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marA, a Regulated Locus Which Controls Expression of Chromosomal Multiple Antibiotic Resistance in *Escherichia coli*

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Stable chromosomal multiple-antibiotic-resistant (Mar) mutants of *Escherichia coli*, derived by exposing susceptible cells to low concentrations of tetracycline or chloramphenicol, express cross-resistance to structurally unrelated antibiotics. The entire resistance phenotype is reversed to susceptibility by insertion of transposon Tn*5* into a locus, designated *marA*, near 34 min on the chromosome (A. M. George and S. B. Levy, J. Bacteriol. 155:541–548, 1983). Strains in which 39 kbp of chromosomal DNA, including *marA*, had been deleted were unable to produce Mar mutants. The deletion strain could be complemented in *trans* by introduction of intact *marA*$^+$ on plasmid F'506. Junction fragments from a strain containing *marA*::Tn*5* were cloned, exploiting kanamycin resistance on Tn*5* for selection. They were used as probes to search a phasmid library of *E. coli* K-12 for recombinants containing the *marA*$^+$ region. Two phasmids which contained regions hybridizing to this probe were identified and shown to complement Δ*marA* in a deletion strain. From one phasmid, several *marA*-containing fragments were cloned: those of ≥7.8 kbp restored the ability to form Mar mutants in a deletion strain. These Mar mutants were shown to be dependent on the cloned *marA* fragment. Chromosomal as well as recombinant Mar mutants showed increased expression of a *marA*-specific mRNA species of about 1.4 kb, which was barely or not detectable in wild-type strains. Exposure of mutants and, to a lesser extent, parental strains to tetracycline or chloramphenicol resulted in elevated levels of mRNA which hybridized to the *marA* probe. These results indicate that the *marA* locus is needed for production of Mar mutants and is regulated, responding to at least two antibiotics to which it controls resistance.

Multiple antibiotic resistance in bacteria is most commonly associated with the presence of plasmids which contain one or more resistance genes, each encoding a single antibiotic resistance phenotype (2, 12). Multiple antibiotic resistance associated with the chromosome has been much less frequently reported (15, 17, 30, 34). George and Levy initially described a chromosomal multiple antibiotic resistance system (Mar) which existed in *Escherichia coli* and could be selected by a single drug, e.g., tetracycline or chloramphenicol (13). In addition to resistance to the selective agents, the Mar phenotype included resistance to structurally unrelated agents (13), including nalidixic acid, rifampin, penicillins, and cephalosporins; more recently, resistance to the fluoroquinolones has also been described (5). Insertion of Tn*5* into a single locus, designated *marA*, at 34 min on the *E. coli* map in a Mar mutant caused a complete reversal of the resistance phenotype (14). This article describes the cloning of the *marA* region, characterization of its role in the resistance phenotype, and regulation of its transcription.

MATERIALS AND METHODS

Organisms and basic microbiological methods. The strains of *E. coli* used in this study are listed in Table 1. Unless otherwise noted, cultures were grown in L broth (10 g of tryptone per liter, 5 g of yeast extract per liter, 5 g of NaCl per liter, 2 g of glucose per liter) at 30°C. Selective media were prepared by using antimicrobial agents. Tetracycline hydrochloride, ampicillin, chloramphenicol, nalidixic acid, rifampin, and kanamycin sulfate were obtained from Sigma Chemical Co. (St. Louis, Mo.); norfloxacin was generously provided by Merck and Co., Inc. (Rahway, N.J.). Chromosomal variants were constructed by P1 transduction (26). Conjugation with plasmid F'506 was carried out as previously described (21). The levels of resistance to antimicrobial agents of different strains were compared by streaking the strains side by side onto antibiotic gradient plates (7, 32). Approximate MICs from these plates were determined by interpolation of the relative lengths of growth, assuming a linear gradient across the plate.

Selection of Mar mutants and cloning procedures. Mar mutants were selected by plating overnight cultures onto MacConkey agar (Difco Laboratories, Detroit, Mich.) containing either tetracycline (3 μg/ml) or chloramphenicol (7 μg/ml). Colonies appearing after 2 to 4 days of incubation at 30°C were purified by streaking onto the initial selection medium.

For cloning purposes, the high-copy-number plasmid vector pUC18 (37) and the temperature-sensitive low-copy-number plasmid vector pHSG415 (16) were used. Strains were transformed with plasmid DNA by the method of Kushner (19).

A λ phasmid library constructed with λSE6 and consisting of 13- to 19-kbp partial *Sau*3A fragments from the *E. coli* K-12 derivative W3110 (9) was generously provided by G. Walker (Massachusetts Institute of Technology). Phasmids can be prepared either as plasmids at 30°C or as phage at 42°C (9). The library was initially propagated as phage in *E. coli* PLK1738. For screening of the library, plaques were prepared in the same host strain at 42°C in LB soft agar (26).

