The Salmonella typhimurium mar Locus: Molecular and Genetic Analyses and Assessment of Its Role in Virulence

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The marRAB operon is a regulatory locus that controls multiple drug resistance in Escherichia coli. marA encodes a positive regulator of the antibiotic resistance response, acting by altering the expression of unlinked genes. marR encodes a repressor of marRAB transcription and controls the production of MarA in response to environmental signals. A molecular and genetic study of the homologous operon in Salmonella typhimurium was undertaken, and the role of marA in virulence in a murine model was assessed. Expression of E. coli marA (marAEc) present on a multicopy plasmid in S. typhimurium resulted in a multiple antibiotic resistance (Mar) phenotype, suggesting that a similar regulon exists in this organism. A genomic plasmid library containing S. typhimurium chromosomal sequences was introduced into an E. coli strain that was deleted for the mar locus and contained a single-copy marR- lacZ translational fusion. Plasmid clones that contained both S. typhimurium marR (marRSt) and marA (marASt) genes were identified as those that were capable of repressing expression of the fusion and which resulted in a Mar phenotype. The predicted amino acid sequences of MarRSt, MarASg, and MarBSt were 91, 86, and 42% identical, respectively, to the same genes from E. coli, while the operator/promoter region of the operon was 86% identical to the same 98-nucleotide-upstream region in E. coli. The marRAB transcriptional start sites for both organisms were determined by primer extension, and a marRAB expression transcriptional regulator of unlinked genes whose modulation affects antibiotic resistance (12, 15, 19, 21, 22, 30); and (iv) marB, a gene of unknown function. Previously characterized mar mutants contain mutations in either marR or marO, which disrupt autorepression and lead to increased expression of marA and, consequently, activation of the regulon (2, 12, 19, 36).

The marRAB operon is induced by a variety of chemical agents, including tetracycline, chloramphenicol, tetracycline, β-lactams, and quinolones (14, 20). Apparently, reduced accumulation accounts, at least in part, for this resistance, through activation of efflux pumps coupled with decreased synthesis of outer membrane porins (14, 15, 20, 34, 38). Mutations contributing to high-level quinolone resistance in E. coli include those located at mar (29), and a multiple antibiotic resistance (Mar) phenotype has been observed in clinical quinolone-resistant gram-negative organisms (28), suggesting that nonspecific resistance systems may play a role in the outcome of antibiotic therapy for this class of compounds.

Genes encoded at the mar locus that are involved in multidrug resistance have been identified. A 1.1-kbp marRAB operon regulates multiple antibiotic resistance (12, 19, 36) by controlling expression of at least 10 unlinked genes (22, 44, 48, 51), two of which, acrA and acrB, are absolutely required for resistance (44). The marRAB operon consists of (i) an operator-promoter region (marO) from which divergent transcripts are generated; (ii) marR, encoding a repressor of the marRAB operon (12, 50, 53); (iii) marA, encoding a positive transcriptional regulator of unlinked genes whose modulation affects antibiotic resistance (12, 15, 19, 21, 22, 30); and (iv) marB, a gene of unknown function. Previously characterized mar mutants contain mutations in either marR or marO, which disrupt autorepression and lead to increased expression of marA and, consequently, activation of the regulon (2, 12, 19, 36).

Since there are few informative mutations in the E. coli marR (marREc) and marA (marAEC) genes, comparing these genes with functionally related homologs could provide additional information about how these genes and their products carry out their roles in the mar system. Toward this end, we have determined, by genetic and molecular methods, the extent to which the Salmonella typhimurium mar locus is related to its counterpart in E. coli.

We also addressed the question of whether marA is a virulence factor for animal infection. A number of observations...
Plasmids

