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The *merG* Gene Product Is Involved in Phenylmercury Resistance in *Pseudomonas* Strain K-62

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The physiological function of a new gene, hereby designated *merG*, located between *merA* and *merB* on the broad-spectrum *mer* operon of *Pseudomonas* strain K-62 plasmid pMR26 was investigated. The 654-bp *merG* gene encodes a protein with a canonical leader sequence at its N terminus. The processing of the signal peptide of this protein was dose-dependently inhibited by sodium azide, a potent inhibitor of protein export. These results suggest that the mature MerG protein (ca. 20 kDa) may be located in the periplasm. Deletion of the *merG* gene from the broad-spectrum *mer* operon of pMR26 had no effect on the inorganic mercury resistance phenotype, but rendered the bacterium more sensitive to phenylmercury than its isogenic wild-type strain. *Escherichia coli* cells bearing pMU29, which carries a deletion of the *merG* gene, took up significantly more phenylmercury than the bacteria with the intact plasmid pMRA17. When the *merG* gene in a compatible plasmid was transformed into the *E. coli* strain carrying pMU29, the high uptake of and high sensitivity to phenylmercury were almost completely restored to their original levels. These results demonstrate that the *merG* gene is involved in phenylmercury resistance, presumably by reducing in-cell permeability to phenylmercury.

Bacterial resistance to organomercurials has mostly been shown to result from the cleavage of the carbon-mercury linkage by the organomercurial lyase enzyme, followed by the reduction of Hg^{2+} to Hg^0 by the mercuric reductase enzyme. The genetics and biochemistry of organomercurial resistance have been extensively studied and reviewed (1, 5, 14, 19, 26, 27). These studies focused predominantly on mercury resistance (*mer*) operons. Most of the *mer* operons encoding a broad-spectrum resistance phenotype have been shown to consist of a regulatory gene (*merR*), an operator-promoter region, and at least four structural genes, *merT*, *merP*, *merA*, and *merB* (7, 8, 10, 11, 17, 21).

Pseudomonas strain K-62, a bacterial strain with broad-spectrum mercury resistance isolated from phenylmercury-polluted soil, has been shown to have a resistance to phenylmercury approximately a thousand times higher than that of other bacteria, such as *Escherichia coli* and *Pseudomonas aeruginosa* (30). Two separate organomercurial lyase enzymes, designated S-1 and S-2, each with somewhat different properties and substrate specificities (28, 29), were previously believed to be the elements contributing to *Pseudomonas* strain K-62's resistance to phenylmercury. Genetic studies of resistance determinants, however, have shown that the mercury resistance of this soil strain had to do with pMR26 and pMR68, two of the six plasmids (8). Two *mer* operons in this soil strain were mapped on a 26-kb plasmid (pMR26), and one was cloned into the *Sac*I site of the cloning vector, pBluescriptII (8). The resultant plasmid, pMRA17, expressed a typical broad-spectrum mercury resistance phenotype. DNA sequence analysis of a 6.6-kb *Sac*I fragment, encompassing the whole region required for the broad-spectrum mercury resistance, of pMRA17 revealed that it comprised six open reading frames (ORFs), five of which were identified as *merR*, *merT*, *merP*, *merA*, and *merB* (10). The order and function of *mer* genes concerned with the mercurial resistance on the 6.6-kb fragment of pMRA17 are basically the same as those of the broad-spectrum *mer* operon of pDU1358, except that it has an additional ORF located between *merA* and *merB* genes (10).

The additional ORF, however, had no homology with the *mer* genes known to date. Deletion of this ORF rendered the bacterium more sensitive to phenylmercury than its isogenic wild-type strain, while surprisingly retaining full resistance to Hg^{2+} (10). The purpose of this paper is to characterize and define more precisely the roles of this ORF, hereby designated *merG*, in phenylmercury resistance.