Preparation of deletion strains. The DNA with the 39-kbp

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TABLE 1. Bacterial plasmids and strains with relevant properties

Plasmid or strain	Relevant properties[a]	Source or reference
Plasmids		
pUC18	Multicopy vector; Amp^r	37
pHSG415	Temperature-sensitive low-copy-number vector; Amp^r Cm^r Kan^r	16
pHHM83	Amp^r; *c*I857	This study
pKan1	pUC18::(*marA*::Tn*5*/5.05 kbp[b]) Amp^r Kan^r	This study
pKan2	pUC18::(*marA*::Tn*5*/8.7 kbp) Amp^r Kan^r	This study
ph2	λSE6::(*marA*$^+$ region/12.4 kbp) Kan^r	This study
ph3	λSE6::(*marA*$^+$ region/13.1 kbp) Kan^r	This study
pHHM183	pHSG415::(*marA*$^+$ region/9-kbp *PstI* fragment from ph3) Insert in orientaton 1; *marA*$^+$ Amp^s Cm^r Kan^r	This study
pHHM184	Same as pHHM183 but 9-kbp *Pst*I fragment inserted in the opposite orientation	This study
pHHM201	Spontaneous mutant to *marA* of pHHM183 in the CH164 background	This study
pSPC7.8	pHSG415::(*marA*$^+$ region/7.8-kbp *Hpa*I-*Pst*I fragment from ph3)	This study
pSPC4.8	pHSG415::(*marA*$^+$ region/4.8-kbp *Hpa*I-*Bst*EII fragment from ph3)	This study
pSPC2.8	pHSG415::(*marA*$^+$ region/2.8-kbp *Hpa*I-*Pvu*II fragment from ph3)	This study
F'506	F' plasmid containing 31 to 37 min of the *E. coli* chromosome	26
E. coli strains		
AG100	*argE3 thi-3 rpsL xyl mtl supE44* Δ(*gal-uvrB*)	13
AG102	Mar derivative of AG100	13
AG1025	AG102 containing *marA*::Tn*5*	13
PLK1738	Trp^r *trpA his-29 ilv pro-2 arg427 thyA deo tsx gyrA rac zdd-230*::Tn*9* Cm^r; deletion of approximately 39 kbp from 33.6 to 34.3 min (Δ*marA*)	Peter Kuempel
HH74	Same as PLK1738 but *manA4 recA* Cm^r	This study
HH84	HH74 transformed with pHHM83. Amp^r Cm^r *c*I857	This study
MM294	*supE44 hsdR endA1 pro thi*	29
HH180	Same as MM294 but contains *zdd-230*::Tn*9* and Δ*marA*	This study
CH164	Same as AG100 but contains *zdd-230*::Tn*9* and Δ*marA*	This study
HH196	Second-step Mar mutant from HH180(pHHM184); expresses Tc^r at 10 μg/ml	This study

[a] Some mutants, independent from the ones listed in Table 1, are named by their parent strain, followed by the abbreviation and the highest concentration of the drug on which they were selected as well as by the number of steps necessary to reach that level of resistance. Drug resistance phenotype abbreviations: Amp, ampicillin; Cm, chloramphenicol; Kan, kanamycin; Tc, tetracycline.

[b] Size of DNA insert.

deletion from strain PLK1738 was transferred to additional strains by P1 transduction (26), taking advantage of the nearby Tn*9* for selection. In this way, CH164 was constructed from AG100 as a test strain for introduction of potential *marA* clones. Since the efficiency of transformation with CH164 was too low for it to effectively serve as an initial recipient for transformation of ligation mixtures, we similarly constructed HH180, a Δ*marA* derivative of the r$^-$ m$^+$ strain MM294, to serve as the initial recipient. Once obtained in HH180, the various plasmid clones were then transferred into CH164 for assessment of *marA* activity.

DNA preparation. Chromosomal DNA was isolated by the method of Marmur and Doty (24); plasmid DNA was prepared by the modified alkaline lysis procedure (22), with or without further purification by cesium chloride-ethidium bromide density equilibrium centrifugation (3). λ phage or phasmid DNA was purified by the method of Yamamoto et al. (36).

DNA analyses. Restriction endonucleases and T4 DNA ligase were obtained from New England BioLabs (Beverly, Mass.) and used according to the manufacturer's specifications. Agarose gel electrophoresis was performed as previously described (22), using Tris-borate-EDTA buffer and 0.8 to 1.2% agarose (International Biotechnologies Inc., New Haven, Conn.).

DNA labelling. DNA fragments extracted from agarose gels by a freeze-squeeze extraction method (1) were radiolabelled with [^{32}P]dCTP (New England Nuclear) by the random priming method (11), using a kit obtained from Boehringer Mannheim Biochemicals (Indianapolis, Ind.). Labelled probes were separated from unincorporated radionucleotides by passage through a Sephadex G-50 spin column (Boehringer Mannheim Biochemicals). Probes were stored at −20°C and denatured for 5 min at 95°C prior to use.

