JOURNAL OF BACTERIOLOGY, Aug. 1999, p. 4842–4847 Vol. 181, No. 16
0021-9193/99/$04.00+0

The Periplasmic Murein Peptide-Binding Protein MppA Is a Negative Regulator of Multiple Antibiotic Resistance in *Escherichia coli*

HONGSHAN LI AND JAMES T. PARK*

Department of Molecular Biology and Microbiology, Tufts University School of Medicine, Boston, Massachusetts 02111

Received 25 February 1999/Accepted 2 June 1999

MppA is a periplasmic binding protein in *Escherichia coli* essential for uptake of the cell wall murein tripeptide L-alanyl-γ-D-glutamyl-*meso*-diaminopimelate. We have found serendipitously that *E. coli* K-12 strains carrying a null mutation in *mppA* exhibit increased resistance to a wide spectrum of antibiotics and to cyclohexane. Normal sensitivity of the *mppA* mutant to these agents is restored by *mppA* expressed from a plasmid. As is observed in the multiple antibiotic resistance phenotype in *E. coli* cells, the *mppA* null mutant overproduces the transcriptional activator, MarA, resulting in expression of the membrane-bound AcrAB proteins that function as a drug efflux pump. Reduced production of OmpF similar to that observed in the multiple antibiotic resistance phenotype is also seen in the *mppA* mutant. These and other data reported herein indicate that MppA functions upstream of MarA in a signal transduction pathway to negatively regulate the expression of *marA* and hence of the MarA-driven multiple antibiotic resistance. Overproduction of cytoplasmic GadA and GadB and of several unidentified cytoplasmic membrane proteins as well as reduction in the amount of the outer membrane protein, OmpP, in the *mppA* null mutant indicate that MppA regulates a number of genes in addition to those already known to be controlled by MarA.

mppA codes for the precursor of a periplasmic binding protein essential for import of murein tripeptide into *Escherichia coli* (29). MppA utilizes membrane and cytoplasmic component(s) of the oligopeptide permease to transfer its bound ligand into the cytoplasm of the cell (29). However, very little free murein tripeptide is transported into the cell (28); in fact, nearly all murein tripeptide is transported into the cell via the AmpG permease (18) in the form of *N*-acetylglucosaminyl-β-1,4-anhydro-*N*-acetylmuramyl-tripeptide (15), which is then cleaved by AmpD amidase (14, 16) to release the murein tripeptide. This caused us to wonder why *E. coli* has a minor pathway via MppA to transport free murein tripeptide (29). Could the binding protein, complexed with the tripeptide, serve a signaling function reporting a change in the periplasmic environment where MppA resides rather than simply providing a small additional amount of tripeptide to the cell? A clue to such a possible function arose when we observed that the *mppA* mutant was significantly more resistant to tetracycline and cefoxitin than the isogenic wild type. Results presented in this report demonstrate that in the absence of MppA, the cell exhibits all of the properties associated with the multiple antibiotic resistance (MAR) phenotype (1). MAR is primarily controlled by the multiple antibiotic resistance (*mar*) operon (1). The *mar* locus consists of two divergently positioned transcriptional units that flank the operator *marO* in *E. coli* (2) and in *Salmonella typhimurium* (36). One unit encodes MarC, a putative integral inner membrane protein whose function is unknown. The other unit is an operon comprising *marRAB*, encoding the MarR repressor, which binds to *marO* and negatively regulates expression of *marRAB* (24, 33); MarA, a transcriptional activator (1), which activates expression of other genes, notably, *acrAB*, encoding the multidrug efflux pump (1, 21) and the *mar* regulon itself (9, 33); and MarB, coding for a small protein of unknown function.

A reduction in the amount of the outer membrane porin, OmpF, is associated with MAR (3) and is also observed in the *mppA* mutant. Another outer membrane protein, the OmpP protease, is present in greatly reduced amounts in the *mppA* mutant. The *mppA* null mutant also overproduces cytoplasmic glutamate decarboxylases GadA and GadB and several inner membrane proteins that may or may not be associated with MAR. Thus, MppA appears to play a central role in the regulation of a number of proteins including those responsible for MAR.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The *E. coli* K-12 strains used in this study are listed in Table 1. All bacterial strains were grown at 37°C with shaking at 240 rpm in L medium (10 g of tryptone, 5 g of yeast extract, and 5 g of NaCl per liter) supplemented with 50 μg of diaminopimelic acid (Dap) per ml when required. The antibiotics ampicillin (100 μg/ml), kanamycin (25 μg/ml), and chloramphenicol (10 μg/ml) were used for selective media. Plasmid pMLD1285 (*ptrc*::*mppA*$^+$) (Amp^r) expresses the *mppA* gene under the control of the isopropyl-β-D-thiogalactoside (IPTG)-inducible *trc* promoter (p^{trc}) (29). Unless otherwise stated, 0.5 mM IPTG was present in cultures of cells containing pMLD1285, although sufficient expression of *mppA* occurs in the absence of IPTG to allow growth of a *dapD2 mppA* null mutant with murein tripeptide as the source of Dap (29).

MICs of antibiotics. The MICs of various antibiotics were determined by plating several hundred cells on L-Dap agar containing a range of concentrations of antibiotics that varied by a factor of 2.

