The Periplasmic Protein MppA Requires an Additional Mutated Locus To Repress marA Expression in Escherichia coli

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Escherichia coli strain TP985, which has an insertional mutation in the gene for the periplasmic murein tripeptide binding protein MppA, was previously reported to overproduce MarA and exhibit a multiple-antibiotic resistance (Mar) phenotype (H. Li and J. T. Park, J. Bacteriol. 181:4842-4847, 1999). We found that TP985 contained a previously unrecognized marR mutation which was responsible for the Mar phenotype. Transduction of the mppA mutation from TP985 to another wild-type strain did not affect antibiotic susceptibility. Overproduction of MppA repressed marA transcription in TP985 but not in other mppA or marR mutants. Therefore, TP985 contains an additional unknown mutation(s) that facilitates the repression of marA expression by MppA.

The Escherichia coli marRAB operon specifies two regulatory proteins. MarR is a negative autoregulator of the marRAB operon whereas MarA positively regulates transcription of the operon (1, 7) and other genes in a mar regulon (6, 26). In the absence of an inducer, MarR presumably binds as a dimer to sites I and II in the operator and represses the expression of the marRAB operon (1, 20, 21). In the presence of specific external inducers, however, MarR repression is alleviated, resulting in increased transcription of marRAB (8, 27). A number of inducers of the marRAB operon have been identified; some have been shown to interact directly with MarR and alter MarR-DNA binding activity (3, 21). Mutations in marR that inactivate the MarR repressor function lead to constitutive expression of the marRAB operon (13, 16, 19, 23).

Derepression of the marRAB operon results in an elevated amount of MarA (1). MarA either directly or indirectly affects the transcription of many other genes in the mar regulon, more than 60 to 80 different genes (6, 26). Among those that are regulated by MarA are many genes coding for proteins that are important in antimicrobial resistance, including the OmpF porin and the AcrAB multiple-drug efflux system (6, 9, 17, 18). The combination of a reduced outer-membrane permeability and increased efflux capacity in the Mar mutants provides Escherichia coli with resistance to multiple antibiotics and other antimicrobial compounds, including dyes, detergents, organic solvents, and oxidative stress agents (1, 22, 25, 29). This MarA-dependent phenotype is referred to as the multiple-antibiotic resistance (Mar) phenotype.

Recently, it was reported that a null mutation in the E. coli gene mppA, which encodes a periplasmic murein tripeptide binding protein, resulted in the overproduction of MarA and thus a Mar phenotype (15). Further, overexpression of MppA from a plasmid complemented the mppA null mutant and resulted in the repression of marA transcription and the reversal of the Mar phenotype. These observations led the authors to conclude that MppA was a negative regulator of the marRAB operon (15). In the present study, we found that both MarR and MppA repressed marA expression in TP985 and that TP985 contained a mutation in marR. We also found that TP985 contained a mutation in another gene(s) that allowed MppA to repress marA expression.

E. coli strains listed in Table 1 were grown in Luria-Bertani broth at 37 or 30°C. For strain AT980 and its derivatives, diaminopimelic acid (Sigma) was added to a final concentration of 50 μg/ml. For plasmid maintenance and marker selection, antibiotics purchased from Sigma were used at the following concentrations: kanamycin, 50 μg/ml; ampicillin, 100 μg/ml; chloramphenicol, 22 μg/ml; and gentamicin, 15 μg/ml. DNA manipulations were performed according to standard procedures. PCR amplification was performed with colonies of AT980 or TP985 or with chromosomal DNA of MG1655 as the template by using Taq or Pfx DNA polymerase according to the instructions of the manufacturer (Gibco BRL, Life Technologies). Northern and Western blot analyses were performed as described previously (6, 28).

TP985 has a single base pair deletion within the marR gene. TP985 contains a null mutation in mppA and was found to overproduce MarA (15). We observed that MarR expressed from pACMarR, a low-copy wild-type marR expression vector (2), caused the repression of marA transcription in TP985 (data not shown). This complementation data suggested that MarR was either not expressed or not functional in TP985. To address this question, the DNA fragments comprising marOR from nucleotides 1201 to 1901 (GenBank accession number M96235) were separately amplified by PCR from the chromosomes of TP985 and AT980 and sequenced. There was a deletion of an adenine at nucleotide 1816 near the 3′ end of marR in TP985 compared to the sequence of the parent strain AT980. The deletion resulted in the change of Asn-126 to Thr and generated a frameshift, resulting in the loss of the 18

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C-terminal amino acids of MarR. Interestingly, an identical mutation was reported in a clinically isolated Mar mutant (16).