MATERIALS AND METHODS

Bacterial strain, plasmids, and growth conditions. The bacterial strain and plasmids used in this study are listed in Table 1. The *E. coli* strain was grown at 37°C in Luria-Bertani medium (22). When necessary, the medium was supplemented with 100 μg of ampicillin or 25 μg of kanamycin per ml.

Plasmid construction. To construct a plasmid containing the broad-spectrum resistance *mer* operon of pMRA17, the plasmid pMRA17 was digested with *Bgl*II, and the resulting 5′ overhang was filled with deoxynucleoside triphosphates (dNTPs) by DNA polymerase I (Klenow fragment). After digestion with *Eco*RV, the 4.7-kb fragment was inserted into the *Sma*I site of the cloning vector pBluescriptII SK+. The resultant plasmid was designated pMR96. Plasmid pMC89 was constructed by digesting pMR96 with *Afl*III-*Nhe*I, and the resulting 5′ overhang was filled with dNTPs by DNA polymerase I (Klenow fragment). The 7.2-kb fragment was religated with a DNA ligation kit.

pMEV89 containing the *merG* gene was constructed by cloning the 1.2-kb *Hin*dIII-*Bam*HI fragment of pMC89 into the *Hin*dIII-*Bam*HI site of a cloning vector, pSTV29. pMUE74 carrying the *merG* gene of pMRA17 was constructed as follows. A 697-bp fragment containing the *merG* gene was PCR amplified with primer 1 ($5'^{3325}$GCTCTAGAGGGCACCAAAACAGGG^{3348} 3′) and primer 2 ($5'^{3998}$GAAGATCTCTTTGTGCCTTGCCGG^{4021} 3′) containing restriction sites for *Xba*I and *Bgl*II, respectively. pMRA17 was used as the template. After digestion with *Xba*I and *Bgl*II, the fragment was cloned into the *Xba*I-*Bgl*II site of plasmid pTUE1122, which contains DNA sequence encoding six consecutive histidine residues immediately posterior to the *Bgl*II site (Table 1). The structures of the relevant genes and restriction sites in the constructed plasmid used in this study are schematically illustrated in Fig. 1.

DNA sequencing. The complete sequence of the 654-bp *merG* gene was determined from both strands by using the dideoxy chain-termination DNA sequencing method of Sanger et al. (24). Sequence analysis was also performed with a DNA autosequencer (model 373A; Applied Biosystems, Inc., Foster City, Calif.) with a *Taq* dye terminator cycle sequencing kit.

Maxicell analysis. Maxicells were used to specifically label plasmid-encoded polypeptides. The transformants of *E. coli* CSR603 carrying the plasmids of interest were incubated aerobically at 37°C in K medium (M9 medium supplemented with 1% Casamino Acids and 0.1 μg of thiamine per ml). After 50 s of UV irradiation (50 J/m^2), the cells were treated with D-cycloserine (150 μg/ml),

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TABLE 1. Bacterial strain and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Source or reference
Strain		
E. coli XL1-Blue	*recA1 endA1 gyrA96 thi hsdR17 supE44 relA1 lac*/[F′::Tn*10 proAB*$^+$ *lacI*^{q} *lacZM15 traD36*]	2
Plasmids		
pBluescriptII SK+	Ap^r ColE1 replicon, cloning vector	Stratagene
pSTV29	Cm^r pACYC replicon, cloning vector	4, 25
pTUE1122	Ap^r *tac* promoter, 6xHis, expression vector	16
pMR26	Broad-spectrum mercury resistance plasmid from *Pseudomonas* strain K-62	8, 10
pMRA17	6.6-kb *Sac*I fragment of pMR26 containing *merRTPAGB* cloned into pBluescriptII SK+	8, 10
pMR96	4.7-kb *Bgl*II-*Eco*RV fragment of pMRA17 containing *merRTPAGB* cloned into pBluescriptII SK+	This study
pMC89	*merB*-deleted plasmid, containing *merRTPAG*, constructed from pMR96	This study
pMU29	*merG*-deleted plasmid containing *merRTPAB* cloned into pBluescriptII SK+	10
pMRD141	*merAGB*-deleted plasmid containing *merRTP*, constructed from pMRA17	9, 10, 31
pMEV89	1.2-kb *Hin*dIII-*Bam*HI fragment of pMC89 containing *merG* cloned into pSTV29	This study
pMUE74	697-bp PCR products of *merG* cloned into pTUE1122	This study

