Roles Played by MerE and MerT in the Transport of Inorganic and Organic Mercury Compounds in Gram-negative Bacteria

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In order to clarify the physiological roles played by MerP, MerT and MerE in Gram-negative bacteria, we constructed the plasmids pTP4 and pTPE21, which contained the genes *merR*, *merT* and *merP*, from the *Pseudomonas* K-62 plasmid pMR26, or the same genes with the *merE* gene of Tn*21* from the *Shigella flexneri* plasmid NR1 (R100), respectively. Cells containing pTP4 showed increased hypersensitivity to Hg(II), but maintained a normal sensitivity to $CH_3Hg(I)$. However, cells with pTPE21 exhibited increased hypersensitivity to Hg(II) and $CH_3Hg(I)$. Cells with pTP4 accumulated appreciably more Hg(II) than control cells, but no significant difference was observed in their uptake of $^{14}CH_3Hg(I)$. In contrast, the cells containing pTPE21 accumulated significantly larger amounts of Hg(II) and $^{14}CH_3Hg(I)$ than either control cells or cells with pTP4. These results suggest that the *mer* operons have evolved to redirect uptake of mercurials into dedicated, specific and relatively high-affinity transport systems comprising the small periplasmic protein MerP and two inner membrane proteins, MerT and MerE.

Key words —— *merE*, *merT*, *merP*, methylmercury transport, broad-type mercurial transporter

INTRODUCTION

Resistance to inorganic and organic mercury compounds (mercurials) is one of the most widely observed resistance phenomena in Gram-positive and -negative bacteria. Gram-negative bacteria typically have loci conferring resistance to mercurials. At a minimum, these *mer* operons contain mercuric reductase (MerA) for reducing reactive inorganic Hg(II) to volatile, relatively inert monoatomic Hg(0) vapor, a periplasmic Hg(II)-binding protein (MerP) and an inner membrane Hg(II) transport protein (MerT), which are under control of the metal-responsive positive or negative regulators MerR or MerD, respectively.[1–4] In Gram-negative bacteria, *merE* is located at the end of the operon immediately following *merD*, and *merE* is also frequently found in Gram-positive *mer* operons.[1, 3] Gram-negative bacteria encode a MerE polypeptide of 78 aa (8.0 kDa), which is predicted to form two transmembrane-spanning α-helices with a cysteine pair positioned in approximately the middle of the first helix.[5] That first cysteine pair is also predicted to be in the same position in MerT, a well-known mercury transporter.[6, 7] However, physiological information regarding MerE is limited. In a previous paper, we demonstrated that MerE localizes in the membrane cell fraction and functions as a broad-type mercury transporter, which governs transport of $CH_3Hg(I)$ and Hg(II) across the bacterial membrane.[8]

In order to determine how MerT, MerP and MerE cooperate in mercurial uptake, we constructed pTP4, which contains *merR*, *merT* and *merP* from the *Pseudomonas* K-62 plasmid pMR26, and pTPE21, which contains *merR*, *merT*, *merP* from pMR26 and the *merE* gene of Tn*21* from the *Shigella flexneri* plasmid NR1 (R100). We demonstrated that the *mer* operons have evolved to redirect mercurial uptake into a dedicated, specific and relatively high-affinity transport system consisting of the small periplasmic protein MerP and two inner membrane proteins, MerT and MerE.

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MATERIALS AND METHODS

Bacterial Strains, Plasmids and Growth Conditions —— *Escherichia coli* (*E. coli*) XL1-Blue carrying the cloning vector pKF19K was grown at 37°C in Luria-Bertani (LB) medium. This strain was used for routine plasmid propagation. When necessary, the medium was supplemented with 25 μg/ml kanamycin.

Enzymes and Reagents —— Restriction enzymes, a DNA ligation kit and Taq polymerase were obtained from Takara Shuzo Corp. (Kyoto, Japan). $^{14}CH_3HgCl$ was obtained from Amersham (Bucks, U.K.). Nonradioactive mercurials were of analytical-reagent grade and were purchased from Wako Chemicals (Tokyo, Japan).

Plasmid Construction —— Plasmid pTP4 was constructed as follows. Plasmid pMRA17, contai ning *merR*-o/p-*merT-merP-merG-merB1* (accession no. D83080), was used as the template for PCR amplification of a 1.1-kb fragment containing the *merR*-o/p-*merT-merP* genes from pMR26. The primers used were 239UPstmerR (5′-AACTGCAGCTAAGCTGTGGAAGCCCCTG-3′) and 244LXbamerP (5′-GCTCTAGAGCGATGCTGCCGTTA-3′), which contain restriction sites for *Pst*I and *Xba*I, respectively. After digestion with *Pst*I and *Xba*I, the fragment was cloned into the corresponding sites in pKF19k.