Tolerance to cyclohexane. The test was done essentially as described by White et al. (39). L-Dap agar plates, dried at 37°C for several hours, were spotted with 5 μl of cultures that contained about 10^5 late-log- or stationary-phase cells per ml. After the drop had dried thoroughly, the agar was flooded with cyclohexane to a depth of 2 or 3 mm and incubated at 30°C overnight in a sealed container. Tolerance was indicated by a visible lawn of growth. Sensitive strains produced no visible lawn.

Preparation of cytoplasmic and membrane protein fractions. Cytoplasmic and membrane proteins were separated by the method of Koski et al. (19), with some modifications. Cells were grown in 40 ml of L-Dap medium from a 1% inoculum. When needed, 0.5 mM IPTG was added in early log phase, and growth was

* Corresponding author. Mailing address: Department of Molecular Biology and Microbiology, Tufts University School of Medicine, 136 Harrison Ave., Boston, MA 02111. Phone: (617) 636-6753. Fax: (617) 636-0337. E-mail: jpark02@emerald.tufts.edu.

TABLE 1. *E. coli* strains used

Strain	Genotype or description	Source or reference
AT980	*dapD2 relA1 spoT1 thi-1* Hfr(defective)	*E. coli* Genetic Stock Center (Yale University, New Haven, Conn.), isolate 4545
AG100K	AG100 Δ*marORAB*::Kan^r	23
HSL5	AT980 Δ*marCORAB*::Kan^r	P1(AG100K) × AT980
HSL6	AT980 Δ*acrAB*::Tn*903* Kan^r	P1(JZM120) × AT980
TP982	AT980 *oppB*::mini-Tn*10*Cm *ampG*::Kan	29
HSL2	AT980 *oppB*::mini-Tn*10*Cm	P1(TP982) × AT980
TP985	AT980 *mppA*::mini-Tn*10*Cm	29
HSL3	TP985 Δ*acrAB*::Tn*903* Kan^r	P1(JZM120) × TP985
HSL4	TP985 Δ*marCORAB*::Kan^r	P1(AG100K) × TP985
AG100	*argE3 thi-3 rpsL xyl mtl supE44* Δ(*gal-uvrB*)	10
AG102	AG100 *marR1*	10
JZM120	*argE3 hisG4 leuB6* Δ(*gpt-proA*)*62 thr-1 thi-1 ara-14 galK2 lacY1 mtl-1 xyl-1 kdgK51 tsx-33 recB21 recC22 sbcB15 supE44 rpsL31 rac* Δ*acrAB*::Tn*903* Kan^r	21
JF568	*aroA357 ilv-227 metB65 his-53 purE41 cyc-1 xyl-14 lacY29 rpsL77 tsxN63*	7
JF701	JF568 *ompC264*	7
JF703	JF568 *ompF254 aroA*$^+$	7

continued for approximately 4 h. Cells were harvested at 4°C when the optical density at 600 nm of cultures reached 0.7 to 1. Cells were washed with 10 ml of cold 10 mM HEPES-KOH buffer (pH 7.4) (buffer A). The cell pellet was suspended in 3 ml of the same buffer and disrupted by sonication with cooling. The unbroken cells were removed by centrifugation at 3,000 × *g* for 5 min, and the supernatant was centrifuged at 4°C for 60 min at 180,000 × *g*. The supernatant (cytoplasmic and periplasmic proteins) was stored on ice. The pellet (cell envelope fraction) was washed once with cold buffer A, resuspended in 1 ml of buffer A containing 1% (wt/vol) sodium lauryl sarcosinate (sarcosyl), and incubated at room temperature for 30 min. The anionic detergent sarcosyl solubilizes the proteins of the inner membrane while leaving the major outer membrane proteins insoluble (6). The sarcosyl-insoluble outer membrane proteins and peptidoglycan were sedimented by centrifugation at 180,000 × *g* as before, and the supernatant containing the cytoplasmic membrane proteins was saved. The pellet was washed once with 1 ml of buffer A, reextracted with sarcosyl for 5 min, pelleted by centrifugation as before, and finally resuspended in 200 μl of distilled water.

Protein techniques. Proteins were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) according to the method of Laemmli (20). For N-terminal amino acid sequencing, the proteins were transferred from the SDS-polyacrylamide gel to Immobilon-P by the Western blotting method of Towbin et al. (38). The blots were washed in 50 mM Tris-HCl (pH 7.5)–0.15 M NaCl–0.05% (vol/vol) Tween 20, rinsed with distilled water, and stained with 0.025% aqueous Coomassie brilliant blue R-250 for 5 min. The blot was destained with water and then rinsed quickly with 40% methanol–10% acetic acid to facilitate visualization and excision of the band of interest. The N-terminal amino acid sequences of the proteins in the excised bands were determined at the Tufts University Core Facility with an Applied Biosystems model 477A pulsed/liquid-phase sequencer coupled to an on-line high-performance liquid chromatography model 120A analyzer. Data were analyzed by the Genetics Computer Group program.

The Western blots were immunostained with anti-MarA polyclonal rabbit antibody (32), and the MarA band was visualized with a Renaissance chemiluminescence detection kit as instructed by the manufacturer (NEN Life Sciences Products, Boston, Mass.).

