Role of MerC, MerE, MerF, MerT, and/or MerP in Resistance to Mercurials and the Transport of Mercurials in *Escherichia coli*

Yuka Sone,^a Ryosuke Nakamura,^a Hidemitsu Pan-Hou,^b Tomoo Itoh,^a and Masako Kiyono^{*,a}

^aDepartment of Public Health and Molecular Toxicology, School of Pharmacy, Kitasato University; 5–9–1 Shirokane, Minato-ku, Tokyo 108–8641, Japan; and ^bFaculty of Pharmaceutical Sciences, Setsunan University; 45–1 Nagaotoge-cho, Hirakata, Osaka 573–0101, Japan.

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The characteristics of bacteria take up mercury into cells *via* membrane potential-dependent sequence-divergent members of the mercuric ion (Mer) superfamily, *i.e.*, a periplasmic mercuric ion scavenging protein (MerP) and one or more inner membrane-spanning proteins (MerC, MerE, MerF, and MerT), which transport mercuric ions into the cytoplasm, have been applied in engineering of bioreactor used for mercurial bioremediation. We engineered bacteria to express MerC, MerE, MerF, or MerT with or without MerP to clarify their individual role and potential in transport of mercurial. By immunoblot analysis using specific polyclonal antibody, the proteins encoded by *merC*, *merE*, *merF*, *merT* or *merP*, were certainly expressed and identified in the membrane fraction. Bacteria expressing MerC, MerE, MerF or MerT in the absence of MerP transported significantly more C₆H₅Hg(I) and Hg(II) across bacterial membrane than their isogenic strain. *In vivo* expression of MerP in the presence of all the transporters did not cause apparent difference to the C₆H₅Hg(I) transport, but gives an apparently higher Hg(II) transport than that did by MerE, MerF or MerT but not by MerC. Among the four transporters studied, MerC showed more potential to transport Hg(II) across bacterial membrane than MerE, MerF and MerT. Together these findings, we demonstrated for the first time that in addition to MerE and MerT, MerF and MerC are broad-spectrum mercury transporters that mediate both Hg(II) and phenylmercury transport into cells. Our results suggested that MerC is the most efficient tool for designing mercurial bioremediation systems, because MerC is sufficient for mercurial transport into cells.

Key words *merC*; *merE*; *merF*; *merP*; *merT*; mercurial transporter

Mercury is the most toxic heavy metal because of its high affinity for the sulfhydryl ligands in amino acids, which alters the structure of proteins after binding, often leading to a loss of function.¹⁾ Mercury affects ecosystems and human health, and the increasing prevalence of this metal in the environment is a growing problem. Thus, there is an urgent need to develop cost-effective, sustainable, and environmentally friendly methods that facilitate the removal of mercury from contaminated sites.

Bioremediation using bacteria or plants is often regarded as a relatively inexpensive and efficient method for cleaning up waste, sediments, and soils contaminated with toxic mercury.²⁾ In general, the rehabilitation of metal-contaminated soils by plants requires a long time. McGrath and Zhao reported that several months were required to reduce the mercury content of contaminated soils by half.³⁾ The expression of bacterial mercury transporters in plants may improve mercury uptake, thereby shortening the time required to complete the purification process.

Bacteria can be used for bioremediation because they take up mercury *via* membrane potential-dependent sequence-diverged members of the mercuric ion (Mer) superfamily, *i.e.*, a periplasmic Hg(II)-scavenging protein (MerP) and one or more inner membrane-spanning proteins (MerC, MerE, MerF, and MerT), which transport Hg(II) into the cytoplasm.^{4–11)} These proteins have two, three, or four transmembrane domains (TMD), but TMD I and II are found in all members of the superfamily.¹²⁾ In all these proteins, one pair of cysteine residues is predicted to reside within the inner membrane, with or

without a second pair of cysteine residues on the cytoplasmic face. Hg(II) is thought to pass into the cytoplasm by a series of exchange reactions between paired cysteine thiols in MerP and MerT led to the so-called “baseball glove” model. The baseball glove model consists of binding of Hg(II) initially by pair of vicinal cysteines in MerP, followed by sequential passing of Hg(II) from glove to glove in MerT.⁵⁾ The mechanism of transport of Hg(II) across the bacterial membrane mediated by *merC*, *merE*, *merF* or *merT* has been well studied.^{6,11,13–16)} However there is no direct experimental evidence concerning bacterial transport of organomercury until recently.

