— **Minireview** —

Genetic Engineering of Bacteria for Environmental Remediation of Mercury

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(Received February 8, 2006)

To prevent environmental mercury poisoning incidents, an effective technology for treating mercury-polluted environments is urgent. Recently, with advances in biotechnology, bioremediation utilizing microorganisms to remove mercurials from contaminated sites has become one of the most rapidly developing fields of environmental restoration. A number of bioremediation strategies, including biotransformation, biosorption, and bioprecipitation of mercurials, have been developed to treat mercurial-polluted environments and mercury-containing waste. To construct bacteria that are capable of specifically accumulating mercury, we have genetically engineered *Escherichia coli* to express a mercury transport system and organomercurial lyase enzyme simultaneously, and overexpress polyphosphate, a strong chelator of essential divalent metals. The mercury accumulation system was designed so that overexpressed polyphosphate would serve as a mercury accumulator; the mercury transport system would make the bacterial cell specifically accumulate mercury; and the intracellular accumulation process would allow the bioaccumulation system to be less sensitive to ambient conditions. The applicability of the new engineered bacteria in the environmental remediation of mercurials is evaluated and discussed in this review.

Key words —— mercurial bioremediation, pMKB18, polyphosphate, mercury-resistant gene, polyphosphate kinase gene

INTRODUCTION

Mercury has been recognized as one of the most toxic heavy metals in the environment and has been released into environment in substantial quantities through natural events and anthropogenic activities. The effects of this heavy metal on the ecosystem and human health are growing concerns. Several physically and chemically based technologies have been utilized to remove mercury from polluted sites, although these treatments are extremely expensive, environmentally disruptive, labor intensive, and/or require input of external chemical additives and generate concentrated waste streams that must be disposed.[1–3] Biological remediation of mercury contamination may replace or be used in conjunction with engineering-based methods, potentially reducing both the cost and environmental impact. Mercury-resistant bacteria are now considered a potential approach to biological remediation. Bioremediation strategies including biotransformation, biosorption, and bioprecipitation of mercurials have been developed and rarely been applied to remediation of mercurials in the environment.[4–6] The adsorptive treatment and bioprecipitation process are generally sensitive to the ambient conditions, *e.g.*, pH and the presence of other metals or metal chelators. In particular, they lack specificity, which may cause difficulties in the recovery of the desired metals. Biotransformation through bacterial reduction and volatilization of mercurials mediated by the bacterial mercury-resistant (*mer*) gene, *merA*, for the environmental remediation of mercury pollution has been one of the most actively studied processes.[7–11] However, this *merA*-mediated process still causes public anxiety because of the release of mercury vapor into the ambient air that will redistribute in the ecosphere and be recycled to the environment.

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To help solve this environmental problem, a new mercury-scavenging mechanism that can be expressed in living cells and accumulate mercury from contaminated sites without releasing mercury vapor into the ambient air is necessary in place of the mercury reduction mechanism mediated by *mer* genes.

Advances in genetic engineering techniques have opened up new avenues to move toward the goal of genetically engineered bacteria to function as designer biocatalysts, in which certain functional proteins from different bacteria are brought together in a single bacterial cell with the aim of performing a specific reaction. To develop a potential biocatalyst to recover and accumulate mercurials from contaminated sites without releasing mercury vapor into the ambient air, the *Pseudomonas* K-62 *mer* genes should first be used to evaluate the organization of *mer*-genes and their functions.