RESULTS

MAR phenotype of the *mppA* null mutant. We found serendipitously that the *mppA* mutant, TP985, was significantly more resistant to tetracycline and cefoxitin than its isogenic parent. This caused us to examine the sensitivity of the *mppA* mutant to a wider spectrum of antibiotics. The *mppA* mutation in TP985 resulted in a fourfold increase in resistance to penicillin G and nalidixic acid as well as to tetracycline and cefoxitin compared to its wild-type parent AT980 (Table 2; compare lines 1 and 5). Introduction of pMLD1285, expressing *mppA*, restored the mutant strain to wild-type sensitivity (Table 2, line 8). The antibiotic resistance of AG102, which has a point mutation in *marR*, the repressor gene in the *marRAB* operon, and consequently overexpresses the transcriptional activator, MarA (2), was three- to sixfold greater than that of its parent, AG100, under our conditions (data not shown).

Interestingly, AG102 (*marR1*) does not exhibit increased resistance to kanamycin. Likewise, TP985 (*mppA*::Cm) is not more resistant to kanamycin than its isogenic parent (data not shown).

The *marA* overexpression mutant, AG102 (*marR1*), has been shown to grow under a lake of cyclohexane (1, 39). We found that TP985 (*mppA*::Cm) is similarly able to grow under

TABLE 2. MIC test

Line	Strain	Relevant genotype	Concn (μg/ml)			
			Tetracycline	Nalidixic acid	Cefoxitin	Penicillin G
1	AT980	Wild type	2	2	2	20
2	HSL5	Δ*marCORAB*	2	2	1	20
3	HSL6	Δ*acrAB*	0.5	0.5	1	10
4	HSL2	*oppB*::Cm	1	2	2	20
5	TP985	*mppA*::Cm	8	8	16	80
6	HSL4	*mppA*::Cm Δ*marCORAB*	2	2	2	10
7	HSL3	*mppA*::Cm Δ*acrAB*	0.5	1	2	20
8	TP985	*mppA*::Cm/pACYC177 *mppA*$^+$	4	2	2	ND[a]
9	TP985	*mppA*::Cm/pACYC177	8	8	16	ND

[a] ND, not done.

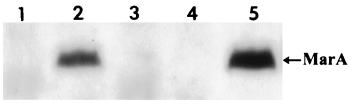

FIG. 1. Overproduction of MarA by the *mppA* mutant and by a *marR* mutant as determined by Western blotting with anti-MarA antibody. Lanes: 1, AT980; 2, TP985 (*mppA*::Cm); 3, TP985/pMLD1285 (*mppA*$^+$); 4, AG100; 5, AG102 (*marR1*).

a lake of cyclohexane and that the plasmid expressing *mppA* restores its sensitivity to cyclohexane (data not shown). Thus, an *mppA* mutant mirrors the resistance of a *marA* overexpression strain, strongly indicating that MppA plays a role in the negative regulation of MAR.

The *mar* operon is required for the Δ*mppA* MAR phenotype. As shown in Table 2 (line 6 versus lines 5 and 1), in the absence of the *mar* operon, the antibiotic sensitivity of TP985 returns to that of its parent. Thus, the lack of MppA cannot activate the MAR phenotype unless the *mar* operon is present. This result indicates that MppA acts exclusively on the *mar* operon to regulate MAR.

Opp permease is not involved in the negative regulation controlled by MppA. MppA, in its murein tripeptide transport function, utilizes the membrane and cytoplasmic components of the Opp permease, OppBCDF (29). However, strain HSL2, carrying mini-Tn*10*Cm in *oppB*, does not express the MAR phenotype (Table 2, line 4). This suggests that OppBCDF is not involved in the regulatory function of MppA.

Increased abundance of MarA and involvement of AcrAB in the MAR phenotype of TP985 (*mppA*::Cm). It is known that MAR is associated with an increase in MarA, which then activates the *acrAB* operon to produce AcrA and AcrB, which constitute the drug efflux pump primarily responsible for MAR (1, 21, 27). SoxS and Rob can also up-regulate *acrAB* (22) but are not involved here, since, as already shown, in the absence of the *mar* operon, *mppA*::Cm has no effect on antibiotic sensitivity. If MppA is involved in the negative regulation of *marA*, the MarA and AcrAB contents of the *mppA* mutant should increase relative to its parent. Figure 1 is a Western blot of soluble *E. coli* proteins probed with anti-MarA antibody. As can be seen, TP985 (*mppA*::Cm) exhibits a greater than wild-type amount of MarA, and complementing the mutant with pMLD1285 (expressing *mppA*) down-regulates the amount of MarA in the mutant to the wild-type level. AG102 (*marR1*), overexpressing *marA*, produces 50% more MarA than does TP985 (*mppA*::Cm) (Fig. 1, lanes 2 and 5).

AcrB is a cytoplasmic membrane protein with 12 transmembrane segments that, together with AcrA (a periplasmic lipoprotein) and TolC, is the efflux pump largely responsible for MAR (21, 27). JZM120 (Δ*acrAB*) is supersensitive to hydrophobic antibiotics and detergents but only slightly more sensitive to many other antibiotics (21). When the Δ*acrAB* mutation was introduced into TP985 (*mppA*::Cm), the resultant strain HSL3 became as sensitive or slightly more sensitive to antibiotics than the parent strain (Table 2; compare lines 5 and 7 and lines 1 and 3). Δ*acrAB* also rendered AT980 and TP985 (*mppA*::Cm) at least 300-fold more sensitive to SDS, consistent with the report by Ma et al. (21) that deletion of *acrAB* increased the sensitivity of two other *E. coli* K-12 strains to SDS more than 150-fold. Taken together, these results support the notion that MppA affects MAR primarily by regulating *acrAB* expression through its control of *marA*.