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant genotype or phenotype</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pMOB</td>
<td>Apr; smaller derivative of pUC vectors for yaph transposon tagenecassette</td>
<td>Gold Biotechnologies (52)</td>
</tr>
<tr>
<td>pUC4K</td>
<td>Apr'; Km'; vector containing kanamycin resistance gene cassette</td>
<td>Pharmacia Biotech Inc.</td>
</tr>
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<td>p144</td>
<td>Apr'; marR&lt;sub&gt;A&lt;/sub&gt; insert in pBluescript SK from χ3181 plasmid library</td>
<td>This study</td>
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<tr>
<td>p145</td>
<td>Apr'; marR&lt;sub&gt;B&lt;/sub&gt;A&lt;sub&gt;B&lt;/sub&gt; insert in pBluescript SK from χ3181 plasmid library</td>
<td>This study</td>
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<tr>
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<td>Apr'; marR&lt;sub&gt;B&lt;/sub&gt;A&lt;sub&gt;B&lt;/sub&gt; insert in pBluescript SK from χ3181 plasmid library</td>
<td>This study</td>
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<tr>
<td>p147</td>
<td>Apr'; marR&lt;sub&gt;B&lt;/sub&gt;A&lt;sub&gt;B&lt;/sub&gt; insert in pBluescript SK from χ3181 plasmid library</td>
<td>This study</td>
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</tr>
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</tr>
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<td>p9</td>
<td>Apr'; 2.2-kbp E. coli marA B&lt;sub&gt;AB&lt;/sub&gt; insert in pBR322; marA B&lt;sub&gt;AB&lt;/sub&gt; expressed from the tetracycline promoter</td>
<td>19</td>
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<td>pBluescript SK&lt;sup&gt;+&lt;/sup&gt;</td>
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<td>Ap'; cloning vector</td>
<td>I. Blomfield (5)</td>
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<td>Ap'; pUC18 containing Bacillus subtilis sacB</td>
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<tr>
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<td>Cm&lt;sup&gt;+&lt;/sup&gt;; temperature-sensitive vector</td>
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<td>Cm&lt;sup&gt;+&lt;/sup&gt;; counter selectable temperature-sensitive vector for allelic exchange</td>
<td>This study</td>
</tr>
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<td>p162</td>
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<td>This study</td>
</tr>
<tr>
<td>p185</td>
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<td>This study</td>
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</table>

**Materials and Methods**

**Bacterial strains, plasmids, media, and general reagents.** Bacterial strains and plasmids used in this study as well as their relevant properties are listed in Table 1. Cultures were routinely grown in LB medium (1% Bacto Tryptone, 0.5% Bacto Yeast Extract, 0.5% NaCl [Difco, Detroit, Mich.] at 37°C unless otherwise noted. Also used was lactose-MacConkey agar medium (Difco). Antibiotics (Sigma, St. Louis, Mo.) were added to selective media at the following final concentrations (unless otherwise noted): ampicillin, 50 μg/ml; kanamycin, 40 μg/ml; and chloramphenicol, 20 μg/ml. Enoxacin (40), a fluoroquinolone, was obtained from an in-house synthesis. SAL was from Sigma and when used was added to a final concentration of 2.5 mM. α<sup>-32</sup>PldATP was from Amersham (Arlington Heights, Ill.).

**General molecular biological techniques.** All molecular biology methods were performed following established protocols (4) unless otherwise noted. Restriction enzymes were purchased from Boehringer Mannheim (Indianapolis, Ind.) and were used as recommended by the manufacturer. Dephosphorylation of DNA was accomplished by using calf intestinal alkaline phosphatase (GibcoBRL, Gaithersburg, Md.). DNA was routinely introduced into bacteria by transformation using the TSS method (11) unless otherwise noted. PCR amplifications of DNA were accomplished by using calf intestinal alkaline phosphatase (Gibco-BRL). Custom oligonucleotides were commercially prepared (Gibco-BRL). Southern hybridization analysis was performed under stringent conditions (4), using the Genus system (Boehringer Mannheim). The marR<sub>B</sub>A<sub>AB</sub>B<sub>AB</sub> probe used for Southern analysis was a 0.7-kb SalI-PvuII DNA fragment from p9 (corresponds to nucleotides 1641 to 2341 of the published E. coli marR<sub>B</sub>A<sub>AB</sub>B<sub>AB</sub> sequence [12]). It contains the 3' half of E. coli marR, the entire marA<sup>+</sup>, and 27 nucleotides of the 5' of marB. The probe was hybridized to BamHII digested plasmid DNA following electrophoresis on a 0.8% agarose gel and transferred to a Nytran membrane (Schleicher & Schuell, Keene, N.H.).