and the maxicell proteins were then labeled with [^{35}S]methionine (1,000 Ci/mmol; New England Nuclear) according to the original protocol (23). For induction of the *mer* gene-encoded polypeptides, 1 μM $HgCl_2$ was added to the cells during the 1-h period of labeling with [^{35}S]methionine. Gel electrophoresis was performed by the method of Laemmli (12), and sodium salicylate was used for detection of the ^{35}S-labeled polypeptide (3).

Protein purification and analysis. An *E. coli* XL1-Blue strain carrying pMUE74 was cultured in Luria-Bertani medium supplemented with 100 μg of ampicillin per ml at 37°C for 2 h, and 1 mM isopropyl-β-o-thiogalactopyranoside (IPTG) was added for a further 30 min of cultivation. After incubation with IPTG, the cells were incubated at 37°C in the absence or presence of various concentrations of sodium azide, an inhibition of protein transport (18). After 2 h, the cells were harvested by centrifugation at 10,000 × *g* for 1 min and resuspended in 1% sodium dodecyl sulfate (SDS) sonication buffer (1% SDS, 100 mM phosphate buffer [pH 7.8], 300 mM NaCl). The cells were disrupted by boiling for 5 min, and the cell debris was removed by centrifugation at 10,000 × *g* for 5 min at 4°C. The resultant supernatant was gently mixed with Ni-nitrilotriacetic acid (NTA) resin (Qiagen) at room temperature for 16 h. The mixture was centrifuged, and the pellet was washed three times with 1% SDS sonication buffer. The pellet was then mixed with SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer (2% SDS, 60 mM Tris-HCl [pH 6.8], 5% mercaptoethanol, 5% sucrose, 0.002% bromophenol blue) containing 100 mM EDTA. After incubation for 5 min at 100°C, the supernatant obtained by centrifugation was resolved by SDS-PAGE (17.5% polyacrylamide gel). Gel electrophoresis was performed by the method of Laemmli (12), and Coomassie brilliant blue was used for protein staining. After purification, the N-terminal amino acid sequences of the 25- and 21-kDa proteins were determined, respectively, with an HP G1005A protein sequencing system (Hewlett-Packard).

Mercury resistance assay. Resistance of bacteria to $HgCl_2$ and phenylmercuric acetate (PMA) was determined on petri dishes according to the method of Foster et al. (6). The zones of inhibition of growth around the disks were measured after incubation at 37°C for 16 h.

Mercury volatilization assay. A qualitative detection of nonradioactive mercury volatilization by the cells was done according to the X-ray film method described by Nakamura and Nakahara (15). Cells were grown, induced with 1 μM Hg^{2+}, and harvested. Cells were resuspended in 0.15 ml of 70 mM phosphate buffer (pH 7.0) containing 0.5 mM EDTA, 0.2 mM magnesium acetate, 5 mM sodium thioglycolate, and 184 μM $HgCl_2$ or 149 μM PMA in a microplate. The plate was covered with X-ray film in the darkroom. The foggy area on the film was the result of the reduction of Ag^+ emulsion by the mercury vapor formed from $HgCl_2$ and PMA.

Mercury uptake assay. Bacterial cells were grown in Luria-Bertani medium in the absence or presence of 0.5 μM $HgCl_2$ or PMA. The late-log-phase cells were harvested and resuspended in Luria-Bertani medium containing 100 μg of chloramphenicol per ml, 100 μM EDTA, and 5 μM $^{203}Hg^{2+}$ (136 mCi/mmol) or 50 μM $^{14}C_6H_5Hg^+$ (30 mCi/mmol). After incubation at 37°C, aliquots (0.5 ml) were removed periodically and filtered on a Whatman GF/B glass microfiber filter (0.45-μm pore diameter). The filters were washed three times with 5 ml of Luria-Bertani medium, and the radioactivity on the filter was measured with a Beckman gamma scintillation counter (GAMMA-5500) or liquid scintillation spectrometer (Aloka LSC-3500). The standard deviations of measurement were less than 10%.