Increased expression of *acrAB* in the *mppA* null mutant. Recently, Rhee et al. reported the crystal structure of MarA bound to the *marO* sequence and proposed a consensus sequence for MarA binding based on 10 *mar* regulon promoters (30). We recognized a near-perfect MarA binding site 27 nucleotides upstream of promoter 1 of the *acrAB* operon. The presence of a MarA binding site upstream of the *acrAB* promoter strongly suggests that overexpression of MarA in TP985 (*mppA*::Cm) should lead to increased levels of the AcrAB proteins that constitute the efflux pump and hence to the MAR phenotype.

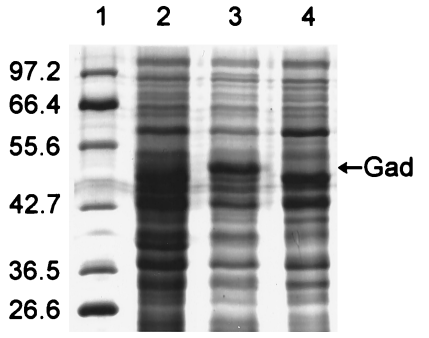

FIG. 2. SDS–10% acrylamide gel of *E. coli* soluble proteins showing overproduction of Gad by the *mppA* mutant TP985. Lanes: 1, molecular weight standards (sizes indicated in kilodaltons); 2, AT980; 3, TP985 (*mppA*::Cm); 4, TP985/pMLD1285 (*mppA*$^+$).

A cytoplasmic enzyme, glutamate decarboxylase (Gad), is overproduced in TP985 (*mppA*::Cm). The cytoplasmic proteins from TP985, its isogenic parent strain AT980, and TP985/pMLD1285 (p*trc*::*mppA*$^+$) were separated by SDS-PAGE. A remarkably intense band (representing up to 17% of the total soluble protein) was present in TP985 (Fig. 2, lane 3). In contrast, this band was only faintly detectable in the soluble fraction from AT980 and TP985/pMLD1285 (p*trc*::*mppA*$^+$) (Fig. 2, lanes 2 and 4). To identify the protein in this band, the N-terminal amino acid sequence of this protein band was determined. The sequence revealed that the band consisted of two proteins, GadA and GadB. These proteins represent isotypes of glutamate decarboxylase differing only in five amino acid residues, three of which are located within the first six N-terminal residues (34). The N-terminal sequence analysis MD(K or Q)K(L or Q)(L or V) indicated that the sample contained a mixture of these two nearly identical proteins. From the quantitation of the amino acids recovered in the N-terminal amino acid analysis, it was concluded that the band contained about 25% more GadA than GadB. To confirm that overexpression of *gadA* and *gadB* indeed occurs in the *mppA* mutant, Gad enzymatic activity, assayed by the method of Rice et al. (31), was at least eightfold higher in TP985 (*mppA*::Cm) than in AT980 and TP985/pMLD1285 (data not shown). We also observed overproduction of Gad in AG102.

To ascertain if *gadA* and *gadB* overexpression in TP985 could be a consequence of *marA* overexpression, we initially examined the promoter regions upstream of *gadA* and *gadBC* for putative MarA binding sites. Both genes were found to have potential, though imperfect, MarA binding sites about 60 nucleotides upstream of the coding sequence. This observation is consistent with activation of the glutamate decarboxylase genes by MarA. However, when the Gad content of HSL4, the *mppA* Δ*mar* double mutant, was compared with that of the *mppA* mutant, both were found to equally overproduce Gad. From this result, it seems clear that MppA regulation of Gad does not depend upon the *mar* operon. Whether overexpression of Gad is related to the MAR phenotype is not clear at present.

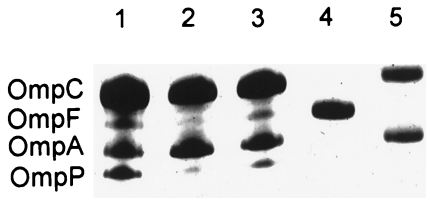

FIG. 3. Reduced amounts of OmpF and OmpP in the *mppA* mutant as revealed by SDS-PAGE on a 12.5% acrylamide gel. Lanes: 1, AT980; 2, TP985 (*mppA*::Cm); 3, TP985/pMLD1285 (*mppA*$^+$); 4, JF701 (*ompC*); 5, JF703 (*ompF*).

Outer membrane protein changes associated with the *mppA* mutation. The outer membrane proteins from TP985 (*mppA*::Cm), its isogenic parent AT980, and TP985/pMLD1285 (p*trc*::*mppA*$^+$) were compared by SDS-PAGE. Outer membranes prepared from strains lacking OmpC or OmpF were used as standards (Fig. 3, lanes 4 and 5). As can be seen in Fig. 3, the OmpF content in TP985 was decreased significantly, as is known to occur in *marR* mutants such as AG102 (*marR1*) (4). MicF is known to inhibit the formation of OmpF by hybridizing to its mRNA (26). MicF is up-regulated by MarA and is therefore responsible for the reduced OmpF content of this mutant (4). When plasmid IV (26), also known as pmicB21, bearing the *micF* promoter fused to *lacZ* was introduced into AT980 and TP985, the *mppA* mutant was found to produce about 50% more β-galactosidase than AT980, indicating that more *micF* message was produced. This may account for the reduced amount of OmpF in TP985 (data not shown).