ORGANIZATION OF *Pseudomonas* K-62 *mer* GENES

Pseudomonas K-62, a bacterial strain with broad-spectrum mercury resistance isolated from phenylmercury-polluted soil, has been shown to have approximately a 1000-fold higher resistance phenotype to phenylmercury than other bacterial strains such as *Escherichia coli* (*E. coli*) and *Pseudomonas aeruginosa*.[12] Although the biochemical basis of resistance noted for this soil strain was demonstrated to be due to enzymatic degradation of organomercurial to Hg^{2+} and reduction of the yielded Hg^{2+} to elemental mercury (Hg^0) catalyzed by organomercurial lyase and mercuric reductase,[13–15] respectively, there is no direct experimental evidence to explain this hyperresistant phenotype. Recently, in a genetic study of the mercury resistance determinants of *Pseudomonas* K-62, we have demonstrated for the first time that the mercurial resistance of this soil strain is conferred by pMR26 and pMR68, two of the six plasmids in the bacterial cell.[16] In addition, we found that the plasmid pMR26 contained two *mer* gene clusters (*mer* operons) that mapped about 1 kb apart. One of the two *mer* operons conferred bacterial resistance to both inorganic and organic mercury, and the other *mer* operon conferred bacterial hypersensitivity to organomercurials.[16] Both the *mer* operons have been cloned into the *Sac*I site of the cloning vector pBluescript II, and the resultant plasmids were designated pMRA17 and pMRB01, respectively.[16] DNA sequence analysis of the two

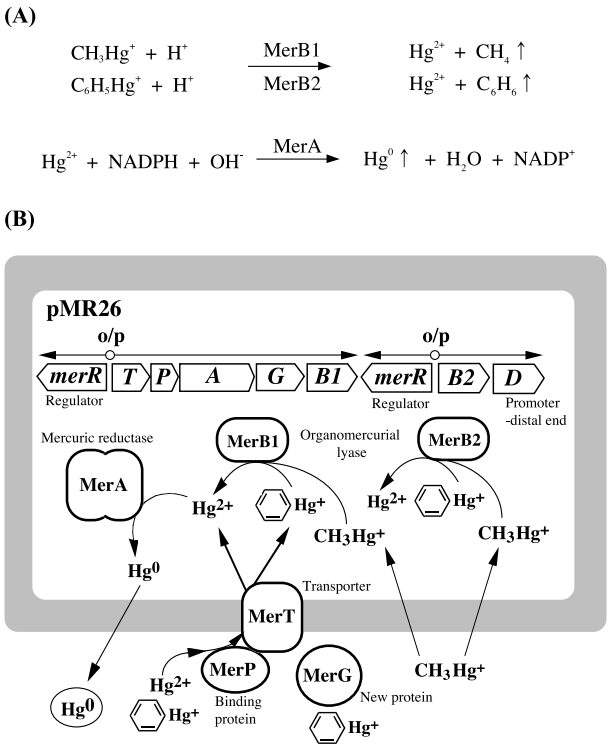

Fig. 1. Detoxificaion of Organomercurial and Hg^{2+} (A) and Genes and Corresponding Proteins of the Mercury Resistance System of pMR26 from *Pseudomonas* K-62 (B)

mer operons in pMR26 revealed that it comprised six open-reading frames (ORFs) in the first operon (pMRA17), five of which were identified as *merR*, *merT*, *merP*, *merA*, and *merB1* in that order,[17] and three ORFs in the second *mer* operon (pMRB01) are referred to as *merR*, *merB2*, and *merD*[18] by comparison with the DNA and amino acid sequences of previously sequenced *mer* operons. The mercurial-resistance determinants of *Pseudomonas* K-62 have thus finally been completely clarified (Fig. 1).

FUNCTION OF *Pseudomonas* K-62 *mer* GENES

The function of *Pseudomonas* K-62 *mer* genes involved in mercurial resistance on plasmid pMR26 (Fig. 1) was found to be basically the same as those in the narrow- and broad-spectrum *mer* operons.[19–22] As currently viewed,[23] *merR* is a regulatory gene that controls transcription of the structural genes both negatively and positively. *MerD* is associated with a transcriptional coregulatory function. *MerT*, *merP*, *merA*, and *merB* code for membrane Hg^{2+}-transport

protein, periplasmic Hg^{2+}-binding protein, mercuric reductase, and organomercurial lyase, respectively. In most cases, *merB* is mapped immediately downstream from *merA*. An additional ORF designated *merG*, located between *merA* and *merB1* on pMRA17, had no significant homology with the published *mer* genes and appears to be a new *mer* gene that may be involved in phenylmercury resistance.