**Genomic library construction.** The pBluescript SK<sup>-</sup> S. typhimurium χ3181 plasmid library was constructed as follows. A partial Sau3AI digest of chromosomal DNA was electrophoresed through a 0.8% agarose-Tris-borate-EDTA gel. A gel slice containing 3- to 8-kbp DNA fragments was excised, and the DNA purified by using a Qiagen gel extraction kit (Qiagen, Chatsworth, Calif.). This DNA was ligated to BamHII-digested and dephosphorylated pBluescript SK<sup>-</sup> plasmid DNA and transformed into library-competent E. coli DH5α competent cells (GIBCO-Life Technologies Inc., Gaithersburg, Md.) as instructed by the

**Table 1. Bacterial strains and plasmids.**
Construction of S. typhimurium χ3181 marA::kan. An S. typhimurium marA::kan strain was produced by allelic exchange using pSac3, a temperature-sensitive, counterselectable plasmid derivative of pSC101 whose construction is described below. p147 (Table 1), carrying the marK and marR genes, was digested with SalI, which cleaves in the middle of the mramA fragment. This site was blunt ended with T4 DNA polymerase and ligated to the agarose-gel purified 1.6-kbp PstI fragment of pUC4K, containing a kanamycin resistance determinant. This produced plasmid p182. pSac3 was constructed by ligation of a BamHI-AhoI DNA frag and with the BamHI- and SalI-digested pB307 (5) (both restriction sites located in the MCS). pSac3 was then digested with PstI and blunt ended with T4 DNA polymerase. The XhoI-HindIII fragment from p162 containing marA::kan was similarly rendered blunt ended and ligated (G187) into pSac3, resulting in p197. This plasmid was introduced into S. typhimurium LT2 and subsequently into χ3181 by standard transformation protocols. Allelic exchange of marA::kan form p185 into the chromosome was accomplished by a modification of the methods of Hamilton et al. (26) and Blomfield et al. (5). χ3181(p185) was grown at 30°C to stationary phase, and dilutions were plated out at 44°C on prewarmed LB agar containing kanamycin and chloramphenicol. After overnight incubation, surviving colonies were presumed to have the plasmid integrated in the chromosome. Independent colonies were inoculated into LB medium containing kanamycin and incubated at 30°C overnight; they were then diluted 1:50 in LB medium and grown 6 h at 44°C and finally plated onto prewarmed (to 44°C) solid medium containing 1% tryptone, 0.5% yeast extract, 6% sucrose, and kanamycin. Sucrose-resistant survivors that were kanamycin resistant and chloramphenicol sensitive were presumed to be cured of the plasmid. Successful allelic exchange was verified by PCR amplification, using a pair of oligonucleotide primers that flank marA, MCS1 (see above) and M939 (CAAGCAGCGGCGAGACGTTGAG), whose sequences correspond to nucleotides 2017 to 1848 and 1321 to 1344, respectively, in Fig. 2. The χ3181 marA::kan strain was designated PD188.

Antibacterial susceptibility testing. Comparison of antibiotic susceptibility levels was performed by using gradient plates as described previously (19). Results are expressed as percent growth across the gradient.

Virulence studies. Female, 31- to 53-day-old BALB/c mice (Charles River, Bar Harbor, Maine) were used for animal experiments. Inoculation was done as described by Gulig and Curtiss (24). Briefly, mice were starved for 6 h prior to oral inoculation with 50 μl of 10% sodium bicarbonate and 500 μl of bacteria suspended in saline containing 0.1% gelatin. The 50% lethal dose (LD50) was determined by the method of Reed and Muench (45), using four to five mice per bacterial challenge dose.

Nucleotide sequence accession number. The GenBank accession number for the nucleotide sequence shown in Fig. 2 is U54648.

RESULTS

Analysis of phenotypic antibiotic resistance in S. typhimurium. SAL induces Par in E. coli (47), in part by activation of the marRAB operon. Representative S. typhimurium strains, including the avirulent LT2 derivative LB5010 along with the mouse-virulent strains, were assessed for a SAL-inducible Mar phenotype. SAL was found to induce antibiotic resistance in all S. typhimurium strains, including the avirulent LT2 derivative LB5010, indicating that a MarA regulatable resistance system exists in this species. Interestingly, the mouse-virulent strains containing p9 showed a greater increase in antibiotic resistance than did the E. coli and S. typhimurium laboratory strains.
TABLE 2. Induction of antibiotic resistance in E. coli and S. typhimurium by SAL and plasmid p9 expressing marA

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant feature</th>
<th>Plasmid</th>
<th>Chloramphenicol (0–50 μg/ml)</th>
<th>Enoxacin (0–0.6 μg/ml)</th>
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<tr>
<td></td>
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<td>-SAL</td>
<td>+SAL</td>
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<tr>
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<td>18</td>
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<td>pBR322</td>
<td>65</td>
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<td>MC4100</td>
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<td>p9</td>
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<td></td>
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<tr>
<td>S. typhimurium</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td>pBR322</td>
<td>&lt;5</td>
<td>&lt;5</td>
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<td>Standard lab strain</td>
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</tr>
<tr>
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<td>&gt;95</td>
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<td>p9</td>
<td>&gt;95</td>
<td>&gt;95</td>
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</tbody>
</table>