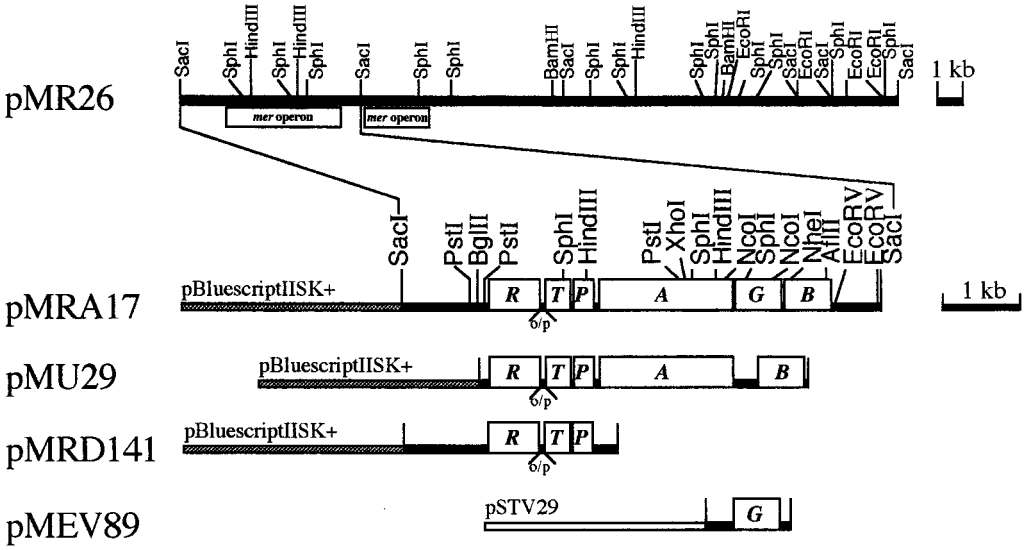

FIG. 1. Structure of the relevant *mer* genes and restriction endonuclease sites in plasmid pMRA17 and its derivatives. *R*, *merR* (regulatory gene); *T*, *merT* (encodes mercury transport protein); *P*, *merP* (encodes periplasmic mercury-binding protein); *A*, *merA* (encodes the enzyme mercuric reductase); *G*, *merG* (new gene for phenylmercury resistance); *B*, *merB* (encodes the enzyme organomercurial lyase). o/p, operator-promoter region.