In the present study, to clarify the individual role and potential of MerC, MerE, MerF, or MerT in transport of mercurial, the *merC* or *merE* gene from Tn21 in the *Shigella flexneri* plasmid NR1 (R100), *merF* gene from the *Pseudomonas fluorescens* plasmid pMER327/419 or *merT* gene from *Pseudomonas* K-62 plasmid pMR26 with or without a periplasmic Hg(II)-binding protein gene, *merP* from pMR26 were cloned into *E. coli* under control of mercury inducible regulatory part, *merR*-operator/promoter (o/p) genes from the *mer*-operon of pMR26 (Table 1).

Here we showed for the first time that in addition to MerE and MerT, MerC and MerF are broad-spectrum mercury transporters that mediate both C₆H₅Hg(I) and Hg(II) transport into cells. In addition, our results showed that coexpression of MerP with the four mercury transporters did not cause apparent difference to the C₆H₅Hg(I) transport, but gave higher Hg(II) transport than the expression of MerE, MerF or MerT alone. Among the four transporters, MerC showed highest potential for Hg(II) transport across the bacterial membrane.

The authors declare no conflict of interest.

*To whom correspondence should be addressed. e-mail: kiyonom@pharm.kitasato-u.ac.jp

Table 1. Strains and Plasmids Used in This Study

Strains and plasmids	Description of relevant feature(s)	Reference or source
Strains		
<i>E. coli</i> XL1-Blue	<i>recA1 endA1 gyrA96 thi hsdR17 supE44 relA1 lac</i> [F':Tn10 <i>proAB</i> ⁺ <i>lacI</i> ^a <i>lacZM15 traD36</i>]	Bullock <i>et al.</i> (1987)
Plasmids		
pE4	<i>merR</i> -o/p- <i>merE</i> in pKF19k	Kiyono <i>et al.</i> (2009)
pF17	<i>merR</i> -o/p- <i>merF</i> in pKF19k	This study
pT5	<i>merR</i> -o/p- <i>merT</i> in pKF19k	This study
pC7	<i>merR</i> -o/p- <i>merC</i> in pKF19k	This study
pPE62	<i>merR</i> -o/p- <i>merP</i> - <i>merE</i> in pKF19k	This study
pPF8	<i>merR</i> -o/p- <i>merP</i> - <i>merF</i> in pKF19k	This study
pTP4	<i>merR</i> -o/p- <i>merT</i> - <i>merP</i> in pKF19k	Sone <i>et al.</i> (2010)
pPC20	<i>merR</i> -o/p- <i>merP</i> - <i>merC</i> in pKF19k	This study

MATERIALS AND METHODS

Computer Analyses of Protein Sequences The transmembrane helices of individual proteins were predicted using FASTA-formatted sequences, which were analyzed with the TMHMM program.¹⁷⁾ The topologies of MerE, MerF, MerT, and MerC were analyzed using the SOSUI program, which predicts the N-terminal and C-terminal regions located in the cytoplasm.

Bacterial Strain, Plasmids, and Growth Conditions *Escherichia coli* (*E. coli*) XL1-Blue harboring pKF19K,¹⁸⁾ a cloning vector, was grown at 37°C in Luria–Bertani (LB) medium and used for routine plasmid propagation. The medium was supplemented with 25 µg/mL kanamycin as necessary.