The *merE* region (0.23-kb) of Tn*21* from plasmid R100 (accession no. AF071413) was amplified using the primers 240UXbaSDmerE (5′-GCTCTAGATTCGAAAGGACAAGCGCATGAACGCCCCTGACAAACT-3′) and 243LEcomerE (5′-CGGAATTCTCATGATCCGCCCCGGAAGGC-3′). The PCR fragment containing *merE* was then cloned into the *Xba*I and *Eco*RI sites of pTP4 to yield pTPE21. The integrity of all cloned fragments was confirmed by sequencing.

The structure of the relevant genes and restriction sites used in this study are illustrated schematically in Fig. 1.

Mercurial Resistance Assay —— Bacterial resistance to $HgCl_2$ and CH_3HgCl was determined on Petri dishes, as described previously.[9] *E. coli* XL1-Blue cells carrying control plasmid or the constructs described above were grown in LB broth containing 25 μg/ml kanamycin at 37°C overnight. The cells were harvested and suspended in the original volume of LB broth, and then grown at 37°C until optical density $(OD)_{600}$ = 1.00. LB-agar plates were inoculated with cell culture and then paper disks con-

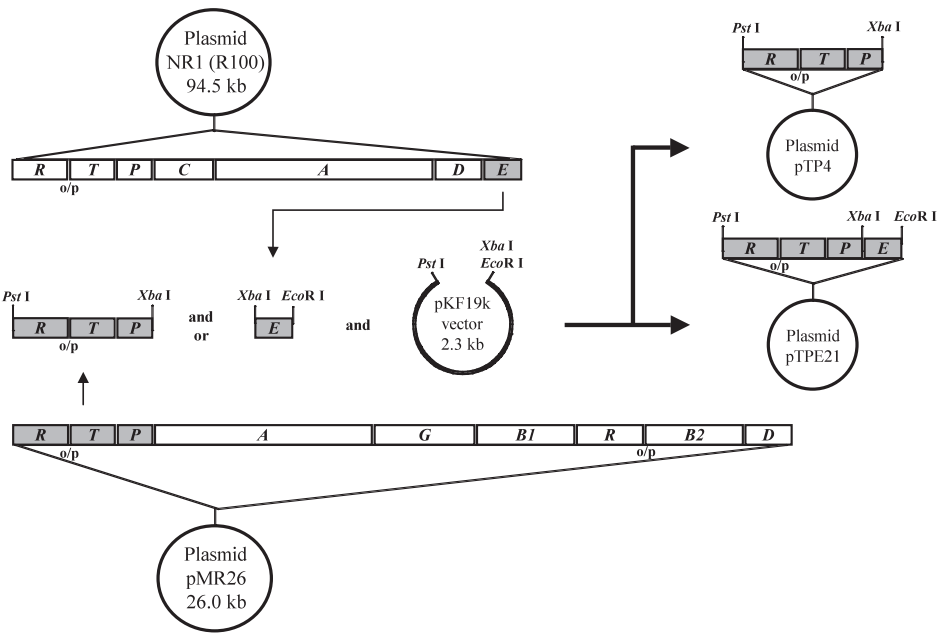

Fig. 1. A Schematic Representation of the Construction of Plasmids pTP4 and pTPE21 from the Plasmids NR1 and pMR26

Structure of the relevant *mer* genes and restriction endonuclease sites in plasmids pTP4 and pTPE21. *R*, *merR* (encodes a regulatory protein); *T*, *merT* [encodes a Hg(II) transport protein]; *P*, *merP* [encodes a periplasmic Hg(II)-binding protein]; *C*, *merC* [encodes a Hg(II) transport protein]; *A*, *merA* (encodes a mercuric reductase); *D*, *merD* (encodes a secondary regulatory protein); *E*, *merE* [encodes a Hg(II) and $CH_3Hg(I)$ transport protein]; *G*, *merG* (encodes a periplasmic phenylmercury resistance protein); *B1* & *B2*, *merB1* & *merB2* (encodes a organomercurial lyase, respectively); o/p, operator-promoter region.

taining $HgCl_2$ or CH_3HgCl were placed on the agar surface. The diameter of each zone of inhibition was measured after incubation at 37°C for 16 hr.