DNA transfer and hybridization. After electrophoresis, agarose gels were treated with 0.25 M hydrochloric acid for 20 min, followed by capillary transfer overnight to a positively charged nylon membrane (GeneScreen Plus; New England Nuclear) using 0.4 N sodium hydroxide (27). Following transfer, the membrane was neutralized with 0.2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) for 2 min and allowed to air-dry at room temperature. Hybridization of radiolabelled DNA probes to membrane-bound DNA was carried out at 68°C overnight and followed the procedure previously described (22). After hybridization, membranes were washed twice in 2× SSC for 5 min at room temperature, followed by two 30-min washes in 2× SSC containing 1.0% sodium dodecyl sulfate (SDS) at 68°C and two 30-min washes in 0.1× SSC at room temperature (22). Filters were dried and subjected to autoradiography, using Kodak XAR-5 film (Eastman Kodak Co, Rochester, N.Y.).

Colony and plaque screening. Bacterial colonies or bacteriophage λ plaques were transferred to nylon membrane filters (Colony/Plaque Screen; New England Nuclear) and treated by the manufacturer's protocol. The filters were air-dried, and hybridization and washing were done as described above, except prehybridization was carried out for 6 h.

RNA analysis. Total cellular RNA was extracted from cells by a hot phenol-acetate method (6, 33). Final RNA pellets were dissolved in sample buffer (50% deionized formamide, 16% formaldehyde, 20% glycerol, 0.02 M borate, 0.02 mM EDTA, 0.005% bromphenol blue, 0.005% xylene cyanol) by vigorous vortexing. The samples were heated to 65°C for 10 min and subjected to electrophoresis in 1% agarose containing 8.3% formaldehyde, 0.02 M borate, 0.02 M EDTA, and 2

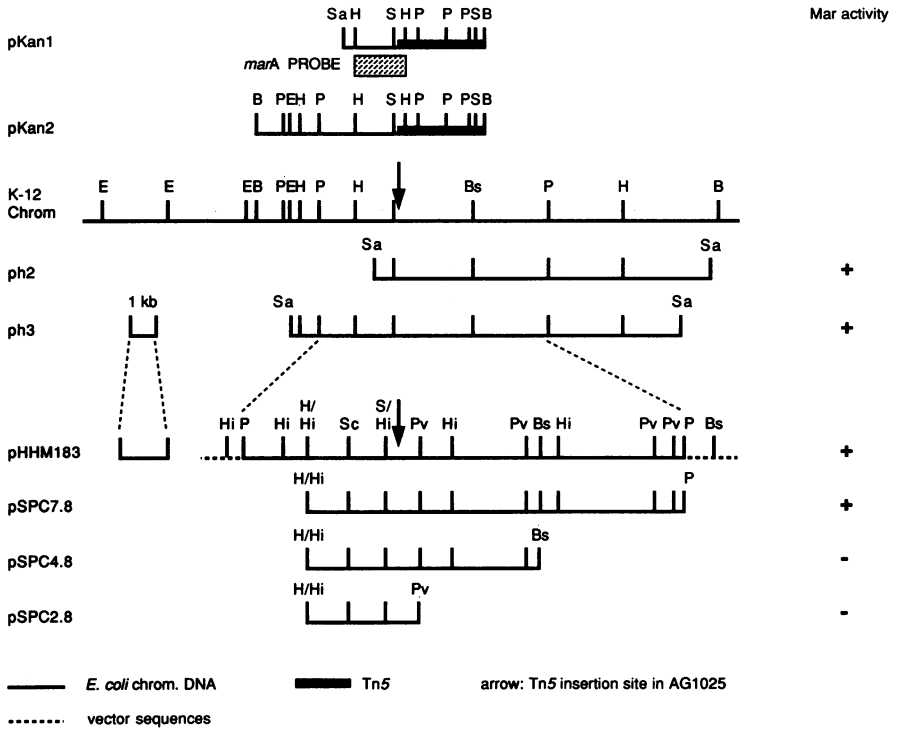

FIG. 1. Restriction endonuclease maps of the *E. coli* K-12 chromosome at 34 min as well as of relevant plasmid and phasmid constructs. Complete inserts and no vector sequences are shown. For the construction of the left branch of the chromosomal map, additional non-Mar active phasmids (unpublished data) were used. Thin lines indicate *E. coli* chromosomal DNA. Thick lines indicate Tn*5*. Dashed lines indicate vector sequences which allow the orientation of the insert to be judged. The arrows indicate the position of Tn*5* in the chromosome of AG1025. Those clones which conferred Mar-like activity to the deletion strain are indicated (+). Abbreviations: B, *Bam*HI; Bs, *Bst*EII; E, *Eco*RI; H, *Hpa*I; Hi, *Hin*dII; P, *Pst*I; S, *Sal*I; Sa, *Sau*3A; Pv, *Pvu*II; Sc, *Sca*I.