Enzymes and Reagents The restriction enzymes, DNA ligation kits, and Taq polymerase were obtained from Takara Shuzo Corp. (Kyoto, Japan). The mercury was analytical reagent grade and was purchased from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan).

Plasmid Construction The plasmids pF17, pT5, and pC7, which carried the *merR*-o/p-*merF*, *merR*-o/p-*merT*, and *merR*-o/p-*merC* genes, respectively, were constructed in pKF19k, as follows, whereas the construction of pE4 has been described previously.¹⁹⁾ The primers UKpnmerF (5'-GGGGTACCA TGA AAG ACC CGA AGA CACT-3') and LEcomerF (5'-CGG AATTCTCATTTTTTTTACTCCA TTGA-3') were used to amplify the *merF* region (0.25 kb) of Tn5053 (accession no. L40585) in the plasmid pUC18F.¹¹⁾ After digesting the polymerase chain reaction (PCR) product with *KpnI* and *EcoRI*, the fragment was cloned into the *KpnI*–*EcoRI* site of pR2,¹⁹⁾ which contained the *merR*-o/p gene of plasmid pMR26 (accession no. D83080) from pKF19k. The cloned fragment was sequenced and the resulting plasmid was designated as pF17. The primers UKpnmerT (5'-GGGGTACCA TGTCTGAAC CACAAAACGG-3') and LEcomerT (5'-CGGAATTCTTAA TAGAAAAATGGAACGA-3') were used to obtain the *merT* region (0.35 kb) of pMR26. After digesting the PCR product with *KpnI* and *EcoRI*, the fragment was cloned into the *KpnI*–*EcoRI* site of plasmid pR2 and the cloned fragment was sequenced, where the resulting plasmid was designated pT5. The primers UKpnmerC (5'-GGGGTACCA TGGGACTGATGA CACGCA T-3') and LEcomerC (5'-CGGAATTCTCACAAG CGCTTGCGGGGA-3') were used to amplify the *merC* region (0.46 kb) of Tn21 (accession no. AF071413). After digesting the PCR product with *KpnI* and *EcoRI*, the fragment

was cloned into the *KpnI*–*EcoRI* site of plasmid pR2 and the cloned fragment was sequenced, where the resulting plasmid was designated as pC7.

The plasmids pPE62, pPF8, and pPC20, which carried the *merR*-o/p-*merP*-*merE*, *merR*-o/p-*merP*-*merF*, and *merR*-o/p-*merP*-*merC* genes, respectively, were constructed in pKF19k as follows, whereas pTP4 was constructed as described previously.²⁰⁾ A 0.5 kb fragment containing the *merP*-*merE* genes from plasmid pTPE21²⁰⁾ was PCR-amplified using the primers UKpnmerP (5'-GGGGTACCA TGA AGA AAC TGT TTG CCT C-3') and LEcomerE (5'-CGGAATTCTCATGATCCGCCC CGGAAGGC-3'), which contained *KpnI* and *EcoRI* sites. After digesting the PCR product with *KpnI* and *EcoRI*, the fragment was cloned into the *KpnI*–*EcoRI* site of vector pR2 and sequenced, where the resulting plasmid was designated as pPE62. The primers UXbaSDmerF (5'-GCTCTAGAT TCG AAA GGA CAA GCG CAT GAA AGA CCC GAA GAC ACT-3') and LEcomerF were employed to amplify the *merF* region (0.25 kb) using Tn5053 as a template, and the primers UXbaSDmerC (5'-GCTCTAGATTCGAAAAGGACAAGCG CATGGGACTGATGACACG CAT-3') and LEcomerC were employed to amplify the *merC* region (0.46 kb) using Tn21 as a template. After digesting the PCR products with *XbaI*–*EcoRI*, the fragments were cloned into the *XbaI*–*EcoRI* sites of pPE62, including one *XbaI* site between the *merP* and *merE* genes. The cloned fragments were sequenced and the resulting plasmids were designated pPF8 and pPC20. The structures of the relevant genes and the restriction sites in the plasmids constructed in this study are shown in Table 1.