Bacterial resistance to Hg^{2+} is known to occur by transport of Hg^{2+} into the cytoplasm of bacterial cells and subsequent enzymatic reduction of toxic Hg^{2+} to less toxic and volatile Hg^0.[19–22] The transport proteins MerP and MerT encoded by *merP* and *merT*, respectively, are required to deplete mercury from the periplasm and to protect the periplasmic face of the inner membrane damage by Hg^{2+}.[24,25] The precise role of *merT* and *merP* in the transport of Hg^{2+} has been studied in great detail. However, it is not yet known whether these *merT* and *merP* genes are involved in the uptake of organomercury into the cytoplasm. It appears reasonable to expect that the transport of organomercury into the cytoplasm is necessary for the organomercurial lyase to act upon it. To clarify whether the transport genes *merT* and *merP* play a role in the transport of organomercurials into the cells, we constructed a deletion plasmid, pMRD141, which lacked the genes conferring the organomercurial lyase and mercuric reductase from pMRA17 and compared the phenotypic properties of bacteria with pMRD141 and pBluescript II, a cloning vector.

Plasmid pMRD141 showed hypersensitivity not only to Hg^{2+} but also to phenylmercury, but still expressed normal sensitivity to methylmercury.[26,27] It has already been shown that expression of *merT* and *merP* in the absence of mercuric reductase activity renders bacteria hypersensitive toward Hg^{2+} based on hyperaccumulation of the mercuric ion.[24,25] A bacterium with pMRD141 took up about three times more Hg^{2+} and about two times more phenylmercury than its isogenic cell with the cloning vector pBluescript II. However, no difference in the uptake of methylmercury was found between the two bacterial cells. The hypersensitivity to phenylmercury is thought to result from the hyperuptake of phenylmercury in the absence of detoxifying enzymes encoded by *merB* and *merA*. These results demonstrate for the first time that *merT* and *merP* genes are involved in the transport of phenylmercury, but do not participate in methylmercury transport.[26–28]

The additional *mer* gene, *merG*, located between *merA* and *merB1* on pMRA17, is 654 bp long, encoding a 217-amino acid polypeptide.[29] The predicted amino acid sequence of the gene product (MerG) has a good leader sequence that contains a short, positively charged region at *N*-terminus, followed by a hydrophobic region and a signal peptide cleavage site (alanine-leucine-alanine-alanine, ALAA) at position 32–35. This characteristic *N*-terminal sequence is homologous to the "leader sequences" of known periplasmic proteins.[29] 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 MerG protein may be located in the periplasm.

Deletion of *merG* from pMRA17 did not impair the Hg^{2+} resistance, but rendered the bacterium more sensitive to phenylmercury than its isogenic strain. Bacterial cells with the *merG*-deleted plasmid (pMU29) took up appreciably more phenylmercury than the cell with intact pMRA17, but no significant difference in the uptake of Hg^{2+} was found between cells with pMU29 and pMRA17.[29] In addition, deletion of *merG* had no effect on the enzymatic activities encoded by *merA* and *merB*.[29] These results demonstrate that the *merG* gene is only involved in phenylmercury resistance, presumably by reducing cell permeability to phenylmercury.