μg of ethidium bromide per ml. Following electrophoresis, the gel was rinsed briefly in distilled water and transferred overnight by capillary action to a nylon membrane with 5× SSC. Following transfer, the membrane was air-dried and heated to 80°C under vacuum for 2 h. Hybridization of radiolabelled DNA probes to the membrane-bound RNA was performed in a solution containing 1% SDS, 1 M sodium chloride, and 10% dextran sulfate at 60°C overnight following a 2-h prehybridization. RNA blots were washed as described above for DNA blots, except that the third and fourth washes were carried out at 60°C. Washed membranes were dried, and autoradiography was performed. The quantitation of bands on the exposed film was done by densitometry using a Scanalytics Masterscan densitometer.

RESULTS

Complementation of strains with *marA* deleted. In order to assess potential clones for Mar activity, we needed a strain with a defective *marA* locus. PLK1738 was an ideal candidate, since it contained a 39-kbp deletion extending from 33.6 to 34.3 min on the *E. coli* K-12 chromosome, thus including the *marA* region at 34 min. Stable Mar mutants could not be derived from PLK1738 (frequency of $<1 \times 10^{-9}$) or from its *recA manA* derivative, HH74 (frequency of $<3 \times 10^{-9}$). Plasmid F'506, which bears the *marA* region of *E. coli*, was mated into HH74. Selection for *manA*$^+$ on F'506 and Cm^r in the recipient resulted in the isolation of the transconjugant HH77, which produced Mar mutants at a frequency of 3×10^{-7}, comparable to results reported for wild-type *E. coli* (13). This finding indicated that the *marA* deletion could be complemented in *trans* by an intact *marA*$^+$ locus on F'506.

Cloning of the *marA* region. Initial attempts to clone the intact *marA* region using ColE1 vectors and large chromosomal fragments from the Mar mutant AG102 were unsuccessful. This failure was probably related to the low level of resistance conferred by the *marA* locus alone (25). Other approaches exploiting Kan^r from AG1025, a Tn*5*-inactivated, antibiotic-susceptible derivative of AG102 (i.e., *marA*::Tn*5*), in a multicopy plasmid also failed. Therefore, we cloned only a portion of the Tn*5* consisting of the Kan^r determinant and junctional DNA from the chromosome of AG1025. We took advantage of the single *Bam*HI site in Tn*5* and used chromosomal DNA from AG1025 digested with *Bam*HI or partially digested with *Sau*3A and ligated these fragments into the single *Bam*HI site in the polylinker region of pUC18. Two different plasmid clones were obtained, pKan1 and pKan2 (Fig. 1). Both contained 3.2 kbp from Tn*5* to the left of its internal *Bam*HI site and included flanking *E. coli* chromosomal DNA. pKan1, derived from partially digested *Sau*3A DNA, contained 2 kbp of chromosomal DNA, while pKan2, derived from *Bam*HI-digested DNA, contained 5.5 kbp of additional flanking DNA.

To isolate the intact, uninterrupted *marA* locus, we used the 2-kbp *Hpa*I junction fragment from pKan1, containing 187 bp from the IS*50*L of Tn*5* and 1.85 kbp of chromosomal DNA, as a probe (Fig. 1). We used a λ phasmid library derived from partial *Sau*3A digests of *E. coli* W3110 (9) to isolate a chromosomal DNA fragment bearing the genes. The isolation was performed in host strain PLK1738, in

which the *marA* region has been deleted and which does not hybridize with the 2.0-kbp probe. Several phasmids which hybridized with the probe were isolated. Two of these phasmids, ph2 and ph3, were mapped with restriction endonucleases (Fig. 1) and found to contain 12.4 and 13.1 kbp of insert DNA, respectively. By using different fragments of pKan1 and pKan2 as probes, the restriction map of the phasmids was found to correspond to the map of the intact chromosome of *E. coli* (data not shown) as displayed in Fig. 1. The restriction map of the *marA* region permitted its alignment with the restriction map of the *E. coli* chromosome generated by Kohara and coworkers (18). Using restriction sites for *Bam*HI, *Eco*RI, *Pst*I, and *Pvu*II, we were able to unambiguously locate the Tn*5* insertion site in AG1025 to 1,636.7 kb of the map of Kohara et al. (18).

Assay of phasmids for *marA* activity. For detection of *marA* activity of recombinant phasmids, we used the *marA* deletion strain HH84, which contained the temperature-sensitive λ^- repressor *c*I857 on plasmid pHHM83. The latter is a pNT204 replicon (31) containing a 2.4-kbp Amp^r-mediating fragment from a pMF45 (23) derivative, cloned into the single *Bam*HI site within the Tc^r determinant of pNT204. This strain construction allowed replication and functioning of the phasmid as a plasmid at the permissive temperature (30°C) and further permitted extraction of phasmid DNA from lytic phage once the culture was shifted to 42°C. Destruction of the Tc^r determinant on pNT204 allowed assessment of Mar activity on agar containing tetracycline.