* Extent of growth across a gradient of chloramphenicol or enoxacin.

removes ~39 kb of DNA including marRAB; it is also lysogenic for a recombinant lambda phage containing a marR'-lacZ protein fusion under the control of the mar operator/promoter region. Expression of the fusion is constitutive (Lac') due to the absence of a source of MarR repressor. Introduction of a plasmid containing marR results in repression of the mar operon and a Lac' phenotype, identified as white colonies on lactose-MacConkey indicator medium. The presence of marA on the same plasmid confers increased antibiotic resistance and restores SAL-inducible antibiotic resistance. Accordingly, B318 was used as a reporter strain for the detection of plasmids carrying the S. typhimurium marR and marA genes. A plasmid library consisting of random χ3181 chromosomal DNA was introduced into this strain, and nine white (Lac') colonies were obtained. Plasmid DNA was isolated from the nine transformants and analyzed by Southern blot analysis for the presence of mar sequences, using an E. coli marRAB probe. Five clones gave positive signals (data not shown). Of the five, only two (p145 and p147) also restored SAL-inducible antibiotic resistance in B318. Both B318(p145) and B318(p147) exhibited an almost fourfold increase in intron enoxacin resistance over that of the plasmidless control strain B318. In addition, SAL-induced resistance to enoxacin was more than threefold higher than in the control. Taken together, these results suggested that we had isolated five recombinant plasmids that contained marRSt, and that two of these (p145 and p147) also had a functional marASt gene.

**Genetic analysis of the S. typhimurium mar locus.** γδ transposon mutagenesis was used to identify those sequences contained within the pl147 DNA insert fragment that encoded the marRSt and marASt activities (52). As described in Materials and Methods, the 2.5-kb DNA insert fragment from p147 was subcloned into pMOB yielding p197, and this plasmid was subsequently mutagenized. We identified 96 plasmids that contained transposon insertions spanning the entire cloned DNA insert in p197, and their locations were mapped relative to the MCS. By monitoring the Lac and Par phenotypes of B318 transformants carrying the γδ-mutagenized plasmids, complementation units for the presumptive marRSt and marASt genes were identified. Insertions in an ~400-bp region (located from bp 1002 to 1392 on the linear map in Fig. 1) abolished the ability of p197 to complement the mar deletion in B318 for both repression of the marR'-lacZ fusion as well as stimulation of antibiotic resistance. A second set of insertions that were clustered in an adjacent 400-bp segment disrupted antibiotic resistance enhancement without affecting repression of the marR'-lacZ fusion. These could be interpreted, by analogy to the E. coli system, as evidence for the second cluster of insertions lying in marASt and the first residing in marRSt or adjacent promoter regions, with a polar effect on expression of the downstream marASt gene. All other insertions had no effect on either phenotype.

**DNA sequence analysis of the S. typhimurium mar locus.** The DNA sequences of both strands of portions of the DNA inserts from p197 and p147 were determined (Fig. 2). Five ORFs were identified in the 2,337-nucleotide sequence. They are homologous to the E. coli marRAB genes and flanking ORFs described by Cohen et al. (12). The products of ORF144, ORF129, and ORF71 have significant amino acid sequence similarity to E. coli MarR, MarA, and MarB, respectively, indicating that these ORFs are the marRSt genes. Specifically, the product of ORF144 shows 91% amino acid sequence identity to MarRSt, and is the same length. ORF129 begins 13 nucleotides downstream from the stop codon of ORF144, and the predicted amino acid sequence of its product is 94% identical to MarASt. Predicted helix-turn-helix DNA-binding motifs found in both proteins are identical. ORF71, located 28 nucleotides downstream of ORF129, could encode a protein with 42% amino acid sequence identity (64% similarity when including conserved residues) to MarBSt. In protein database searches, the only significant match to this ORF was MarBSt, suggesting that ORF71 is the marBSt gene. A stretch of 98
nucleotide residues located immediately upstream from the marR coding region (ORF144) is 90% identical with the marR$_{Ec}$ operator/promoter region. Within this region, a putative σ70 promoter was identified as well as imperfect direct repeats (DR1 and DR1') that differ by only one nucleotide in DR1’ from similar elements found in the E. coli sequence. These direct repeats are the proposed MarR-binding regions in the E. coli mar promoter (37). A marbox, a proposed MarA-binding site (35), was found upstream of the putative −35 promoter element; it is 93% identical to its E. coli counterpart.