Preparation of Specific Antibodies Plasmid pMRD141,⁸⁾ which contained *merR*-o/p-*merT*-*merP* from pMR26, was used as the template for the PCR amplification of a 0.3 kb *XbaI*–*BglII* fragment containing *merP*. The primers used were UP1117Xba (5'-GCTCTAGAT TCCATT TTTCTA TTA-3') and LP1412Bgl (5'-GAAGATCTCTTCTTCAGC TCAGAT-3'), which contained restriction sites for *XbaI* and *BglII*, respectively. After digestion with *XbaI* and *BglII*, the fragment was cloned into the corresponding sites in plasmid pTUE1122,²¹⁾ which had a 6× His tag-encoding sequence, and the recombinant plasmid pMUP1122 was transformed into *E. coli* cells. *E. coli* transformants that carried pMUP1122 were grown in 200 mL of LB broth supplemented with 50 µg of ampicillin per mL at 37°C for 3 h in the exponential growth phase, before 1 mM isopropyl-β-D-thiogalactopyranoside was added to the culture and the cells were incubated for a further

3 h at 37°C. The MerP-His-tagged recombinant protein was purified by affinity chromatography using Ni-nitrilotriacetic acid agarose beads (Qiagen, Chatsworth, CA, U.S.A.), according to the manufacturer's instructions. One milliliter of purified MerP-His-tagged protein (0.2 mg/mL) was mixed well with 2 mL of complete Freund's adjuvant (Difco) and 3 mL of the emulsion was injected into a healthy rabbit. After 2 weeks, a mixture of the same amount of protein and 2 mL of incomplete Freund's adjuvant (Difco) was injected. Two weeks after the second immunization, antisera against the relevant antigen MerP were obtained.

To obtain a large volume of MerF or MerT-His₆-tagged protein for antibody production, the proteins were expressed using a baculovirus-silkworm expression system (Katakura Industries Co., Ltd., Saitama, Japan). The primers UXhomerF (5'-CCGCTCGAGATGAAAGACCCGAGACAC-3') and LXbamerFhista (5'-GCTCTAGATTAGTAGGTGATGGTGA TGT TTT TTT ACTCCATTGAAT-3'), or UXhomerT (5'-CCGCTCGAGATGTCTGAACCAAAAAC-3') and LXbamerThista (5'-GCTCTAGATTAGTAGGTGATGGTGA TGA TAG AAAAATGGAACGAC-3'), were used to produce *merF* (0.25 kb) from Tn5053 or *merT* (0.35 kb) from pMR26, respectively. The PCR fragments of *merF* or *merT*-his tag were inserted into the *XhoI*-*XbaI* site in the transfer vector pM01 (Katakura Industries Co., Ltd., Saitama, Japan) and sequenced. The transfer vector and linearized genomic DNA from the ABv baculovirus (*Bombyx mori* nucleopolyhedrovirus; CPd strain; Katakura Industries Co., Ltd., Saitama, Japan)²² were co-transfected into *B. mori*-cultured cells (BmN).²³ After propagating the recombinant baculovirus containing the *merF*- or *merT*-his₆-tagged gene in BmN cells, the virus was used to infect silkworm pupae. Six days after inoculation, the infected pupae (40 g) were homogenized in 140 mL of homogenization buffer (20 mM Tris-HCl, pH 8.0, 0.15 M NaCl, 1 mM ethylenediamine tetraacetic acid (EDTA), 1 mM ethylene glycol bis(2-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), 10% glycerol, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride, and 10 mM benzamidine) and centrifuged for 1 h at 100000×*g* and 4°C, and the supernatant was discarded. The pellet was resuspended in 140 mL of homogenization buffer with 1% Triton X-100. After incubation with stirring for 1 h at 4°C, the suspension was centrifuged, as described above, and the pellet was discarded. The supernatant was applied to a Ni-agarose column and the column was washed with wash buffer (25 mM Tris-HCl, pH 8.0, 0.25 M NaCl, 1 mM EDTA, 5% glycerol, and 0.5% Triton X-100). The MerF- or MerT-His₆-tagged protein was then eluted using elution buffer (100 mM Tris-HCl, pH 8.0, 0.3 M NaCl, 1 mM EDTA, 5% glycerol, 0.3% Triton X-100, and 0.4 M imidazole). Fractions that contained the MerF- or MerT-His₆-tagged protein were pooled and frozen at -80°C until further use. The MerF- or MerT-His₆-tagged protein was injected into rabbits, and the anti-MerF and anti-MerT antibodies were prepared by Operon Biotechnology (Tokyo, Japan).