The phasmids were introduced as phage by transduction into deletion strain HH84, which was unable to produce Mar mutants (frequency of $<6 \times 10^{-9}$). They were selected for, maintained, and tested phenotypically in the recipients via the phasmid marker (Kan^r) at 30°C, when they replicated as low-copy-number plasmids. HH84 containing ph2 or ph3 was able to yield Mar mutants at a frequency of approximately 2×10^{-6} upon tetracycline selection at 3 μg/ml. Resistant colonies from the phasmid-containing strains were isolated and subjected to curing by growth at 42°C overnight in the absence of phasmid selection. Many Kan^s derivatives which did not contain the phasmids were obtained, as judged by the strains' inability to produce bacteriophage particles and by direct examination of total cellular DNA. Several of these were compared with the original Kan^r isolates which had not been carried through the curing procedure. The phasmidless derivatives lost the *marA* phenotype, as judged by loss of tetracycline and norfloxacin resistance, while Kan^r cultures still expressed resistance to these antibiotics.

Phasmid DNA was isolated from wild-type and resistant cultures of HH84(ph2) and HH84(ph3) and transduced back into HH84. After kanamycin selection of transductants containing the phasmids, these transductants were isolated and tested for antibiotic resistance on gradient plates containing tetracycline. Phasmids derived from multiply resistant strains, but not from sensitive ones, were able to confer increased tetracycline resistance relative to HH84 (Fig. 2). We noted that the intrinsic tetracycline resistance of HH84 was lower than those of other wild-type *E. coli* strains, possibly as a result of the presence of pHHM83 containing the *c*I857 gene. Upon curing of the phasmids derived from multiply resistant strains, the increased tetracycline resistance was lost (Fig. 2). These results indicate that the DNA cloned into ph2 and ph3 was capable of restoring *marA* activity in the deletion mutant and that the resistance phenotype selected was linked to the phasmid.

Subcloning of MarA activity into a low-copy-number cloning vector pHSG415. DNA fragments from wild-type ph3 were used for subcloning *marA*$^+$ into the low-copy-number vector pHSG415. Mar activity was tested in CH164, a Δ*marA* strain genetically related to the original AG100 Mar mutants. Subclones containing either the 9-kbp *Pst*I or 7.8-kbp *Hpa*I-*Pst*I fragment, but none containing any smaller fragments (Fig. 1), produced Mar mutants.

Expression of the Mar phenotype by these cloned DNAs was confirmed by the following three criteria (Fig. 3): (i) the ability of strains bearing the wild-type chromosomal fragment to produce at least second-step Mar mutants of CH164 upon transfers in low concentrations of tetracycline; (ii) the appearance of immediate multiple resistance after introduc-

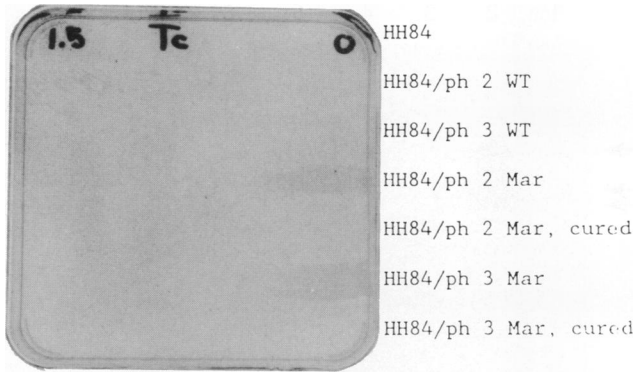

FIG. 2. Resistance phenotypes of *E. coli* HH84 containing phasmids bearing the *marA*$^+$ or *marA* region on nutrient agar gradient plate containing 0 to 1.5 μg of tetracycline (Tc) per ml. WT, wild type.

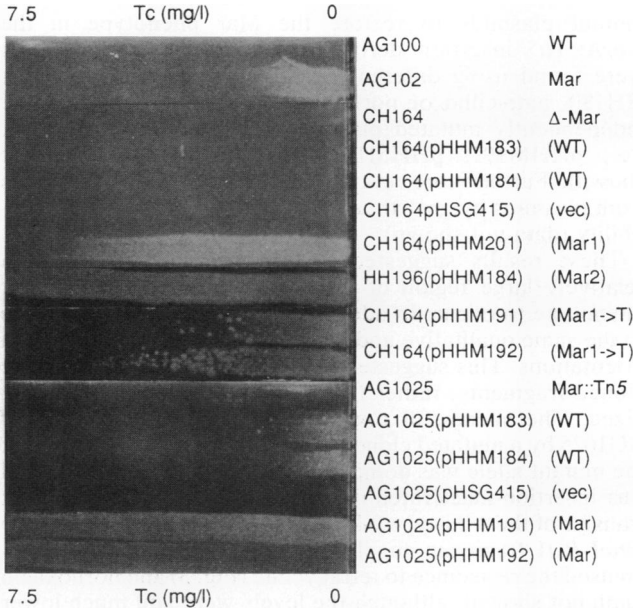

FIG. 3. Resistance phenotypes of *E. coli* K-12 constructs, deficient in *marA* (by deletion or by Tn*5* insertion), with or without recombinant plasmids. Growth on nutrient agar gradient plates containing 0 to 7.5 μg of tetracycline (Tc) per ml is shown. WT, wild type; Mar, Mar mutants; Mar1 or Mar2, first- or second-step Mar mutants resistant to 3 or 10 μg of tetracycline per ml, respectively; Mar1→T, mutant plasmid transferred into fresh deletion host; vec, vector. The strains shown had never been in contact with tetracycline before this experiment.