ORF221 and ORFA flank the marRAB$_{Ec}$ region on either side and are similar to ORFs that neighbor the marR$_{Ec}$ operon. ORF221 is located 258 nucleotides upstream of an oriented opposite ORF144 (marR$_{Ec}$). ORF221 could encode a protein that has 91% amino acid sequence identity with MarC (21a) from E. coli (originally proposed to encode two adjacent ORFs, ORF64 and ORF157 [12]) and is similar in location (211 nucleotides upstream) and orientation to marR$_{Ec}$. Database searches also identified an E. coli ORF of unknown function located upstream of adhe (32) whose product exhibits 74% amino acid sequence similarity to that encoded by ORF221. ORF221 encodes a predicted basic (pI = 9) protein with a hydrophobic profile that suggests the presence of six membrane-spanning domains (data not shown).

Located 188 nucleotides downstream of ORF71 (marB$_{Ec}$) is the C-terminal end of a putative ORF, designated ORF31. Translation of this sequence generates a polypeptide that has 70% residue identity to the C-terminal region of E. coli ORF266, which is located downstream of marB$_{Ec}$. Thus, sequence comparison with the E. coli mar locus suggests that the marR$_{Ec}$ operon, as well as its surrounding regions, is organized similarly.

The conclusions from the sequence data for marR$_{Ec}$ and marA$_{Ec}$ are corroborated by the genetic data described earlier. The γ6 insertions that identified the marR complementation unit in p197 (insertions 85, 33, 9, and 8) are located in ORF144; those presumed to identify marA (insertions 60, 45, 79, and 10) are found in ORF129 (Fig. 2). Clear roles for ORF221 and marB$_{Ec}$ in either antibiotic resistance or mar regulation did not emerge from this analysis. For example, γ6 insertions 66, 43, 41, 77, and 70, which are located in ORF221 (Fig. 2), had no effect on the resistance phenotype or regulation of marR’-lacZ (Fig. 1). Truncation of marB did not affect either antibiotic resistance or repression of the marR’-lacZ fusion. Sequence analysis of the p147 DNA showed that only two-thirds of marB is present on this clone whereas pl45 contains the entire marB gene (data not shown). Nonetheless, B318 containing either plasmid had the same antibiotic resistance phenotype and reporter fusion expression patterns.

Expression of marRAB and ORF221 by Northern analysis. Since SAL induces marRAB transcription in E. coli (13, 39), we investigated whether this is also true for S. typhimurium. Total RNA from S. typhimurium χ3181 and E. coli MC4100 grown in the absence and presence of SAL was analyzed by Northern blot analysis, using probes specific for marRA and ORF221 (Fig. 3). With the marR$_{Ec}$ probe, a ~1.1-kbp band was identified from S. typhimurium RNA (data not shown). This size is consistent with the predicted size of 1.1 kbp of the marR$_{Ec}$ operon from the putative initiation codon of MarR$_{Ec}$ to a rho-independent-like stem-loop structure located downstream of marR$_{Ec}$ (residues 985 to 2091 in Fig. 2). When RNA from the SAL-treated culture was examined, the intensity of this band increased greatly (Fig. 3d). By comparison, a ~1.0-kbp marR$_{Ec}$-specific band, identified by a specific E. coli probe, was also clearly more intense in the RNA sample obtained from SAL-treated cells than in that obtained from untreated cells (Fig. 3b). The data indicate that SAL induces the transcription of marRAB in both species.

In addition, expression of the S. typhimurium ORF221 transcript was also induced by SAL, although to a much lesser extent than was marRAB. With the S. typhimurium ORF221 probe, bands at −0.7, 1.0, and 1.2 kbp increased in intensity in the presence of SAL, indicating some accumulation of mRNAs derived from the ORF221 region (Fig. 3c). By contrast, no marked effect of SAL treatment on induction of marC$_{Ec}$ mRNA was observed (Fig. 3a).