Mercurial Uptake Assay —— *E. coli* XL1-Blue cells carrying the control plasmid or the constructs described above were grown in LB broth containing 25 μg/ml kanamycin at 37°C overnight. The cells were harvested and suspended in the original volume of LB broth, and then grown at 37°C until OD_{600} = 1.00. Mid-exponential phase cells were harvested and resuspended in LB broth containing 100 μg/ml chloramphenicol and 100 μM EDTA.

For the $HgCl_2$ uptake assay, cell suspensions were incubated at 37°C with 10 μM $HgCl_2$. Aliquots (0.5 ml) were harvested and washed three times in LB broth containing 100 μg/ml chloramphenicol and 100 μM EDTA. After digestion with concentrated nitric acid for 1 hr at 90°C , the total mercury was measured with an atomic absorption spectrometry analyzer HG-310 (HIRANUMA, Mito, Japan). For the CH_3HgCl uptake assay, the cell suspension was incubated at 37°C with 5 μM $^{14}CH_3HgCl$ (2.11 GBq/mmol). Aliquots were removed periodically and filtered using a Whatman GF/B glass microfiber filter (0.45 μm). The filters were washed three times with LB broth containing 100 μg/ml chloramphenicol and 100 μM EDTA, and then the radioactivity on the filter was measured with a liquid scintillation spectrometer (PerkinElmerA310001). The standard deviations of measurement were less than 10%.

RESULTS

Effect of MerT, MerP and MerE on Mercury Resistance and Mercury Uptake

To evaluate the physiological roles played by MerT, MerP and MerE in bacterial cells, recombinant plasmids were constructed containing the *merR-merT-merP* genes, with or without *merE* (Fig. 1). Bacteria containing pTP4, *i.e.*, expressing MerT and MerP, showed a greater hypersensitivity to Hg(II) than control cells carrying the control plasmid pKF19k (Fig. 2A). Bacteria containing pTPE21, *i.e.*, expressing MerT, MerP and MerE, exhibited greater hypersensitivity to Hg(II) than control cells, but almost the same level of resistance as cells containing pTP4. In contrast, cells with pTPE21 showed significantly higher sensitivity to $CH_3Hg(I)$ than those carrying pTP4, which had similar sensitivity to pKF19k-containing control cells

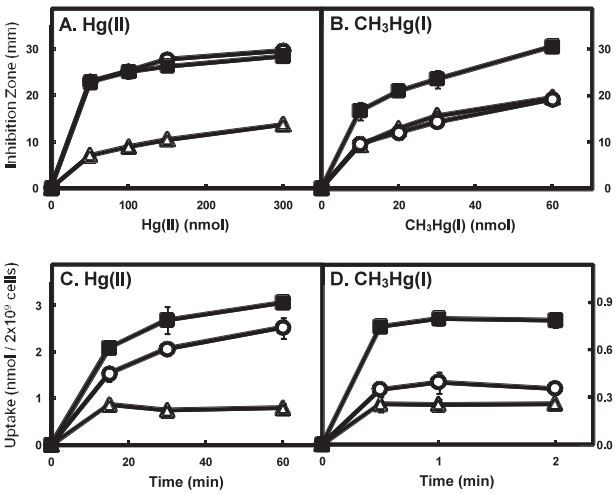

Fig. 2. Bacterial Susceptibility to Mercuric Chloride (A) or Methylmercuric Chloride (B), and Bacterial Uptake of Hg(II) (C) or $^{14}CH_3Hg(I)$ (D)

E. coli XL1-Blue with control vector pKF19 (△), pTP4 (○), or pTPE21 (■) was grown, prepared and assayed as described in Materials and methods. All values represent the means of determinations performed in triplicate from three separate experiments. Values are expressed as means ± S.D.

(Fig. 2B).

The accumulation of Hg(II) and $CH_3Hg(I)$ by cells containing the control vector pKF19k, pTP4 or pTPE21 was examined further. As shown in Fig. 2C, cells with pTP4 accumulated approximately twice as much Hg(II) as control cells and the expression of MerE (pTPE21) resulted in an even greater increase than with pTP4. Cells with pTPE21 accumulated significantly more $^{14}CH_3Hg(I)$ than cells carrying pTP4 or the control (pKF19k). No difference in the accumulation of $^{14}CH_3Hg(I)$ was observed between pTP4- and pKF19k-containing cells (Fig. 2D).