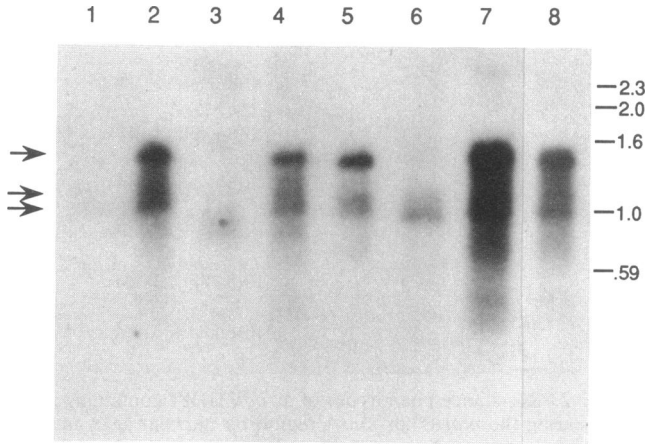

FIG. 4. Autoradiogram of RNA isolated from various *E. coli* strains. Mar mutants were grown in the presence of tetracycline (2 μg/ml). RNA was subjected to electrophoresis in 1.0% agarose, blotted to a nylon membrane, and hybridized to the 2.0-kbp *marA* probe. The strains examined are AG100 (lane 1), AG100-Tc2.5-1 (lane 2), AG100-Tc2.5-1-Kan (lane 3), AG100-Tc50-4 (lane 4), AG100-Cm7-1 (lane 5), CH164(pHHM183) (lane 6), CH164(pHHM 201) (lane 7), and CH164(pHHM201) (lane 8) (shorter exposure of lane 7). The positions of radioactive molecular weight standards (λ *Hin*dIII digest; 1-kb ladder from Bethesda Research Laboratories) are shown to the right of the gel. Arrows point to RNA bands of 1.4, 1.1, and 1.0 kb. The relative amounts of RNA loaded in each lane were estimated by visualization of the rRNA subunits by ethidium bromide staining.

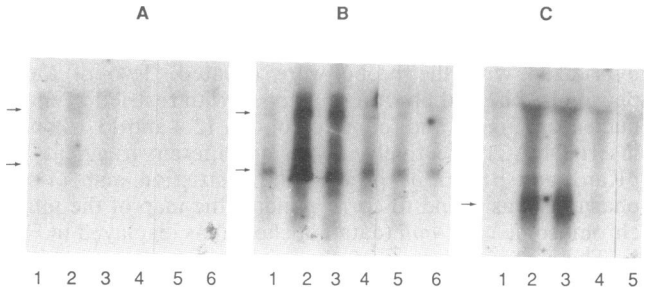

FIG. 5. Expression of the *marA* region in different derivatives of AG100. RNA was separated by electrophoresis in 1.0% agarose, transferred to a nylon membrane, and hybridized to the 2.0-kbp *marA* probe. Results from an autoradiogram are presented for AG100 (A), AG100-Tc2.5-1 (B), and AG100-Tc2.5-1-Kan (C). RNA was isolated 1 h after the addition of the following compounds to logarithmic-phase cultures growing in LB broth at 30°C with vigorous shaking: no additions (lanes 1), tetracycline (2 μg/ml) (lanes 2), chloramphenicol (5 μg/ml) (lanes 3), nalidixic acid (2 μg/ml) (lanes 4); ampicillin (10 μg/ml) (lanes 5), and norfloxacin (0.2 μg/ml) (lanes 6). Arrows point to 1.4- and 1.0-kb RNA bands in panels A and B and to the 0.7-kb RNA band in panel C.

tion of an already mutated plasmid into a deletion host without challenge by antibiotics; and (iii) the ability of mutant plasmids to restore the Mar phenotype in the *marA*::Tn*5* insertion derivative, AG1025. Similar results were found using different components (the deletion host HH180, ampicillin or norfloxacin gradient plates, and the independently mutated plasmids derived from pHHM183, i.e., pHHM191, pHHM192, and pHHM193) (data not shown). Furthermore, curing multiply resistant derivatives from plasmids by growth at 42°C restored wild-type susceptibility (data not shown).