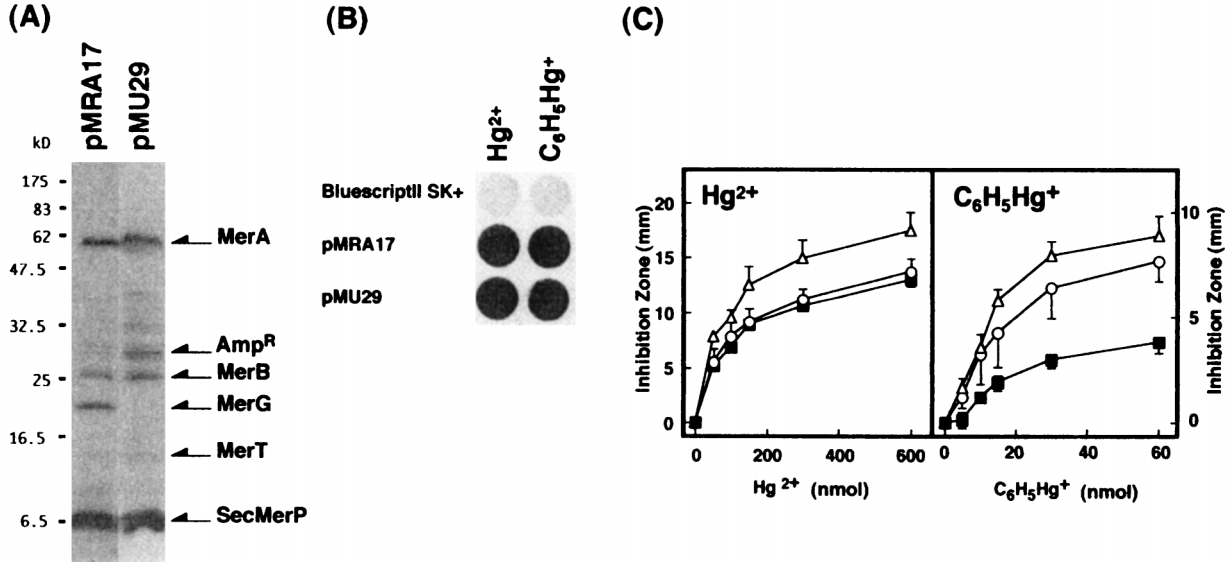

FIG. 2. *mer* polypeptides (A), mercury volatilization (B), and mercury resistance encoded by deletion plasmid pMU29. (A) *E. coli* CSR603 harboring pMRA17 or pMU29 was cultured, induced with 1 μM Hg^{2+}, UV irradiated, and labeled with [^{35}S]methionine. Labeled cells were collected and lysed, and proteins were analyzed on SDS-polyacrylamide gels. Molecular mass markers are indicated to the left. The arrows marked MerA, MerB, MerG, MerT, and SecMerP indicate the polypeptides of the respective genes. (B) Cells were grown, induced with 1 μM Hg^{2+}, and harvested. Volatilization of mercury from mercurials was detected by the X-ray film method as described in Materials and Methods. (C) Resistance of *E. coli* XL1-Blue with pMRA17 (■), pMU29 (○), and pBluescriptII SK+ (△) to mercuric chloride and phenylmercuric acetate was determined by measuring the diameter of the inhibition zone (minus the 6.5-mm disk diameter) on petri dishes after overnight growth at 37°C. All values are the means of triplicate experiments.

Nucleotide sequence accession number. The nucleotide sequence of *merG* has been deposited in the EMBL/GenBank/DDBJ Nucleotide Sequence Database under accession no. D83080.

RESULTS

Evidence that *merG* codes for phenylmercury resistance. Plasmid pMRA17 carries a 6.6-kb *Sac*I-fragment of the *Pseudomonas* strain K-62 plasmid pMR26, which encodes a typical broad-spectrum mercurial resistance based on the degradation of organomercury and reduction of the resultant Hg^{2+} to Hg^0 (10). A new gene, *merG*, which specified a protein with a deduced molecular mass of 20 kDa, was found in the broad-spectrum *mer* operon of pMR26.

To define the roles of *merG*, a series of deletion plasmids were constructed (Fig. 1 and Table 1). As shown in Fig. 2A, the 20-kDa protein was not detected in maxicells carrying *merG*-deleted plasmid pMU29. pMU29, however, still expressed its ability to volatilize mercury, not only from Hg^{2+}, but also from $C_6H_5Hg^+$ (Fig. 2B). Deletion mutation in the *merG* region did not impair Hg^{2+} resistance but significantly decreased phenylmercury resistance (Fig. 2C). These results suggest that *merG* may be involved in phenylmercury resistance.

DNA sequence and properties of *merG*. The nucleotide sequence of the 950-bp *Nco*I-*Nhe*I fragment of pMEV89 which contained the entire *merG* gene was carefully redetermined and is identical to that previously reported for the *merG* region (10). (Note that an error was made at position 277 of the *merG* gene region. There should have been a "c" at this position, but it was inadvertently omitted in the previous report [10].) There is a 654-bp *merG* gene, preceded by a potential ribosome binding site. From the DNA sequence data, the protein encoded by this gene has been predicted to have molecular mass of 23.8 kDa, which is about 4 kDa larger than that detected in maxicell analysis (Fig. 2A).