Together, the results obtained suggest that the high phenylmercury resistance noted for *Pseudomonas* K-62 may be achieved by the two functional organomercurial lyase enzymes encoded by *merB1* and *merB2*; alteration in cellular permeability to phenylmercury encoded by *merG*, since both *merB* and *merG* genes were identified on pMR26; and an anticipant *mer* operon located on plasmid pMR68, because following elimination of pMR26 from *Pseudomonas* K-62, there remains the ability to volatilize both inorganic and organic mercurials and its mercurial-resistant phenotypes.[16]

GENETICALLY ENGINEERED BACTERIA FOR MERCURY REMEDIATION

To recover and accumulate mercury from mercurial-contaminated sites in bacterial cells using the *Pseudomonas* K62 *mer* operon, it is better to delete the *merA* and *merG* genes from the operon. However, deletion of *merA* from the *mer* operon would render the host cells hypersensitive toward both in-

organic and organic mercury based on hyperaccumulation of the mercurials. Therefore, an additional mercury-scavenging mechanism that can be expressed in the bacterial cells is necessary.

Polyphosphate, a linear polymer of many tens or hundreds of orthophosphate residues in anhydrous linkage, appears to be a potential candidate because polyphosphate is capable of chelating divalent essential metals such as Mn^{2+}, Mg^{2+}, and Ca^{2+} up to about 40% of the dry weight of the cells.[30–32] The conservation and ubiquitous distribution of this polymer may play an important role in the living cells. In view of these findings, it is of interest to determine whether polyphosphate could serve as a chelator for mercurials and reduce mercury toxicity when mercury is taken up into bacterial cells. As expected, we found that polyphosphate is able to react with divalent mercuric ion *in vitro* but not with organomercuric ion.[33] The reaction between polyphosphate and mercuric ion is chelation, because the reaction was almost abolished by the addition of EDTA to the reaction medium. This result suggests that polyphosphate can serve as an intracellular mercury accumulator. Recently, an *E. coli* strain has been generated by fusion of a *merA-merG*-deleted broad-spectrum *mer* operon from *Pseudomonas* K-62 with a bacterial polyphosphate kinase gene (*ppk*) determining a polyphosphate kinase, a key enzyme for polyphosphate synthesis from *Klebsiella aerogenes* in the vector pUC119.[33] A large amount of the *ppk*-specified polyphosphate was identified in the mercury-induced bacterium with the fusion plasmid designated pMKB18 (*merR*-o/p-*merT-merP-merB1-ppk*) but not in the cells without mercury induction. These results suggest that the synthesis of polyphosphate catalyzed by *ppk*-specified polyphosphate kinase encoded by the *ppk* gene as well as the expression of the *mer* genes is mercury inducible and regulated by *merR*. The *E. coli* strain with pMKB18 was more resistant to both Hg^{2+} and $C_6H_5Hg^+$ than its isogenic strain with the cloning vector pUC119.[34] These results show that *merT*, *merP*, *merB1*, and *ppk* are expressed in bacterial cells and also demonstrate that the *ppk*-specified polyphosphate reduces the cytotoxicity of Hg^{2+}, probably *via* a chelation mechanism. Phenylmercury in the growth medium appears to be transported into the bacterial cells mediated by *merT-merP*, then degraded to Hg^{2+} by organomercurial lyase encoded by *merB1*, and finally the resultant Hg^{2+} is chelated with the *ppk*-specified polyphosphate. Due to chelation with polyphosphate, the resultant Hg^{2+} or the transported Hg^{2+} is never free in the cytoplasm, and the bacteria therefore express a resistant phenotype to both mercurials. Based on these observations, our newly designed plasmid pMKB18 may serve as a useful strategy for bioremediation of mercurials in the environment.