These results suggested that in the deletion strain, a relatively large region of DNA was required for the Mar phenotype to occur. In addition, active fragments gave rise to the same qualitative and quantitative Mar activity in both orientations. This suggested that a promoter from within the cloned fragments, rather than from the vector, was recognized. The successful reversion to multiple resistance of AG1025 by a mutated cloned fragment (Fig. 3) suggested that the mutant allele was dominant in *trans* to the chromosomal Tn*5* insertion-inactivated region. In addition, we found that transfer of the wild-type 9-kb *Pst*I fragment (Fig. 3) or 7.8-kb *Hpa*I-*Pst*I fragment on plasmids into AG1025 slightly increased the resistance to tetracycline (Fig. 3) and norfloxacin (data not shown), although the levels were still much lower than those observed when fragments bearing mutant *marA* regions were introduced. This finding suggested some form of *trans* effect with several extrachromosomal wild-type copies of the *marA*$^+$ region.

Restriction endonuclease analysis of subcloned fragments. Plasmids pHHM183 and pHHM184 as well as their Mar mutant derivatives pHHM191→193 and pHHM201 were subjected to restriction endonuclease analysis. All plasmids had restriction endonuclease profiles which were unchanged from the wild type, e.g., pHHM183 (Fig. 1).

RNA transcription from *marA*. We examined the RNA isolated from the susceptible strain AG100 and its derivatives, Mar mutants AG100-Tc2.5-1, AG100-Tc50-4, and AG100-Cm7-1, as well as the *marA*::Tn*5* strain AG100-Tc2.5-1-Kan, for hybridization with the 2.0-kbp *Hpa*I junction fragment from pKan1 (Fig. 4). Three bands measuring 1.4, 1.1, and 1.0 kb were present in the mutants but were also expressed to a much less degree in the wild type and were visible only when the autoradiogram was overexposed. In mutants, the 1.4-kb band predominated, whereas in the wild type, the 1.0-kb band was the most prominent. It is not known whether the 1.1- and 1.0-kb bands are breakdown products of the 1.4-kb transcript. A similar pattern of hybridization was observed for both the low (first-step) and high (fourth-step) tetracycline-selected mutants as well as the first-step chloramphenicol-selected mutant. Moreover, the higher-level mutant (e.g., AG100-Tc50-4) did not have increased levels of *marA*-specific RNA relative to the first-step mutant (AG100-Tc2.5-1) from which it was derived (Fig. 4). The same pattern of altered transcription in the mutants was also observed for plasmid-containing strains (Fig. 4), although the amount of mRNA was greater. The plasmid-containing Mar mutants produced six- to eightfold-more hybridizing mRNA than the chromosomal Mar mutants did. The strains containing plasmids (4 to 6 copies per cell) with a wild-type Mar region now produced visible amounts of hybridizable mRNA.

The transcripts which hybridized with the 2.0-kbp *Hpa*I probe and the 9-kbp *Pst*I fragment from ph3 were also compared. The 9-kbp probe did reveal some additional weak RNA bands, but none of these bands increased in the mutant compared with the wild type; only the three bands already detected by the 2.0-kbp *Hpa*I fragment showed an increase (data not shown). Therefore, the failure to obtain Mar mutants in deletion strains with subclones smaller than 7.8 kbp suggests that other regions on the cloned fragments are involved in the resistance phenotype and are expressed constituitively. Their transcription patterns were not detectably altered in the mutant.

Inducibility of *marA* transcripts. Various antibiotics whose susceptibility levels are altered in Mar mutants were tested for their ability to induce RNA which hybridized to the Mar probe. AG100, AG100-Tc2.5-1, and AG100-Tc2.5-1-Kan were grown to late logarithmic phase, at which time the antibiotics were added and incubated at 30°C for 1 h prior to RNA extraction. Tetracycline and chloramphenicol caused an increase in *marA* hybridizable RNA (Fig. 5). Ampicillin, nalidixic acid, and norfloxacin did not. The greatest level of induction was in the Mar mutant: 16-fold for tetracycline and 9-fold for chloramphenicol. The wild-type strain also showed inducibility of the same RNA by these compounds (fourfold for tetracycline and twofold for chloramphenicol). The Tn*5*-inactivated mutant also showed induction responses to tetracycline and chloramphenicol of 15- and 10-fold, respectively; however, the size of the transcript was smaller, approximately 0.7 kb.

These results suggest that compounds such as tetracycline and chloramphenicol are capable of inducing transcription from the *marA* region even in susceptible *E. coli* and that mutation to Mar involves the enhanced ability to respond to these agents by increasing transcription. Also, the insertion of Tn*5* that restores wild-type susceptibility levels to a mutant does not interfere with the induction of transcription by tetracycline or chloramphenicol but results in the increased expression of a truncated message.

DISCUSSION

Chromosomal multiple antibiotic resistance in *E. coli* has been shown to rely on the activity of a locus designated *marA* at 34.05 min on the chromosome (13, 14). Previous studies have shown that this locus can affect distant chromosomal genes, such as *micF*, and can indirectly affect *ompF* (6). The function of a wild-type version of this locus (*marA*) has not been determined, nor has it been determined whether it is normally cryptic or silent. Direct cloning of the mutant *marA* locus from a Mar mutant was initially unsuccessful because the level of the *marA*-encoded resistance was not high enough to be exploited for selection (25). However, using a junctional fragment as a probe to a library of *E. coli* K-12, followed by subcloning of hybridizing phasmid fragments into a low-copy-number plasmid vector, we created a collection of *marA*$^+$-containing clones with inserts ranging from 13.1 to 2.8 kbp.