To address whether the truncated form of MarR (herein named MarRΔc) present in TP985 was functional as a repressor, the mar operon from TP985 and wild-type MG1655 were separately cloned into the low-copy vector pBBR1MCS-5 (14) between the EcoRI and BamHI sites to generate the plasmids pMRwt and pMRΔc, respectively. pMRwt and pMRΔc were subsequently transformed into E. coli marRΔc mutant AG112, which has a 5-bp deletion near the 5′ end of marR and therefore overexpresses marA and exhibits the Mar phenotype (24). As shown in Table 2, MarRΔc expressed from pMRΔc did not repress antibiotic resistance in AG112 whereas wild-type MarR expressed from pMRwt did. Therefore, MarRΔc from TP985 was not functional.

The MarR repressor function was also evaluated in strain ASS121, which contains a deletion of the marCORAB locus and a mar promoter-lacZ fusion at the λ att site (27). β-Galactosidase analyses showed that wild-type MarR expressed from pMRwt repressed lacZ expression by nearly 99% whereas MarRΔc expressed from pMRΔc had little effect (less than 9% repression) (Table 3). These data were consistent with the complementation results for AG112, confirming that the truncated MarRΔc present in TP985 did not function as a repressor of the mar operon. The data are consistent with the MarR crystal structure that demonstrates the formation of a dimeric protein through the interaction of the N terminus of one MarR subunit with the C terminus of a second MarR subunit (4). In the crystal structure, Lys-140 and Pro-144, which are both absent in MarRΔc18, were found to be important for the interaction (4). Our data confirm the importance of the C terminus of MarR for its repressor function.

mppA deletion did not affect antibiotic susceptibility. The finding of the marR mutation in TP985 suggested that this mutation was responsible for the Mar phenotype in TP985. However, Li and Park found that mppA expressed in trans repressed marA expression in TP985 and therefore suggested that MppA was a negative regulator of marA expression (15). To clarify this finding further, two types of mppA mutants were generated from wild-type strains AT980 and AG100. One

<table>
<thead>
<tr>
<th>Strain</th>
<th>MICs (µg/ml) of:</th>
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<tbody>
<tr>
<td></td>
<td>TET</td>
</tr>
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<td>AG112(pBBR1MCS-5) Vector control</td>
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</tr>
<tr>
<td>AG112(pMRwt) Wild-type MarR</td>
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<tr>
<td>AG112(pMRΔc) MarRΔc</td>
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</table>

* MICs were determined by using Etests (AB Biodisk). TET, tetracycline; CHL, chloramphenicol; AMP, ampicillin; NAL, nalidixic acid.

**TABLE 2.** Antibiotic susceptibilities of marR mutant AG112 complemented by cloned wild-type or mutant marR

MarRΔc expressed from pMRΔc had little effect (less than 9% repression) (Table 3). These data were consistent with the complementation results for AG112, confirming that the truncated MarRΔc present in TP985 did not function as a repressor of the mar operon. The data are consistent with the MarR crystal structure that demonstrates the formation of a dimeric protein through the interaction of the N terminus of one MarR subunit with the C terminus of a second MarR subunit (4). In the crystal structure, Lys-140 and Pro-144, which are both absent in MarRΔc18, were found to be important for the interaction (4). Our data confirm the importance of the C terminus of MarR for its repressor function.

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**TABLE 3.** β-Galactosidase activities of Pmar-lacZ fusion strains expressing wild-type or mutant marR

α The mean of results from triplicate samples.
A mutant was made by the λ-red method (11), in which the mppA gene on the chromosome of AG100 was replaced by a kanamycin resistance cassette; the cassette was subsequently deleted. The resultant strain AG100ΔM was compared with AG100 for changes in antibiotic susceptibility profiles by using the gradient plate method (10). No difference was observed between strains AG100 and AG100ΔM in the susceptibilities to tetracycline, nalidixic acid, chloramphenicol, rifampin, nor-
magnesium protein and ampicillin (data not shown). Consistent with these results, Northern and Western analyses showed no changes in marA transcription (Fig. 1A) or MarA production in AG100ΔMar (data not shown). To rule out the possibility that the specific mutated mppA construct in TP985 contributed to the observed Mar phenotype in TP985, we transduced the mppA::Cm mutation from TP985 to strains AT980 and AG100 to create strains RB923 (AT980 mppA::Cm) and RB919 (AG100 mppA::Cm), respectively. Analysis of these strains showed that they also were unchanged in their susceptibilities to the tested antibiotics (data not shown).