The application of this new biocatalyst in the environmental remediation of mercurials was next evaluated using an alginate immobilized biocatalyst carrying pMKB18. The immobilized cells engineered to express the mercury transport system, organomercurial lyase, and polyphosphate efficiently removed both organic and inorganic mercury from contaminated wastewater over a wide concentration range of mercurials, probably *via* intracellular accumulation mediated by *ppk*-specified polyphosphate.[35] Almost all of the mercurials that disappeared in the wastewater had accumulated in the alginate beads. The Hg^{2+} and $C_6H_5Hg^+$ coexisting in the medium were also simultaneously and efficiently removed by the immobilized cells carrying pMKB18. The accumulation rate was found to be around 890 and 780 nmol Hg per milligram of cells from Hg^{2+}- and $C_6H_5Hg^+$-contaminated wastewater, respectively. It is generally accepted that immobilization of bacterial cells has many advantages, including conservation of the cells and stabilization of their biological activities. Our results showed that the immobilized cells carrying pMKB18 are able to remove mercurials repeatedly from mercurial-contaminated wastewater without significant loss of their activities.[35] In addition, the high resistance to other ambient metals or a wide range of pH may make it feasible to remove mercury selectively from sites contaminated with various metals. From these results, it is concluded that the immobilized bacterium carrying pMKB18 is useful for simultaneous removal of both inorganic and organic mercurials from contaminated wastewater (Fig. 2).

CONCLUSION

The full mercury resistance of *Pseudomonas* K-62 is conferred by two plasmids, pMR26 and pMR68. The order, number, and function of the genes involved in mercurial resistance on pMR26 are basically the same as those of the broad-spectrum *mer* operon of pDU1358,[36] except that it has an additional *merG* located between *merA* and *merB*, which may be involved in the phenylmercury resistance of *Pseudomonas* K-62 *via* reducing cell permeability

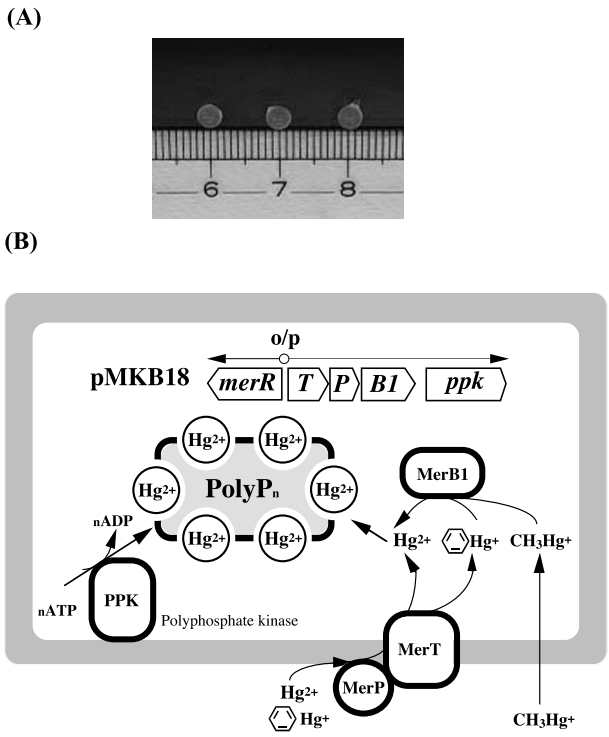

Fig. 2. Alginate Immobilized *E. coli* Carrying pMKB18 (A) and a Schematic Model of the Mercury-Removal System in *E. coli* Carrying pMKB18 (B)

to phenylmercury.

Due to the lack of suitable clean-up technologies, decontamination of mercury in the environment has long been a challenge. A new fusion plasmid, pMKB18, which simultaneously expresses a mercury transport system, organomercurial lyase enzyme, and polyphosphate, was genetically engineered. The alginate immobilized biocatalyst carrying pMKB18 accumulated large amounts of mercury from contaminated wastewater, probably *via* intracellular chelation formation of the transported or the resulting Hg^{2+} without taxing the biocatalyst. The polyphosphate-mediated mercury accumulation by the alginate immobilized biocatalyst carrying pMKB18 could serve as a useful strategy for the simultaneous removal of both mercurials.

Acknowledgements We thank all coworkers who cooperated in the execution of this research at the Department of Analytical Chemistry in Hygiene of Setsunan University.

This work was supported in part by a Grant-in-Aid for Scientific Research (B) (No. 16360267) from the Ministry of Education, Science and Culture, Japan.

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