Transcriptional start site of the marRAB promoter. To more firmly establish our assignments of promoter regions, transcriptional start sites for both the S. typhimurium and E. coli marRAB operons were determined by primer extension analysis. In E. coli, a major extension product terminated 27 bp upstream of the GTG start site of marR, located at nucleotide 1418 of the sequence reported by Cohen et al. (12) (Fig. 4A, lane 1). Major extension products terminating 29 and 30 bp upstream of the marR$_{Ec}$ GTG start codon are shown in Fig. 4B, lane 3. These findings indicate that the putative −10 and −35 regions identified by sequence analysis for the E. coli (12) and S. typhimurium (Fig. 2) marRAB operons are the likely promoter elements for both transcription units.

Construction of S. typhimurium marA mutant strains: Mar phenotype and virulence studies. An S. typhimurium χ3181 marA::kan strain, PD188, was constructed by the allelic exchange method of Hamilton et al. (26; see Materials and Methods) by using p185, a temperature-sensitive marker containing a counterselectable marker and the marA::kan allele. Successful allelic exchange was verified by PCR of the allele. Subsequently, the Mar phenotype of this strain was determined. A reduction in SAL-inducible antibiotic resistance was observed (Table 3), as evidenced by increased susceptibility to ampicillin, chloramphenicol, and tetracycline compared to the isogenic marA$^+$ parent strain. These results are similar to those reported for E. coli Δmar strains (13, 53). Hypersensitivity to chloramphenicol and tetracycline in this strain in the absence of SAL was not observed.

To determine if marA plays an essential role in S. typhimurium virulence, per oral LD$_{50}$ in BALB/c mice for both marA$^+$ and marA::kan strains were determined. The results of these studies showed the LD$_{50}$ for both strains to be 60 CFU by the method of Reed and Muench (45). Thus, the absence of a functional marA does not diminish S. typhimurium virulence in an orally administered infection of BALB/c mice.

The S. typhimurium virulence plasmid plays no role in marA-mediated antibiotic resistance. Since both SAL treatment as well as a marA$^+$-overproducing plasmid induce a more robust antibiotic resistance response in S. typhimurium virulent strains than in E. coli MC4100, it seemed plausible that the S. typhimurium virulence plasmid, pStsSR101, was responsible for this difference. Therefore, the effectiveness of marA in promoting multiple antibiotic resistance was assessed in isogenic strains that either contained (χ3306) or were cured (χ3337) of the 100-kbp virulence plasmid. The marA::kan allele was recombined, by homologous recombination, into both strains as described in Materials and Methods, and all four strains were assayed for sensitivity to several antibiotics. As shown in Table 3, the presence of the virulence plasmid did not affect any of the antibiotic resistance phenotypes in all strains tested.

DISCUSSION

A previous study by Cohen et al. (16) showed that marA-like sequences could be detected in S. typhimurium by Southern blot analysis. In this study, we confirm and extend this obser-
vation to show that *Salmonella typhimurium* encodes a marRAB operon that is structurally and functionally almost identical to that identified in *E. coli*. Transposon insertions in marRSt were polar for marASt, and no promoter sequences are apparent upstream from the coding regions of marASt or marBSt. Moreover, the SAL-inducible marRASt-specific transcript of 1.1 kbp (determined by Northern analysis) extending from the mRNA start site upstream of marR (identified by primer extension) is able to accommodate the three mar ORFs. Thus, as previously shown for *E. coli* (12, 13, 53), the marRSt, marASt, and marBSt genes are organized in an operon.

The high degree of homology of MarASt to MarAEc explains why both are functionally interchangeable between organisms. When present on a multicopy plasmid, marA from either organism was able to confer a Mar phenotype in the heterologous background. The two proteins have identical helix-turn-helix DNA-binding motifs, and it follows that the target promoter sites recognized by these proteins are likely to be conserved between the two organisms. Analogous to what was previously reported for *E. coli* (12, 13, 53), *Salmonella typhimurium* marA strains are attenuated for SAL-inducible antibiotic resistance in comparison to wild-type strains (Table 3); however, such attenuation is not complete. Thus, SAL induces Par by both mar-dependent and mar-independent pathways, as was previously reported for *E. coli* (13). Interestingly, the phenotypic effect of a marA mutation appears to differ among particular strains: *E. coli* AG100 marA::Tn5 strains are hypersensitive to tetracycline and chloramphenicol compared to the wild type (56), while *E. coli* MC4100 marA::Tn10kan and the *S. typhimurium* χ3337, χ3306, and χ3181 marA::kan strains are only slightly or not at all hypersensitive, respectively, compared to their isogenic parental strains (Fig. 3). Although antibiotic resistance phenotypes were generally stronger in the virulent *S. typhimurium* strains, we found that the MarA target sites necessary for antibiotic resistance are located exclusively on the chromosome. They were not present on the *S. typhimurium* virulence plasmid, since a plasmidless strain showed a Mar phenotype identical to a wild-type strain (Fig. 3). Taken together, the data show that MarASt is almost identical to its *E. coli* counterpart in structure and function. In this light, it is interesting that the seven amino acid differences between MarASt and MarAEc are clustered in the last 19 amino acids of the proteins. This finding suggests either that this portion of each protein plays no role in the activities of MarA assessed here.