The fate of MerG protein fused on its C terminus of eight amino acid residues (RSHHHHHH; 1 kDa) in cells with pMUE74 was studied. As shown in Fig. 3, a protein with molecular mass of 21 kDa was predominantly detected in the cells with pMUE74. When the cells were treated with sodium azide, an inhibitor of protein export (18), an additional protein with a molecular mass of 25 kDa was detected in the cells. The appearance of the 25-kDa protein was dose-dependently increased by sodium azide, along with the decrease of the appearance of the 21-kDa protein (Fig. 3). The N-terminal amino acid sequences of the 25- and 21-kDa proteins overlapped completely with those predicted from translation of the DNA

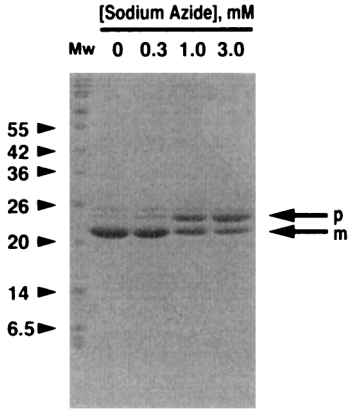

FIG. 3. Effect of sodium azide on the translocation of MerG. *E. coli* XL1-Blue carrying pMUE74 was cultured in Luria-Bertani medium and induced with 1 mM IPTG for 30 min. After a 2-h treatment with sodium azide, the cells were harvested and disrupted by boiling for 5 min, and the resultant supernatant was mixed with Ni-NTA resin (Qiagen) at room temperature for 16 h. After centrifugation, the pellet was washed, mixed with SDS-PAGE sample buffer containing 100 mM EDTA, and boiled for 5 min. The proteins in the sample were resolved by SDS-PAGE, and Coomassie brilliant blue was used for protein staining as described in Materials and Methods. p, the precursor MerG; m, the mature MerG. Mw, molecular mass markers (kilodaltons).

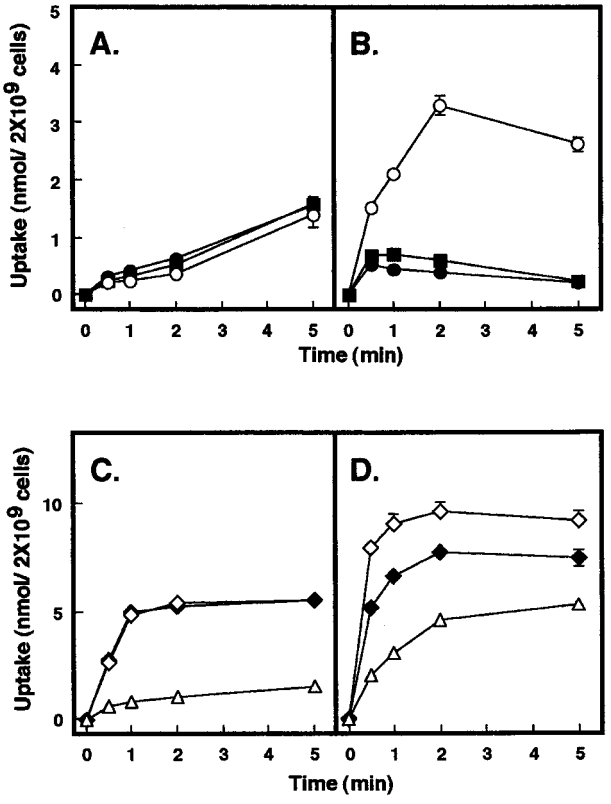

FIG. 4. Effect of *merG* on bacterial uptake of $^{203}Hg^{2+}$ (A and C) and $^{14}C_6H_5Hg^+$ (B and D). *E. coli* XL1-Blue cells with pMRA17 (■), pMU29 (○), pMU29 and pMEV89 (●), pMRD141 (◇), pMRD141 and pMEV89 (◆) and pBluescriptII SK+ (△) were grown, induced, and harvested as described in Materials and Methods. After addition of the radioactive mercurials, aliquots were removed periodically, filtered, and washed. All values are the means of triplicate determinations from three experiments.