The anti-MerC or anti-MerE antibodies were prepared as described previously.¹⁹

Subcellular Fractionation, Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE), and Western Blot Analysis *E. coli* XL1-Blue cells that contained the plasmids pE4, pF17, pT5, pC7, pPE62, pPF8, pTP4, or pPC20 were grown to an optical density of 0.80 at 600 nm with

0.5 μM HgCl₂. The cells were centrifuged, washed with buffer (50 mM Tris-HCl, pH 8.0, 0.15 M NaCl, 1 mM EDTA, and 10% glycerol), and suspended in 1.2 mL of the same buffer. The harvested cells were disrupted by sonication in the presence of 80 μM phenylmethylsulfonyl fluoride, 2 mM benzamidine, and 0.25 μg/mL leupeptin. Undisrupted cells were removed by low-speed centrifugation. The cell homogenate was centrifuged at 104000×*g* for 30 min. After centrifugation, the pellet containing the membrane fraction was suspended in 1.2 mL of wash buffer supplemented with 1% Triton X-100.

The soluble (supernatant) and particulate fractions were boiled with an equal volume of 2× Laemmli's sample buffer for 5 min. SDS-PAGE was performed using 12.5% mini-gels. The proteins were separated by SDS-PAGE and transferred to an Immobilon-P membrane (Millipore, Billerica, U.S.A.). After blocking with de-fatted milk, the membrane filter was incubated with anti-MerE,¹⁹ anti-MerF, anti-MerT, anti-MerC,¹⁹ or anti-MerP antibodies to detect the proteins of interest. The membranes were washed and reacted with peroxidase-conjugated anti-rabbit immunoglobulin G (IgG) (Sigma-Aldrich, MO, U.S.A.). Chemiluminescent ECL reagents (GE Healthcare, Giles, U.K.) were used to detect the antigens. The membranes were washed with PBS plus 0.1% Tween 20 and reacted with peroxidase-conjugated anti-rabbit IgG (Sigma-Aldrich, MO, U.S.A.) for 1 h. After washing, the immunoreactive bands were visualized using an ECL detection kit (GE Healthcare, Chalfont, St. Giles, U.K.).

Mercurial Resistance Assay The mercurial (C₆H₅HgOCOCH₃ or HgCl₂) resistance capacities of *E. coli* strain XL1-Blue cells that carried pKF19k (control vector) or recombinants were determined in liquid medium. Cells that carried the control or constructs were grown overnight at 37°C in LB broth containing 25 μg/mL kanamycin. The cells were harvested and suspended in LB broth (8×10⁷ cells/200 μL/well) containing various concentrations of C₆H₅HgOCOCH₃ or HgCl₂. After incubation at 37°C for 16 h, the absorbance of each culture was measured at A₆₀₀ to quantify the cell growth.

