | β-Galactosidase activity[b] | |
|---|---|---|---|
| | | −SAL | +SAL |
| Wild type | *marA25* | 90 | 1,600 |
| Wild type | *marR75* | 5 | 160 |
| Δ1738 | *marA25* | 115 | 1,670 |
| Δ1738 | *marR75* | 130 | 365 |
| Δ1738/p49 | *marR75* | 4 | 103 |
| Δ1738/p50 | *marR75* | 1 | 70 |

[a] All strains are derivatives of MC4100 (3). Plasmids p49 and p50 are pGB2 derivatives that contain the *mar* operator-promoter region and *marRAB'* (p49) or *marR* (p50). The Δ1738 deletion (12) removes 39 kbp of chromosomal DNA, including the *marRAB* operon (see text).

[b] β-Galactosidase assays were performed as previously described (15), and results are expressed as Miller units.

TABLE 2. Genes required for inducible, *mar*-mediated antibiotic resistance

| Strain[a] | Relevant feature | Growth (% of gradient)[b] | |
|---|---|---|---|
| | | −SAL | +SAL |
| Wild type | Wild type | 21 | 53 |
| Δ1738 | Deletion of *mar* region | 14 | 25 |
| Δ1738/p43 | High-copy-number *marRA* | 31 | 77 |
| Δ1738/p51 | High-copy-number *marR* | 16 | 35 |
| Δ1738/p49 | Low-copy-number *marRA* | 24 | 59 |
| Δ1738/p50 | Low-copy-number *marR* | 16 | 35 |

[a] All strains are derivatives of MC4100. Plasmids p43 and p51 are based on plasmid pBR322, while p49 and p50 are derived from pGB2.

[b] Extent of growth across a gradient of 0 to 0.4 μg of enoxacin per ml.

**Construction of a strain specifically defective in *marRAB* operon expression.** A prediction of the experiments described above is that a mutation that blocks expression of only the *marRAB* operon confers a phenotype identical to that of the Δ1738 deletion with respect to the *mar* functions described above. One such mutation is a transposon insertion in *marR* that has a polar effect on *marA* (13). To obtain such a strain, a mini-Tn*10* insertion in a plasmid-borne *marR* gene was isolated (14) and recombined into the chromosome (22) in place of the normal *marR* gene. This strain was then compared with the original Δ1738 deletion strain for SAL-inducible antibiotic resistance (Table 3). The results indicate that the strain containing the *marR*::Tn*10* allele has antibiotic resistance phenotypes that are very similar to those of the deletion strain, confirming that the defect in inducible antibiotic resistance observed in strains containing the Δ1738 deletion is due mainly to the loss of the *marRAB* operon. We did observe that the absolute level of antibiotic resistance in the insertion strain was very slightly but consistently higher than in the deletion strain, possibly because of leaky expression of *marA* in the former. Consistent with this notion, a *marR*::Tn*10tet marA*::Tn*10kan* strain exhibited levels of enoxacin resistance that were indistinguishable from those of the deletion strain (Table 3).

The *marR*::Tn*10* allele was also transferred by P1 transduc-

TABLE 3. Comparison of *mar* alleles

| Strain[a] | % Growth[b] | | *marR-lacZ* activity[c] | |
|---|---|---|---|---|
| | −SAL | +SAL | −SAL | +SAL |
| Wild type | 27 | 67 | 15 | 270 |
| Δ1738 | 22 | 41 | 305 | 560 |
| *marR*::Tn*10tet* | 24 | 48 | 290 | 515 |
| *marR*::Tn*10tet marA*::Tn*10kan* | 21 | 41 | ND | ND |

[a] All strains are derivatives of MC4100.

[b] Extent of growth across a linear gradient of 0 to 0.3 μg of enoxacin per ml.

[c] β-Galactosidase values are expressed as Miller units (15). ND, not determined.

tion into the wild-type strain containing the *marR-lacZ* fusion, and β-galactosidase levels were measured. As shown in Table 3, the transposon insertion in *marR* resulted in high levels of fusion enzyme expression in the absence of SAL.

The conclusion from these studies is that only the *marR* and *marA* genes are required for SAL-inducible antibiotic resistance associated with the *mar* locus. MarR is clearly a negative regulator of *marRAB* operon expression, as has been suggested previously (1, 5). Moreover, a low-copy-number plasmid that contained *marR* and *marA* under the control of their normal promoter sequences restored wild-type patterns of SAL-inducible antibiotic resistance to the *mar* deletion strain. Taken together, these results implicate MarR as the target for SAL-mediated induction of the *mar* operon. Sensing of SAL could be mediated either by a cellular function encoded by an unlinked gene or by MarR itself. A recent report indicated that in vitro binding of a MalE-MarR fusion protein to a DNA fragment containing the *mar* operator-promoter region is antagonized by SAL and other *mar* inducers, suggesting that the molecular relationship between MarR and SAL may be a direct one (18).

Although systematic experiments focusing on *marB* were not conducted here, we failed to find a requirement for this gene. Mutations that specifically inactivate the chromosomal copy of *marB* are needed to determine if its product plays a role in either regulating or promoting antibiotic resistance.

It is not clear as to why the original cloning experiments of Hächler et al. defined the *mar* locus as a 7.8-kbp region (11). One possible explanation is the difference in tests used to determine *mar* function. The 7.8-kbp segment was identified on the basis of the frequency of appearance of spontaneous *mar* mutations in a deletion strain (11). The assays that we employed involved SAL-inducible antibiotic resistance and *mar-lacZ* reporter fusion expression. Perhaps the development of *mar* mutants, which occurs at high frequencies in wild-type strains but not *mar* deletion strains, requires some larger physical context that includes sequences adjacent to the *marRAB* operon. Differences in strain backgrounds may also play a role. For example, the 39-kbp deletion conferred only a slight increase in antibiotic susceptibility in the MC4100 background used here, while the same deletion results in a much larger degree of hypersensitivity in strain AG100 (21). Nonetheless, the results presented here indicate that the regulatory *marRA(B)* operon is the critical genetic element involved in SAL-inducible antibiotic resistance encoded at the *mar* locus.

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