Another outer membrane protein, that migrates slightly faster than OmpA, was also markedly reduced in quantity in TP985 relative to its isogenic parent. Both proteins were restored to normal levels by expression of *mppA* from pMLD1285 (p*trc*::*mppA*$^+$) (Fig. 3, lane 3). The N-terminal amino acid sequence (SDFFGP) of the protein that migrated slightly faster than OmpA identified it as OmpP (17).

Inner membrane protein changes associated with the *mppA* mutation. The inner membrane protein profile of the *mppA* mutant, TP985, compared by SDS-PAGE with AT980 and TP985/pMLD1285(p*trc*::*mppA*$^+$) is shown in Fig. 4. Compared with AT980 and TP985/pMLD1285, TP985 (*mppA*::Cm) exhibits at least nine proteins at increased levels. Of these nine, AG102 (*marR1*) clearly overexpresses the three major proteins (molecular masses of approximately 50, 43, and 24 kDa), but overexpression of the other proteins was not apparent (data not shown). The up-regulation may not be readily seen in the AG100 background because of strain differences. However, MppA may be involved in negative regulation of genes other than those controlled by the *mar* regulon. To test this more directly, the inner membrane protein profile of the *mppA* Δ*mar* double mutant was compared with that of the *mppA* mutant. As shown by SDS-PAGE in Fig. 5, all of the proteins overproduced by the *mppA* mutant are no longer overproduced when the *mar* operon is absent. Thus, we conclude that negative regulation of the *mar* operon by MppA results in greatly reduced expression of nine or more inner membrane proteins. Incidentally, careful inspection of Fig. 5 also reveals that the *mar* deletion by itself causes overexpression of several proteins relative to its isogenic parent.

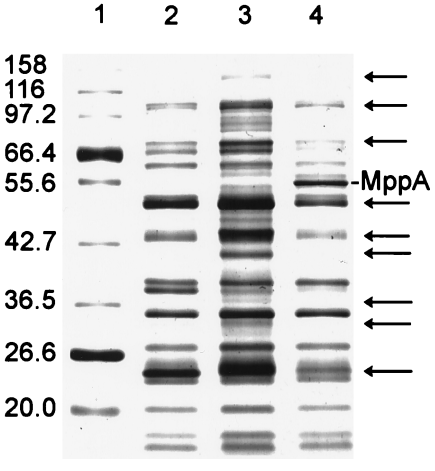

FIG. 4. SDS–7.5% acrylamide gel of inner membrane proteins showing overproduction of inner membrane proteins by the *mppA* mutant. Lanes: 1, molecular weight standards (sizes indicated in kilodaltons); 2, AT980; 3, TP985 (*mppA*::Cm); 4, TP985/pMLD1285 (*mppA*$^+$). Arrows point to inner membrane proteins that are overproduced by TP985.

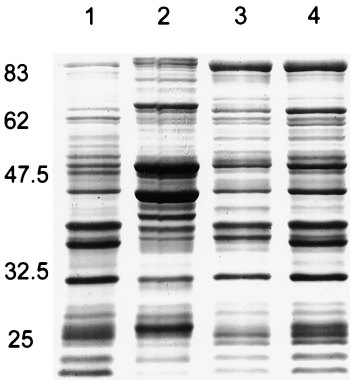

FIG. 5. SDS–10% acrylamide gel of inner membrane proteins showing overproduction of many proteins by the *mppA* mutant and lack of overproduction when the *mar* operon is absent. Lanes: 1, AT980; 2, TP985 (*mppA*::Cm); 3, HSL4 (*mppA*::Cm Δ*marCORAB*); 4, HSL5 (Δ*marCORAB*). Sizes are indicated in kilodaltons.

DISCUSSION

This report demonstrates that the *mppA* null phenotype is very similar to the MAR phenotype that results from *marA* overexpression (1). Compared to the wild-type strain, cells lacking MppA are more resistant to most antibiotics and overproduce MarA and presumably AcrB, the cytoplasmic membrane component of the efflux pump. As in MAR, kanamycin sensitivity is unchanged, the mutant is able to grow in the presence of cyclohexane, and the OmpF porin level is reduced. Reintroduction of the *mppA* gene on a plasmid restores the wild-type sensitivity in all respects. Thus, MppA plays a critical role in the negative regulation of the MAR phenotype.

Comparison of the proteins produced in the presence or absence of MppA has revealed a number of notable changes in the composition of the inner and outer membrane proteins as well as changes in the soluble protein fraction of the cell. In addition to the cytoplasmic protein, MarA, we observed a marked rise in GadA and GadB levels, such that about 17% of the soluble protein of the cell was represented by these glutamate decarboxylases. This overexpression does not require the *mar* operon. The *E. coli* glutamate decarboxylases are pyridoxal phosphate-dependent enzymes that catalyze the alpha-decarboxylation of glutamate to yield γ-aminobutyrate and CO_2. The *E. coli* chromosome contains two genes, *gadA* and *gadB*, encoding this enzymatic activity, and they map at distinct

loci (5). Glutamate decarboxylase activity has been used for rapid identification of *E. coli* since it is absent in enteric organisms other than shigellae (31).