Mercurial Uptake Assay *E. coli* XL1-Blue cells carrying the control or recombinants (as described above) were grown overnight at 37°C in LB broth containing 25 μg/mL kanamycin. The cells were harvested and suspended in the original volume of LB broth and grown at 37°C until OD₆₀₀=1.00. The cells were harvested and resuspended in LB broth containing 100 μg/mL chloramphenicol and 100 μM EDTA. The cell suspensions were incubated for 30 min at 37°C with 5 μM C₆H₅HgOCOCH₃ or 10 μM HgCl₂. Aliquots (0.5 mL) were harvested and washed three times with LB broth containing 100 μg/mL chloramphenicol and 100 μM EDTA. After digestion with concentrated nitric acid for 1 h at 90°C, the total mercury was measured using an atomic absorption spectrometry analyzer HG-310 (Hiranuma, Mito, Japan).

RESULTS

Analysis of the Protein Sequences and Localization of MerE, MerF, MerT, MerC, and MerP The mature MerP is a periplasmic Hg(II)-binding protein of about 80 amino acids, which is synthesized with a cleavable N-terminal leader, and Hg(II) is bound to the sequence GMTCCXC. Hydropathy prediction programs indicated that MerE from *Shigella flexneri* and MerF from *Pseudomonas fluorescens* contained

Fig. 1. Topological Alignment of pMR26 MerP, R100 MerE, pMER327/419 MerF, pMR26 MerT, and R100 MerC Amino Acids According to Their Predicted Hydrophobic Elements (A), and Western Blot Analyses of the Five Proteins (B)

The crude cell extracts (lane 1), soluble fractions (lane 2), and membrane fractions (lane 3) of the transformant strains containing plasmids pE4, pF17, pT5, pC7, pPE62, pPF8, pTP4, and pPC20 were analyzed using anti-MerE, anti-MerF, anti-MerT, anti-MerC, and anti-MerP polyclonal antibodies. *E. coli* XL1-Blue cells containing the plasmids pE4, pF17, pT5, pC7, pPE62, pPF8, pTP4, or pPC20 were generated and grown with Hg(II), as described in Materials and Methods. The arrows indicate MerE (8.0kDa), MerF (8.7kDa), MerT (12.5kDa), MerC (15.0kDa), and MerP (9.5kDa).

two membrane-spanning regions, whereas MerT from *Pseudomonas* strain K-62 had three and MerC from *Shigella flexneri* had four (Fig. 1A). In each case, the first region contained two closely positioned cysteine residues and a nearby distal proline residue (Fig. 1A). With the exception of MerE, each protein also contained an additional pair of cysteine residues, which were predicted to be located in the cytoplasm, with an adjacent proline (Fig. 1A). In MerF and MerC, this region was close to the carboxyl terminal, whereas it was located between TMDs II and III in MerT (Fig. 1A).

To determine how MerE, MerF, MerT, MerC, and MerP cooperate during mercurial uptake, recombinant plasmids were constructed that contained the *merR*-o/p and *merE*, *merF*, *merT*, or *merC* genes, with or without *merP* (Table 1). Mer protein expression in the transformants was measured by immunoblot analysis using polyclonal anti-MerE,¹⁹⁾ anti-MerF, anti-MerT, anti-MerC,¹⁹⁾ and anti-MerP antibodies with SDS-PAGE. The following novel protein bands were identified in the crude cell extracts and membrane fractions from cells that contained pE4, pF17, pT5, pC7, pPE62, pPF8, pTP4, and pPC20 (Fig. 1B, lanes 1 and 3): a band with a molecular mass of 8.0kDa that reacted specifically with the anti-MerE antibody, a band with a molecular mass of 8.7kDa that reacted specifically with the anti-MerF antibody, a band with a molecular mass of 12.5kDa that reacted specifically with the anti-MerT antibody, a band with a molecular mass of 15kDa that reacted specifically with the anti-MerC antibody, and/or a band with a molecular mass of 9.5kDa that reacted spe-

A. Without MerP

B. With MerP

Fig. 2. Mercury Susceptibility of Recombinant *E. coli* Cells That Expressed MerE, MerF, MerT, or MerC without MerP (A) and with MerP (B)