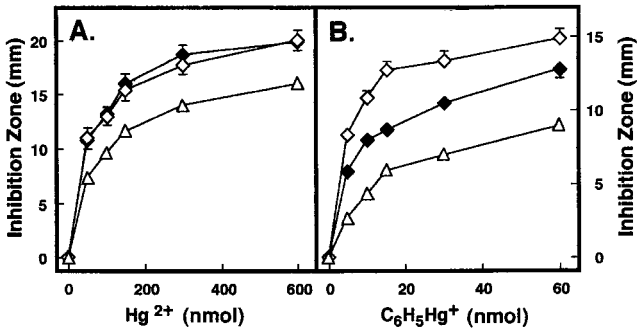

FIG. 5. Mercurial resistance. *E. coli* XL1-Blue cells with pMRD141 (◇), pMRD141 and pMEV89 (◆), and pBluescript II SK+ (△) with mercuric chloride (A) and phenylmercuric acetate (B) were grown at 37°C. After 16 h of incubation, the diameter of the inhibition zone (minus the 6.5-mm disk diameter) on petri dishes was measured. All values are the means of triplicate experiments.

sequence at positions 1 to 5 (MFCDI) and 35 to 39 (AYDFS). These results suggest that the 21-kDa protein is the exported product of the 25-kDa protein encoded by pMUE74.

Role of *merG* in phenylmercury resistance. To study the function of *merG* in mercury resistance, uptake of radioactive mercury by the cells with the *merG*-deleted plasmid pMU29 was examined. No significant difference in the uptake of $^{203}Hg^{2+}$ was found between the cells with pMU29 and pMRA17 (Fig. 4A). However, the cells with pMU29 took up appreciably more $^{14}C_6H_5Hg^+$ than the cells with pMRA17 (Fig. 4B). Transformation of plasmid pMEV89 containing the *merG* gene into the pMU29-bearing cells had no effect on the uptake of $^{203}Hg^{2+}$, but caused a significant reduction in the uptake of $^{14}C_6H_5Hg^+$ (Fig. 4A and B).

The effect of *merG* on bacterial uptake of $^{14}C_6H_5Hg^+$ was next studied in the absence of functional MerB and MerA proteins. pMRD141, containing the intact mercury transport genes (*merT* and *merP*), but not the detoxifying genes (*merA* and *merB*), conferred bacterial hyperaccumulation of both $^{203}Hg^{2+}$ and $^{14}C_6H_5Hg^+$ (Fig. 4C and D). This finding is in agreement with our earlier results (9, 31). Interestingly, when pMEV89 was transformed into the pMRD141-bearing cells, the hyperaccumulation of $^{14}C_6H_5Hg^+$ in these cells was significantly reduced, while the hyperaccumulation of $^{203}Hg^{2+}$ was not (Fig. 4C and D). Figure 5 shows that bacteria carrying pMRD141 consistently showed hypersensitivity, not only to Hg^{2+}, but also to $C_6H_5Hg^+$. When pMEV89 was transferred into the bacterial strain harboring pMRD141, the bacterium was more resistant to $C_6H_5Hg^+$ than was the strain carrying pMRD141 only. However, transformation of pMEV89 into the strain had no effect on the phenotype of hypersensitivity to Hg^{2+} (Fig. 5).

DISCUSSION

From a *Pseudomonas* strain K-62 plasmid, pMR26, we had previously uncovered a new gene which had no homology with any published *mer* genes (10). The new gene was found to be located between the *merA* and *merB* genes on the broad-spectrum *mer* operon of pMR26. In this paper, we have further characterized and defined the physiology of this new gene. The new gene is hereby designated *merG*.