The physiological function of Gad is not known, although a possible role in maintaining pH homeostasis was proposed long ago (8). GadC, which is produced by expression of the *gadBC* operon, is a presumed efflux pump for γ-aminobutyrate and has been shown to be required for development of resistance to acid in *E. coli* (11). Decarboxylation of glutamate consumes a proton, thus raising the cytoplasmic pH. An alternative possible function for Gad was recently suggested by the demonstration that GadB, together with GadC, generates proton motive force that can be converted to ATP (12). The AcrAB drug efflux pump utilizes membrane potential as a source of energy (21). It is tempting to suggest that MAR in *E. coli* is driven, in part, by the membrane potential generated by GadBC and utilized by AcrAB.

The reduction in expression of the outer membrane protein, OmpF, in TP985 (*mppA*::Cm) appears similar to that observed in the MAR phenotype (4). OmpF production involves a posttranscriptional regulatory mechanism mediated by *micF*. Part of the 174-base *micF* RNA is complementary to the 5′ end of *ompF* mRNA and inhibits the translation of *ompF* mRNA by hybridizing with it (26). From studies with a *micF-lacZ* fusion or with strains deleted for the *micF* locus, it was suggested that mutations in both the *tolC* and the *marR* loci increased *micF* expression, causing a posttranslational decrease in the level of OmpF protein (4, 25). The reduction in OmpF content in the *mppA* mutant is moderate but apparent (Fig. 3). We observed a small increase in expression from the *micF-lacZ* fusion plasmid in the *mppA* mutant, which suggests that *micF* may also be overproduced in the *mppA* mutant.

Another outer membrane protein, OmpP, is also significantly reduced in amount in TP985 (*mppA*::Cm) (Fig. 3). OmpP is a protease, 87% identical to the well-known outer membrane protease OmpT (17). Curiously, OmpP is not present in all strains of *E. coli* K-12 (17). The significance of the OmpP level for the MAR phenotype is not apparent.

It is very likely that the MAR, the resistance to cyclohexane, and the diminished amount of OmpF seen in the *mppA* null mutant are related to MarA since these changes also occur in the *marA* overexpression mutant AG102 (*marR1*). It was surprising to discover that at least nine inner membrane proteins are present in greatly increased amounts in the *mppA* mutant and that this requires the presence of the *mar* operon.

How MppA is involved in the regulation of *marA* remains to be determined. Obviously, a periplasmic protein, MppA, cannot directly contact the cytoplasmic protein, MarR, which interacts with *marO* to regulate *mar* expression. One possibility is that a membrane-bound sensor kinase-phosphatase, either directly or via a phosphorelay (35), maintains MarR in the operator-bound, repressor state. Figure 6 compares MarR with several well-studied response regulators and illustrates that MarR contains a sequence element containing the two critical aspartic acid residues and a lysine residue that constitute the signature for two-component response regulators known to undergo reversible aspartyl phosphorylation-dephosphorylation (35). The occurrence of a putative response regulator-like motif in MarR raises the possibility that a sensor kinase is involved in regulation of *marA* by MppA. While we have not eliminated SoxS or Rob as additional transcriptional activators (22, 39) that may contribute to the intrinsic resistance of *E. coli*, the fact that the *mppA* mutant does not express MAR in the absence of MarA clearly indicates that the MAR phenotype of TP985 depends on MarA. Since overproduction of MarA seems sufficient to account for the mutant's MAR phenotype, this leads us to discuss a model in terms of the *mar* operon.

```
MarR: YLSPLD-6-KVLCSI-22-TRMLD-12-NPNDKRG-10-AICE-15-K-6-V-6-LK
PhoB: ILVVED-6-MVCFVL-26-LILLD- 8-IQFIKHL-10-VVML-22-K-6-L-6-VM
ArcA: ILIVED-6-TLKSIF-26-LVIMD- 8-LLLAREL- 7-LMFL-22-K-6-V-6-LL
OmpR: NLVVD-6-LLERYL-26-LMVLD- 8-LSICRRL- 8-IIMV-22-K-6-L-6-VL
CheB: VLSVD-6-IMTEII-28-VLTLD- 8-LDFLEKL- 7-VVMV-24-K-6-E-6-EM
```

FIG. 6. Comparison of the MarR amino acid sequence with the signature sequence of representative two-component response regulators. The two aspartates, one of which becomes phosphorylated, and the lysine that are highly conserved are shown in large bold type. The amino acids shown in smaller bold letters form part of a conserved hydrophobic core (35). MarR contains five charged amino acids in the hydrophobic core, as opposed to only two in CheB and none in the others. The numbers indicate the number of amino acids separating the listed amino acids.

According to this hypothetical model, in the absence of MppA or of murein tripeptide bound to MppA, MarR would be phosphorylated and would be inactive as the repressor of the *mar* operon. The nonphosphorylated form of MarR is predicted to be the active repressor only because purified MalE-MarR fusion protein has been shown to bind tightly to *marO*, and the instability of the aspartyl-phosphate bond makes it unlikely that the fusion protein retained any phosphate (33). However, it must be stressed that the opposite scenario, i.e., the phosphorylated MarR is the repressor and the absence of MppA causes dephosphorylation of MarR to render it inactive, is also possible.