E. coli XL1-Blue cells that contained the vectors pKF19k (open or closed inverted triangles), pE4 (open circles), pF17 (open diamonds), pT5 (open squares), pC7 (open triangles), pPE62 (closed circles), pPF8 (closed diamonds), pTP4 (closed squares), or pPC20 (closed triangles) were grown, prepared, and assayed as described in Materials and Methods. All of the values represent the means of triplicate determinations from three independent experiments. The values are expressed as the mean±standard deviation. The asterisk indicates significant differences from the control value at the same time point (**p*<0.01, *t*-test).

cifically with the anti-MerP antibody. The protein sizes were consistent with those predicted based on the translations of the DNA sequences of the *merE*, *merF*, *merT*, *merC*, and *merP* genes, respectively.

Mercury Resistance and Mercury Uptake The resistance to and accumulation of Hg(II) or C₆H₅Hg(I) by cells containing the vectors pKF19k, pE4 (MerE), pF17 (MerF), pT5 (MerT), pC7 (MerC), pPE62 (MerP-MerE), pPF8 (MerP-MerF), pTP4 (MerT-MerP), pPC20 (MerP-MerC), or pPE62 (MerP-MerE) were examined. Figure 2 shows the mercury susceptibility of recombinant *E. coli* cells that expressed MerE, MerF, MerT, or MerC without MerP (A) and with MerP (B). The cells with MerE or MerF were more sensitive to Hg(II) and C₆H₅Hg(I) than their isogenic cells with the cloning vector (Fig. 2A). The cells with MerT or MerC were more sensitive to Hg(II) and C₆H₅Hg(I) than MerE and MerF (Fig. 2A). The Hg(II) resistance levels of the cells that co-expressed MerP and MerE/MerF were lower than that of the control (Fig. 2B). Cells with MerT-MerP or MerP-MerC were also more sensitive to Hg(II) compared with MerP-MerE and MerP-MerF (Fig. 2B). By contrast, the C₆H₅Hg(I) resistance levels of cells

Fig. 3. Bacterial Uptake of C₆H₅Hg(I) or Hg(II)

E. coli XL1-Blue cells containing the vectors pKF19k (control), pE4 (open bar), pPE62 (closed bar), pF17 (open bar), pPF8 (closed bar), pT5 (open bar), pTP4 (closed bar), pC7 (open bar), or pPC20 (closed bar) were grown, prepared, and assayed as described in Materials and Methods. All of the values represent the means of triplicate determinations from three experiments. The values are expressed as the mean $\pm$ standard deviation. * p <0.05 and ** p <0.01 vs. control; † p <0.01 vs. pE4; ‡ p <0.01 vs. pF17; § p <0.05 vs. pT5; †† p <0.01 vs. pC7.

that expressed MerE, MerF, MerT, or MerC, with or without MerP, were lower than that of the control (Fig. 2B).

As shown in Fig. 3A, the cells that expressed MerE, MerF, MerT, or MerC accumulated significantly more C₆H₅Hg(I) than their isogenic cells with the cloning vector, pKF19k. Coexpression of MerP with MerE, MerF, MerT, or MerC did not cause increased C₆H₅Hg(I) accumulation than the cell expressed transporter alone (Fig. 3A). In contrast, cells that expressed MerE, MerF, MerT, or MerC accumulated significantly more Hg(II) than their isogenic cells with the cloning vector, pKF19k. Among the four transporters, MerC showed more potential to accumulate Hg(II) than MerE, MerF, or MerT. Cells co-expressing MerP with MerE, MerF, or MerT accumulated significantly more Hg(II) than the cells that lacking MerP, however MerP did not cause increased the MerC-specified Hg(II) accumulation (Fig. 3B).