To define whether mutations at both mppA and marR loci have any additional effect on the Mar phenotype, we introduced an mppA deletion into the chromosome of strain AG112 via the λ-red method. The resultant strain AG112ΔM showed no difference compared to AG112 in its susceptibilities to tetracycline, nalidixic acid, and chloramphenicol (data not shown). In addition, no difference in the level of marA transcription (Fig. 1A) or MarA production was detected between AG112 and AG112ΔM (data not shown).

Taken together, the above data show that the mppA null mutation did not result in a Mar phenotype and that the mutation of marR and not the mutation of mppA caused the Mar phenotype in TP985.

MppA restored marA repression in strain TP985 only. Our Northern analysis (Fig. 1C, lane 3) and gradient plate tests (data not shown) confirmed Li and Park’s observation that mppA expression from pMLD1285 repressed marA expression in TP985 (15). However, it was not clear whether such complementation was due to the restoration of MppA or MarR function. Therefore, pMLD1285 was introduced into AG112 and the resulting strain was examined for marA expression. Northern analysis and gradient plate tests showed no change in marA expression (Fig. 1D, lane 3, and Table 4).

One difference between TP985 and AG112 was that TP985 could presumably produce a truncated MarRΔc whereas AG112 could not produce any MarR. Perhaps MppA could facilitate the formation of stable MarRΔc dimers, since MarRΔc itself probably could not form stable dimers. This possibility was ruled out by cotransforming pMRΔc and pMLD1285 into AG112 and showing by Northern analysis that coexpression of MppA and MarRΔc in AG112 did not restore MarRΔc repressor function (Fig. 1B, lane 4). In further studies, the marRΔc gene (cloned from TP985 into temperature-sensitive plasmid pMAK705 to create pXB97) was separately introduced into chromosomes of AG112 and AT980 by homologous recombination (27). The resulting mutants RB928 (AG100 marRΔc) and RB932 (AT980 marRΔc) overexpressed marA as expected (Fig. 1C and D, lanes 4). Overproduction of MppA did not affect marA expression in either of these two backgrounds (Fig. 1C and D, lanes 6). Further, the susceptibilities of both of these strains to tetracycline, nalidixic acid, and ciprofloxacin were unchanged (Table 4 and data not shown).

We also determined whether MppA would repress marA expression in an mppA::Cm and marRΔc double mutant, mppA::Cm was PI transduced from TP985 into RB928 and RB932 to create strains RB955 and RB957, respectively, bearing both the mppA::Cm and marRΔc mutations. No change in the susceptibilities to tetracycline, nalidixic acid, and ciprofloxacin was observed in either strain (Table 4 and data not shown). Northern analysis indicated that the expression of MppA from pMLD1285 in the mppA::Cm and marRΔc double mutants did not repress marA expression (Fig. 1C and D, lanes 9). Only in TP985 did MppA repress marA gene expression (Fig. 1C, lane 3). Based on these results, we hypothesize that TP985 has at least one additional mutation at an unknown locus which responds to the overproduction of MppA in a MarR-deficient strain to cause the repression of marA expression. We are currently trying to identify the additional mutation(s).

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REFERENCES


### Table 4. Antibiotic susceptibilities of E. coli wild-type strains and their mar or mppA mutants

<table>
<thead>
<tr>
<th>Assay</th>
<th>Strain</th>
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<th>Growth on gradient plates (distance in mm)</th>
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<tr>
<td></td>
<td></td>
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<td>TET</td>
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<tr>
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<td>AG100 mppA::Cm marRΔc</td>
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<td>70</td>
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a Each number represents an individual experiment tested on the same gradient plate.

b Data presented here are means of results from three duplicate assays of a representative experiment performed by the gradient plate method. Numbers indicate the distances, in millimeters, that each bacterial culture grew on the gradient plate. The antibiotics were used at various concentrations, in micrograms per milliliter, as follows: tetracycline (TET), 6; nalidixic acid (NAL), 24; and ciprofloxacin (CIP), 0.08 for plate 2, 0.16 for plates 1, 3, and 4.