Strains with a large (39-kbp) chromosomal deletion at 34 min, which included all of *marA*, could not become Mar mutants. We examined the ability of these deletion strains to become Mar mutants when they contained different-sized chromosomal fragments from the *marA*$^+$ region. Mutation to a Mar phenotype was observed at the same frequency as that in wild-type cells, but only in those with clones containing ≥7.8 kbp of chromosomal DNA. Data from smaller subclones suggest that the minimal functional *marA* lies between the *Hpa*I site and the Tn*5* insertion site on the right and includes all the DNA extending at least beyond the *Bst*EII site and possibly as far as the *Pst*I site (Fig. 1). Recent sequencing has revealed a putative operon of little over 1 kb in the region of the Tn*5* insertion site (4). Since only a 1.4-kb *marA*-specific mRNA species (hybridizing to a region close to the Tn*5* insertion site) was increased in cells bearing the ≥7.8-kbp *marA*-containing fragments, it would appear that other genes besides *marA* were needed to produce Mar in the deletion strain.

Mutation from *marA*$^+$ to *marA* led always to increased transcription of the specific 1.4-kb mRNA. In those plasmids bearing the mutated gene(s), the restriction endonuclease fragments were indistinguishable from those of the wild type, implying that the mutation involved only small if not single-base changes. High-level Mar mutants showed no further increase in *marA*-specific mRNA, suggesting that other changes relate to phenotypes with higher resistance. Moreover, increased copy number of the wild-type *marA* region led to detectable amounts of *marA* hybridizable RNA and partial complementation of *marA*::Tn*5*. These findings suggest a regulatory, not structural, change in the *marA* region. Sequence analysis and characterization of wild-type and mutant plasmids will determine the basis for these mutations (work in progress).

Tetracycline and chloramphenicol were able to induce transcription from the *marA*$^+$ region about two- to fourfold. This induction was even greater (16- and 9-fold) in Mar mutants. Tn*5*-inactivated Mar mutants responded in a similar fashion to antibiotic induction but expressed a truncated message of approximately 0.7 kb. Thus, a mutation in the *marA* locus of the wild-type strain produces strains which are not only constitutively antibiotic resistant but also more responsive to antibiotic induction. In addition, inactivation of the *marA* locus by Tn*5* insertion reverses the antibiotic resistance phenotype, but not the inducibility of the region.

Plasmid-encoded multiple antibiotic resistance in bacteria usually arises as a result of insertion of different single-resistance genes into one transposon or one replicon by recombination, transposition, or other mechanisms (2, 12). Usually the different genes are regulated independently, and the mechanisms of resistance are different. In contrast, chromosomal antibiotic resistance mediated by *marA* seems to be a multigenic coordinated phenotype under the control of a single locus (14). A global regulatory role of *marA* is also suggested by the finding that loci far from 34 min are affected by *marA*, such as *micF* and *ompF* (6). Moreover, the present findings show that *marA* itself appears to be regulated.

Other chromosomal multiple antibiotic resistance phenotypes have been described in bacteria, namely, those associated with resistance to β-lactams (34) or different unrelated antibiotics (15, 30). The genetic basis for these mutants has not yet been reported. In *Neisseria gonorrhoeae*, a number of chromosomally mediated resistant clinical isolates have been described (10, 17, 28). The resistance is due to a multigenic system which involves several identified genes, including *mtr*, *penA*, *penB*, and *tet*. These genes can act singly or in combination with one another to cause various levels of resistance to β-lactams, cephalosporins, tetracycline, erythromycin, and in the case of *mtr*, dyes and detergents (17). The nature and regulation of the interaction of the various loci in *N. gonorrhoeae* have not been described. The independent nature of many of the genes in *N. gonorrhoeae* is unlike the *marA* phenotype, where all resistances appear to be controlled by a single regulatory locus.

Multiple drug resistance (Mdr) has also been detected in eucaryotes such as *Saccharomyces cerevisiae* (20) and *Plasmodium falciparum* (35) and in mammalian tumor cells (8). In Mdr mammalian cells, as in Mar mutants, multidrug resistance emerges after challenge of cells with one drug and increases under stepwise elevation of the drug concentration. The corresponding sets of drugs are different for each system. Whether an analog of the P glycoprotein, the major Mdr protein identified, is involved in Mar mutants has not been determined.

The response of Mar to antibiotics, such as tetracycline or chloramphenicol, and the activity against other noninducers, such as quinolones, indicate that the spectrum of antibiotics

and other compounds for which this resistance system acts may not yet be fully understood. Certainly the cross-resistance of the Mar mutants to newer quinolones, selected by antibiotics such as tetracycline or chloramphenicol (5), poses a direct potential clinical problem.

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