DISCUSSION

Bioremediation is an economical and effective technology for dealing with a wide variety of contaminants. However this technology is a slow process that requires a long time to complete the purification. This potential fault may predominantly result from low mercury-uptake activity and thereby limit

its usefulness for practical application. Among the strategies being used to overcome this disadvantage is use of mercury transporter to boost the uptake and transport of mercurial into bacteria or plant cells. Several lines of evidence suggest that accelerated mercury uptake and enhanced mercury accumulation is a particularly promising strategy for improving the bioreactor to be more suitable for use in remediation of mercurials.^{24,25)}

In the present study, the efficiency and potential of the known mercury transporters, MerC, MerE, MerF or MerT, and whether these proteins are also involved in the transport of organomercurial was investigated. The *merC*, *merE*, *merF* or *merT*, genes cloned in *E. coli* were certainly translated into proteins with molecular masses of 15kDa, 8kDa, 8.7kDa or 12.5kDa, respectively, which were located in the membrane fraction, in cells (Fig. 1B). By hydropathy analysis we predicted that MerC, MerE, MerF and MerT had, respectively, four, two, two and three transmembrane regions. Each protein has a cysteine pair located in the first transmembrane regions: Cys-Cys in MerT and MerF, Cys-Pro-Cys in MerE, and Cys-Ala-Ala-Cys in MerC which have been shown to be essential for Hg(II) transport into the cell. In addition, in all four proteins, proline residue is conserved on the C-terminal site of this cysteine motif. These observations are consistent with the

results previously obtained.^{6,11,13–16)}

Bacteria expressing MerC, MerE, MerF or MerT showed more sensitivity not only to Hg(II) but also to C₆H₅Hg(I) than their isogenic control strain independently of MerP (Fig. 2). This hypersensitivity phenotype is thought to result from hyperaccumulation of toxic mercurials in the absence of mercury-detoxifying enzyme. The hypersensitivity phenotype to C₆H₅Hg(I) noted by the recombinants suggest for the first time that the four mercury transporters are involved in the C₆H₅Hg(I) transport across bacterial membrane. Indeed, bacteria expressing MerC, MerE, MerF or MerT in the absence of MerP took up appreciably more C₆H₅Hg(I) and Hg(II) than their isogenic cells without these transporters (Fig. 3). *In vivo* expression of MerP in the presence of MerC, MerE, MerF or MerT did not cause increased C₆H₅Hg(I) uptake than those in the absence of MerP (Fig. 3A). However, cells co-expressing MerP with MerE, MerF, or MerT, but not MerC, took up significantly more Hg(II) than the cells lacking MerP (Fig. 3B). Among the four transporters tested, MerC showed a relatively higher Hg(II) uptake in the absence of MerP (Fig. 3B). In the current model of mercury transport system, MerP has been shown to act primarily as a Hg(II) scavenger, binding Hg(II) and keeping it from other periplasmic proteins which require thiol groups for activity.^{5,26)} In addition MerP may increase the local concentration of Hg(II) in the periplasmic compartment and pass the Hg(II) to inner membrane transporter. Enhancement of Hg(II) uptake mediated by MerE, MerF or MerT in the presence of MerP (Fig. 3B) seems to be primarily resulted *via* the high local concentration of Hg(II) in the periplasmic compartment. However it would be curious why the Hg(II) uptake mediated by MerC did not activate by MerP. Further studies are required to achieve a more complete understanding of this discrepancy.

Bioremediation is highly attractive technology for mercury remediation from the contaminated sites but it is still in its infancy stage. Further study, for example, seeking a molecule that can sequester and retain the transported mercury into living cells but without taxing the cells is required for using mercury transporter in mercury remediation. We have previously shown that polyphosphate, a biomolecule is capable of reducing the cytotoxicity of the transported Hg(II) and retaining more mercury in the living cells but without the cells *via* chelation formation with polyphosphate.^{27–29)}

In conclusion, this study showed for the first time that in addition to MerE and MerT, MerF and MerC are broad-spectrum mercury transporters that mediate the transport of mercuric ions and phenylmercury. Thus, in addition, MerC is the most efficient tool for designing a potential bioreactor used in environmental bioaccumulation of mercurial pollution.

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