DISCUSSION

The seemingly paradoxical Hg(II) uptake system characteristic of Gram-negative mercury resistance loci can be understood in terms of the cellular biochemistry of the detoxification process.[10] The Gram-negative bacterial mercury transport genes *merT* and *merP* have been studied in great detail and they confer resistance to both Hg(II) and $C_6H_5Hg(I)$.[11, 12] Previously, we examined MerT and MerP encoded by the *Pseudomonas* K-62 plasmid pMR26 and found that, although they are involved in the transport of Hg(II) and $C_6H_5Hg(I)$,[6, 7] they do not participate in the

transport of $CH_3Hg(I)$.[9] Recently, we suggested that the *merE* gene encodes a broad-type mercury transporter that mediates transport of both Hg(II) and $CH_3Hg(I)$ across the Gram-negative bacterial membrane.[8] Although almost all mercury resistant Gram-negative bacteria have *merT*, *merP* and *merE* genes in their *mer* operon, little is known about mercurial transport by MerT, MerP and MerE. To define their roles, we constructed recombinant *mer* operons with or without the *merE* gene (Fig. 1) and compared their effects on mercurial transport.

Bacteria containing pTP4, which expresses MerT and MerP, exhibit hypersensitivity to Hg(II) (Fig. 2A) and accumulate appreciably more Hg(II) than cells carrying the control vector pKF19k (Fig. 2C). These results are consistent with our previous observations, which suggest that in the absence of mercuric reductase activity, expression of MerT and MerP renders the host cells hypersensitive to Hg(II), due to hyperaccumulation of toxic Hg(II).[9] Bacteria containing pTPE21 (*i.e.*, express MerT, MerP and MerE) also exhibit the Hg(II) hypersensitive phenotype (Fig. 2A) and accumulate significantly higher levels of Hg(II) than control cells (Fig. 2C). These experimental results clearly demonstrate that MerT, MerP and MerE play significant roles in Hg(II) transport. No difference in Hg(II) hypersensitivity was observed between cells containing pTP4 and pTPE21 at Hg(II) concentration range of 50–300 nM (Fig. 2A). Determination of the hypersensitivity to mercury in the absence of reductase activity has often been used as a method for indirect evaluation of the activity of mercury transport, but this method is not exactly proportional. We then directly examined the uptake of mercurials by the bacterial cells. The cells with pTPE21 took up more Hg(II) than the cells with pTP4 (Fig. 2C). From these results, we communicate that MerE is indeed involved in the Hg(II) transport.

In addition, cells containing pTPE21 also showed significantly greater sensitivity to $CH_3Hg(I)$ than those with pTP4, which exhibited similar levels of $CH_3Hg(I)$ sensitivity as bacteria with the vector control (Fig. 2B). Hypersensitivity to $CH_3Hg(I)$ is thought to result from MerE-mediated hyperaccumulation of $CH_3Hg(I)$. As expected, bacteria carrying pTPE21 accumulated approximately threefold as much $^{14}CH_3Hg(I)$ than cells with control vector alone (Fig. 2D). However, there was no difference in $^{14}CH_3Hg(I)$ uptake between cells carrying pTP4 or the control vector. These results demonstrate that the *merT-merP-merE* genes encode a $CH_3Hg(I)$ transporter, which controls the passage of $CH_3Hg(I)$ across the bacterial membrane. MerE-mediated transport of $CH_3Hg(I)$ may be a universal system in Gram-negative bacteria, since the amino acid sequences of MerE from Tn*21*, Tn*501*, pPB and pDU1358 show a high level of homology (73–78%).

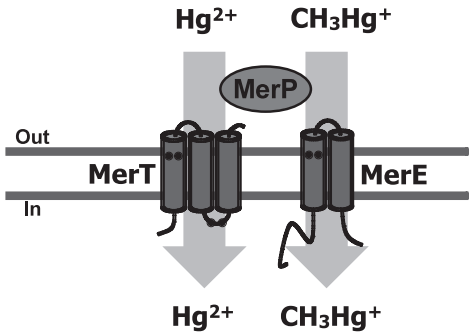

Fig. 3. A Schematic Model for the Mercury Transport System in Gram-negative Bacteria Encoded by MerE, MerT and MerP

In summary, Fig. 3 shows a schematic model of MerT, MerP and MerE-mediated Hg(II) and $CH_3Hg(I)$ transport in Gram-negative bacteria. We have demonstrated that the *merT-merP* gene products are involved mainly in the transport of Hg(II), whereas the *merT-merP-merE* products function primarily in the transport of $CH_3Hg(I)$.

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