Presumably the absence of MppA in the null mutant mimics the unliganded state in the wild type. Hence, in this model for sensing stress, murein tripeptide liganded to MppA is the active negative regulator and senses the normal state. Dissociation of the ligand would activate the signal transduction pathway, inactivate MarR, and trigger MAR. The question then becomes, what stress could dissociate the presumed ligand, murein tripeptide, from MppA? One possible scenario that would reduce the concentration of tripeptide in the periplasm is a defective outer membrane; another possibility would be conditions that shut down AmiA and AmiB, the enzymes that release tripeptide from murein or murein degradation products (37).

In conclusion, we have demonstrated that MppA, a periplasmic protein that binds murein tripeptide, is involved in the negative regulation of *marA* and hence of the MAR phenomenon associated with the *mar* operon as well as expression of many inner membrane proteins. We have proposed, and are now testing, the hypothesis that MppA exerts its control via a signal transduction pathway.

ACKNOWLEDGMENTS

We thank Michael Alekshun, Nicholas Delihas, Ulf Henning, Stuart Levy, Laura McMurry, and Debabrata RayChaudhuri for generously supplying strains, plasmids, antisera, and proteins and for helpful discussions. We especially thank Debabrata RayChaudhuri for critical advice on the manuscript.

This work was supported in part by Public Health Service grant GM51610 from the National Institute of General Medical Sciences.

REFERENCES

1. **Alekshun, M. N., and S. B. Levy.** 1997. Regulation of chromosomally mediated multiple antibiotic resistance: the *mar* regulon. Antimicrob. Agents and Chemother. **41:**2067–2075.
2. **Cohen, S. P., H. Hachler, and S. B. Levy.** 1993. Genetic and functional analysis of the multiple antibiotic resistance (*mar*) locus in *Escherichia coli*. J. Bacteriol. **175:**1484–1492.
3. **Cohen, S. P., S. B. Levy, J. Foulds, and J. L. Rosner.** 1993. Salicylate induction of antibiotic resistance in *Escherichia coli*: activation of the *mar*