In agreement with our previous results (10), deletion of this new gene resulted in a decrease in phenylmercury resistance, but not in a total loss of the resistance or of volatilization activity (Fig. 2B and C). These results clearly demonstrate that *merG* plays a role in the expression of phenylmercury resistance.

An independent determination of the DNA sequence agreed with what was previously reported. There is, however, an error in the DNA sequence in the previous report (10). There should have been a "c" at position 3639, but it was inadvertently omitted. The *merG* gene is 654-bp long, encoding a 217-amino-acid polypeptide with a deduced molecular mass of 23.8 kDa; however, no 23.8-kDa protein was detected in the maxicell analysis (Fig. 2A). There is no doubt that the 20-kDa protein detected by maxicell analysis is encoded by *merG*, because this protein was no longer detected coincidentally along with the gene deleted from the *mer* operon (Fig. 2A). It was this discrepancy that prompted us to characterize the *merG* gene further.

The amino acid sequence of the gene product, predicted from the DNA sequence of *merG*, has a good leader sequence which contains a short positively charged region at the N terminus followed by a hydrophobic region and a signal peptide cleavage site (ALAA) at position 32 to 35. This characteristic N-terminal sequence is homologous to the "leader sequences" of known periplasmic proteins (13). The formation of the 21-kDa protein was significantly inhibited by sodium azide in a dose-dependent manner (Fig. 3). In addition, the 21-kDa protein was predominantly detected in the periplasmic fraction, suggesting that the MerG protein may be located in the periplasm.

It is interesting to note that deletion of the *merG* gene

resulted in a significant decrease in the phenylmercury resistance, but not in a total loss of the resistance (Fig. 2C). Deletion of *merG* did not impair the enzymatic activities encoded by *merA* and *merB*, because the bacteria carrying pMRA17 and pMU29 still volatilized mercury from the $C_6H_5Hg^+$ taken up by the cells (Fig. 2B), and no difference in the rate of $^{14}C_6H_5Hg^+$ disappearance was found between the cells with pMRA17 and those with pMU29 (data not shown). In addition, the radioactivity in the culture medium was constant during the culture of bacteria carrying plasmid pMRD141 and pMEV89 in the presence of $^{14}C_6H_5Hg^+$ (data not shown). These results suggest that the MerG protein-specified phenylmercury resistance seems to be due to the efflux mechanism rather than mercury biotransformation.

Reduced uptake of mercury has been put forward as one of the mechanisms for bacterial resistance to mercurials (20). Several lines of evidence obtained in this study strongly suggest that *merG* is involved in the reduction of cellular permeability to $C_6H_5Hg^+$. (i) Although deletion of the *merG* gene had no apparent effect on the accumulation of Hg^{2+}, it caused an increase in the accumulation of $^{14}C_6H_5Hg^+$ compared to the level in the strain with pMRA17 (Fig. 4A and B). (ii) The high accumulation of $^{14}C_6H_5Hg^+$ was completely abolished when pMEV89 was introduced into the bacterial strain with pMU29 (Fig. 4B). (iii) Transformation of pMEV89 into the bacteria carrying pMRD141 caused a significant reduction in the uptake of $^{14}C_6H_5Hg^+$, but not that of $^{203}Hg^{2+}$ (Fig. 4C and D). And finally, (iv) the hypersensitivity to phenylmercury in the bacteria with pMRD141 was markedly reduced (Fig. 5). Tonomura et al. (30) previously reported that approximately 70% of the phenylmercury taken up by *Pseudomonas* strain K-62 was detected mainly on the cell surface, and they speculated that this soil strain might be less permeable to mercurials than other mercurial-sensitive bacteria. Our results confirmed their speculation.

In conclusion, the *merG* gene is involved in the bacterial expression of phenylmercury resistance. It is plausible that the resistance is the result of a reduction in the cellular permeability to phenylmercury.

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