FIG. 2. Nucleotide sequence of the *S. typhimurium* χ3181 mar locus. Potential ORFs are shown translated below the nucleotide sequence. One-letter amino acid symbols are centered under the nucleotide triplets. All amino acid residues are identical to those in the homologous ORFs of the *E. coli* mar locus determined by Cohen et al. (12), except for those indicated in boldface, in which case a different amino acid is present in *E. coli*. The homologous ORFs are as follows: ORF221 and *E. coli* marC, ORF144 and *E. coli* marR, ORF219 and *E. coli* marA, ORF71 and *E. coli* marB, and ORFA and *E. coli* ORF266. Potential ribosome-binding sites (SD), promoter elements (−35 and −10), imperfect direct repeats (DR1 and DR1’; nomenclature of Cohen et al. [12]), and the marbox (35) are indicated. The marRAB operon transcriptional start site is also indicated, as is the helix-turn-helix motif of MarA. Circled residues in MarR are those that are completely conserved among 14 of 15 members of the MarR family (39a). A putative rho-independent transcription termination motif downstream from marB is identified as IR1. Numbered triangles indicate the specific locations of the ϕ8 transposon insertions shown in Fig. 2. The downward arrow pointing to a P in marB shows the location of an additional proline codon in the *E. coli* gene.

FIG. 3. Northern blot analysis of RNA transcripts from both the *E. coli* MC4100 and *S. typhimurium* χ3181 marRAB regions. (Top) Schematics are shown of the DNA sequence interval (base-pair numbered line) and ORFs (arrows) of the *E. coli* region, corresponding the numbering system of Cohen et al. (12), and that shown for *S. typhimurium* in Fig. 2. Probes used are indicated by rectangular boxes. (Bottom) Northern blots of RNA isolated from exponentially growing cells incubated without (−SAL) or with (+SAL) 2.5 mM SAL for 1 h and hybridized to the corresponding DNA probes indicated above. Locations of 16S and 23S rRNAs are indicated along with locations of RNA molecular size standards.
strain. Presumably, the effect of that transposon insertion is either to produce an unstable MarA protein or to add at the C terminus of the protein γ6-encoded amino acids which are incompatible with MarA function.

MarRSt was found to be functionally similar to MarREc because it repressed a marR-‘lacZ fusion in the heterologous E. coli background in a manner that could be antagonized by SAL. Moreover, this feature was the basis for cloning the marRAB locus. Although we did not directly assess marRSt function in S. typhimurium, the ability of SAL to induce transcription of marRABSt suggests that MarRSt is expressed and represses marRABSt transcription. This protein can also be considered the newest member of the MarR family of phenolic-binding regulatory proteins (54), of which there are now 16 (39a).

An alignment of these sequences has revealed residues which are either completely or frequently conserved among members of this family. None of the 11 amino acid differences between MarRSt and MarREc affects one of these conserved positions. Thus, these 11 substitutions collectively do not affect MarR function.

MarB has no known function in E. coli. In this regard, it is interesting that the divergence between the E. coli and S. typhimurium marB sequences is much greater (42% identical) than was observed for MarR and MarA (>91% identical). The significance of this finding is unclear, but it is consistent with the lack of any clear obligatory role for MarB in the activities analyzed here.

To put these comparisons into a larger context, the structures and organizations of neighboring sequences were also investigated. The marRABSt operon is flanked by ORFs homologous to those found at the same positions in E. coli (12, 21a). MarC from E. coli has 91% amino acid identity to S. typhimurium ORF221. White et al. (56) suggest that this locus is involved in multidrug resistance. In mar deletion strains, a plasmid containing DNA encoding this ORF along with marRAB increased resistance levels two- to threefold above those induced by plasmids expressing only marA and marB (56). By analogy, ORF221 may play a similar role. However, one observation made for ORF221 and its role in antibiotic resistance is noted here. The E. coli strain deleted for the mar locus and containing p197, encoding ORF221St and marORF221St, showed decreased susceptibility to enoxacin compared to a plasmidless strain. This level of resistance was unchanged when ORF221 was disrupted by the transposon γ6. Thus, in the heterologous system used here, we found no role for ORF221 in antibiotic resistance.