operon and a *mar*-independent pathway. J. Bacteriol. **175:**7856–7862.
4. **Cohen, S. P., L. M. McMurry, and S. B. Levy.** 1988. *marA* locus causes decreased expression of OmpF porin in multiple-antibiotic-resistant (Mar) mutants of *Escherichia coli*. J. Bacteriol. **170:**5416–5422.
5. **De Biase, D., A. Tramonti, R. A. John, and F. Rossa.** 1996. Isolation, overexpression, and biochemical characterization of the two isoforms of glutamic acid decarboxylase from *Escherichia coli*. Protein Expr. Purif. **8:**430–438.
6. **Filip, C., G. Fletcher, J. L. Wulff, and C. F. Earhart.** 1973. Solubilization of the cytoplasmic membrane of *Escherichia coli* by ionic detergent sodium-lauryl sarcosinate. J. Bacteriol. **115:**717–722.
7. **Foulds, J., and T.-J. Chai.** 1978. New major outer membrane protein found in an *Escherichia coli tolF* mutant resistant to bacteriophage Tulb. J. Bacteriol. **133:**1478–1483.
8. **Gale, E. F.** 1946. The bacterial amino acid decarboxylases. Adv. Enzymol. **6:**1–32.
9. **Gambino, L., S. J. Gracheck, and P. F. Miller.** 1993. Overexpression of the MarA positive regulator is sufficient to confer multiple antibiotic resistance in *Escherichia coli*. J. Bacteriol. **175:**2888–2894.
10. **George, A. M., and S. B. Levy.** 1983. Amplifiable resistance to tetracycline, chloramphenicol, and other antibiotics in *Escherichia coli*: involvement of a non-plasmid-determined efflux of tetracycline. J. Bacteriol. **155:**531–540.
11. **Hersh, B. M., F. T. Farooq, D. N. Barstad, D. L. Blankenhorn, and J. L. Slonczewski.** 1996. A glutamate-dependent acid resistance gene in *Escherichia coli*. J. Bacteriol. **178:**3978–3981.
12. **Higuchi, T., H. Hayashi, and K. Abe.** 1997. Exchange of glutamate and γ-aminobutyrate in a *Lactobacillus* strain. J. Bacteriol. **179:**3362–3364.
13. **Hiles, I. D., and C. F. Higgins.** 1986. Peptide uptake by *Salmonella typhimurium*: the periplasmic binding protein. Eur. J. Biochem. **158:**561–567.
14. **Höltje, J.-V., U. Kopp, A. Ursinus, and B. Wiedemann.** 1994. The negative regulator of β-lactamase induction AmpD is a N-acetyl-anhydromuramyl-L-alanine amidase. FEMS Microbiol. Lett. **122:**159–164.
15. **Jacobs, C., L.-J. Huang, E. Bartowsky, S. Normark, and J. T. Park.** 1994. Bacterial cell wall recycling provides cytosolic muropeptides as effectors for β-lactamase induction. EMBO J. **13:**4684–4694.
16. **Jacobs, C., B. Joris, M. Jamin, K. Klarsov, J. van Beemen, D. Mengin-Lecreulx, J. van Heijenoort, J. T. Park, S. Normark, and J.-M. Frere.** 1995. AmpD, essential for both β-lactamase regulation and cell wall recycling, is a novel cytosolic N-acetylmuramyl-L-alanine amidase. Mol. Microbiol. **15:**553–559.
17. **Kaufmann, A., Y.-D. Stierhof, and U. Henning.** 1994. New outer membrane-associated protease of *Escherichia coli* K-12. J. Bacteriol. **176:**359–367.
18. **Korfmann, G., and C. C. Sanders.** 1989. AmpG is essential for high-level expression of AmpC β-lactamase in *Enterobacter cloacae*. Antimicrob. Agents Chemother. **33:**1946–1951.
19. **Koski, P., M. Rhen, J. Kantele, and M. Vaara.** 1989. Isolation, cloning, and primary structure of a cationic 16kDa outer membrane protein of *Salmonella typhimurium*. J. Biol. Chem. **264:**18973–18980.
20. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:**680–685.
21. **Ma, D., D. N. Cook, M. Alberti, N. G. Pon, H. Nikaido, and J. E. Hearst.** 1995. Genes *acrA* and *acrB* encode a stress-induced efflux system of *Escherichia coli*. Mol. Microbiol. **16:**45–55.
22. **Ma, D., M. Alberti, C. Lynch, H. Nikaido, and J. E. Hearst.** 1996. The local repressor AcrR plays a modulating role in the regulation of *acrAB* genes of *Escherichia coli* by global stress signals. Mol. Microbiol. **19:**101–112.
23. **Maneewannakul, K., and S. B. Levy.** 1996. Identification of *mar* mutants among quinolone-resistant clinical isolates of *Escherichia coli*. Antimicrob. Agents Chemother. **40:**1695–1698.
24. **Martin, R. G., and J. L. Rosner.** 1995. Binding of purified multiple antibiotic-resistance repressor protein (MarR) to *mar* operator sequences. Proc. Natl. Acad. Sci. USA **92:**5456–5460.
25. **Mirsa, R., and P. R. Reeves.** 1987. Role of *micF* in *tolC*-mediated regulation of OmpF, a major outer membrane protein of *Escherichia coli* K-12. J. Bacteriol. **169:**4722–4730.
26. **Mizuno, T., M. Chou, and M. Inouye.** 1984. A unique mechanism regulating gene expression: translational inhibition by a complementary RNA transcript (micRNA). Proc. Natl. Acad. Sci. USA **81:**1966–1970.
27. **Okusu, H., D. Ma, and H. Nikaido.** 1996. AcrAB efflux pump plays a major role in the antibiotic resistance phenotype of *Escherichia coli* multiple-antibiotic-resistance (Mar) mutants. J. Bacteriol. **178:**306–308.
28. **Park, J. T.** 1993. Turnover and recycling of the murein sacculus in oligopeptide permease-negative strains of *Escherichia coli*: indirect evidence for an alternative permease system and for a monolayered sacculus. J. Bacteriol. **175:**7–11.
29. **Park, J. T., D. RayChaudhuri, H. Li, S. Normark, and D. Mengin-LeCreulx.** 1998. MppA, a periplasmic binding protein essential for import of the bacterial cell wall peptide L-alanyl-γ-D-glutamyl-*meso*-diaminopimelate. J. Bacteriol. **180:**1215–1223.
30. **Rhee, S., R. G. Martin, J. L. Rosner, and D. R. Davies.** 1998. A novel DNA-binding motif in MarA: the first structure for an AraC family transcriptional activator. Proc. Natl. Acad. Sci. USA **95:**10413–10418.
31. **Rice, E. W., C. H. Johnson, M. E. Dunnigan, and D. J. Reasoner.** 1993. Rapid glutamate decarboxylase assay for detection of *Escherichia coli*. Appl. Environ. Microbiol. **59:**4347–4349.
32. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
33. **Seoane, A. S., and S. B. Levy.** 1995. Characterization of MarR, the repressor of the multiple antibiotic resistance (*mar*) operon of *Escherichia coli*. J. Bacteriol. **177:**3414–3419.
34. **Smith, D. K., T. Kassam, B. Singh, and J. F. Elliott.** 1992. *Escherichia coli* has two homologous glutamate decarboxylase genes that map to distinct loci. J. Bacteriol. **174:**5820–5826.
35. **Stock, J. B., A. M. Stock, and J. M. Mottonen.** 1990. Signal transduction in bacteria. Nature **344:**395–400.
36. **Sulavik, M. C., M. Dazer, and P. F. Miller.** 1997. The *Salmonella typhimurium mar* locus: molecular and genetic analyses and assessment of its requirement for virulence. J. Bacteriol. **179:**1857–1866.
37. **Tomioka, S., T. Nikaido, T. Miyakawa, and M. Matsuhashi.** 1983. Mutation of the N-acetylmuramyl-L-alanine amidase gene of *Escherichia coli* K-12. J. Bacteriol. **156:**463–465.
38. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76:**4350–4354.
39. **White, D. G., J. D. Goldman, B. Demple, and S. B. Levy.** 1997. Role of the *acrAB* locus in organic solvent tolerance mediated by expression of *marA*, *soxS*, or *robA* in *Escherichia coli*. J. Bacteriol. **179:**6122–6126.