A 95-bp segment of the operator-promoter region beginning at the marRStTGT start codon and extending upstream is 86%
identical at the nucleotide level to the same region in *E. coli* and contains nearly identical regions implicated in MarR and MarA binding (35, 37). To better compare the operator/promoter regions, we identified *marRAB* transcription start sites by primer extension, thereby empirically defining the promoter sites of both organisms. The start site for *E. coli* conforms to the expected site defined by putative −10 and −35 regions upstream of *marRAB*, originally identified by Cohen et al. (12). The *S. typhimurium* promoter sites are similarly situated (Fig. 2).

SAL-mediated induction of *marRAB* transcription appears identical in both organisms. By Northern blot analysis, SAL was shown to induce the accumulation of *marRAB* mRNA (Fig. 3). Also, since treatment of *E. coli mar* mutants with tetracycline has been reported to elevate mRNA levels of both *marRAB* and the upstream *marC* (12), it was possible that treatment with SAL would do the same. While induction of the upstream transcript by SAL was more pronounced in *S. typhimurium* than in *E. coli*, where little effect was observed, this induction was much smaller than that of the *marRAB* transcript (Fig. 3). The similarity to *E. coli* of nucleotide sequences of the operator/promoter regions, Northern data on inducibility of the operon by SAL, and structural and functional similarities of MarR to its *E. coli* counterpart all suggest that regulated control of *marRAB* expression in *S. typhimurium* is nearly identical to that of *E. coli*.

When grown in the presence of SAL or when containing plasmids expressing *MarAsc*, *S. typhimurium* strains that are capable of infection in BALB/c mice were less susceptible to different classes of antibiotics than were less virulent *S. typhimurium* and *E. coli* strains (Table 2). This finding suggested a possible correlation between MarA-mediated antibiotic resistance and virulence potential. Therefore, the contribution of *marA* in virulence was assessed by using a well-established murine model of infection (24). However, the *S. typhimurium* *marA* strain did not differ from the wild-type control in the level of killing of BALB/c mice. We note that our LD₅₀ values were much lower than those reported for an isogenic strain (24); however, this difference does not affect the result since our two strains exhibited indistinguishable LD₅₀ values. Thus, we could not implicate *marA* in *S. typhimurium* virulence.

If *marA* is involved in virulence, it is possible that its effect is masked by the activation of other overlapping regulons that engender a phenotypic antibiotic resistance. Some of the genes controlled by *marRAB* are also members of the redox-sensitive *soxRS* regulon (2, 22) in which constitutive mutations also confer a Mar phenotype (23, 39). The regulatory overlap is presumably due to the strong homology between the direct activators of both regulons, SoxS and MarA (12). The *soxRS* regulon protects the cell against both redox-cycling compounds (43, 55) and the nitric oxide free radical (NO) (41, 42), as well as NO-generating activated macrophages (41, 42). Thus, some genes common to both the *marRAB* and *soxRS* regulons are likely to be expressed in vivo, as a consequence of *soxRS* activation. *marRAB* may therefore be redundant when *S. typhimurium* is in contact with macrophages, where it is known to escape humoral defenses (18). In addition, recent evidence indicates that overexpression of another MarA homolog, Rob, also results in multiple antibiotic resistance (3), presumably by activating promoters common to the MarA regulon. Experiments designed to evaluate strains containing combinations of *marRAB*, *soxRS*, and *rob* mutations for attenuation of virulence may identify overlapping roles for these genes in pathogenesis.

Studies focusing on MarA-regulated intrinsic antibiotic resistance can now be conducted in two different organisms. Although the natural physiological role of the two operons is unclear (here the *Salmonella mar* operon is not implicated as a virulence factor), their close similarities suggest that an analysis of MarA target genes from both organisms may identify functions that collectively best a concerted physiological response. Given the interchangeability of *marRAB* and *marA*, it seems reasonable to consider these genes allelic. The functional similarities between *marR* and *marA* suggest that these genes can be compared similarly. Such observations should contribute to our understanding of how these interesting regulatory proteins carry out their specific roles in modulating intrinsic antibiotic resistance.

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**REFERENCES**


