Sequence and Analysis of a Plasmid-Encoded Mercury Resistance Operon from *Mycobacterium marinum* Identifies MerH, a New Mercuric Ion Transporter

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In this study, we report the DNA sequence and biological analysis of a mycobacterial mercury resistance operon encoding a novel Hg$^{2+}$ transporter. MerH was found to transport mercuric ions in *Escherichia coli* via a pair of essential cysteine residues but only when coexpressed with the mercuric reductase.

Narrow-spectrum mercury resistance (i.e., resistance to mercuric salts) requires transport of mercuric ions across the membrane by specific transporters, typically MerT and MerP (10), cytoplasmic reduction to elemental mercury Hg(0) by mercuric reductase (MR) and passive diffusion of the volatile metal out of the cell (2). In broad-spectrum mercury resistance, the organomercurial lyase MerB catalyzes the breakdown of carbon-mercury bonds and, with the transport and reductase proteins, confers resistance to a wide range of organomercurials (12). Both mechanisms are subject to tight regulation by a mercury-responsive transcriptional regulator, MerR (5).

Although mercury resistance has been extensively studied and characterized in different bacterial genera, there are few reports of mycobacterial mercury resistance genes, and no mer operons have been described in mycobacteria. Most studies consist of the isolation and identification of mercury-resistant mycobacterial strains in which the mercuric reductase gene has been detected by PCR and MR activity has been shown by volatilization of radioactive mercury (11, 21). Some strains were also positive for amplification of the *merB* gene. Mercury resistance was also demonstrated in mycobacteria expressing *mer* genes from *Tn501*, and mycobacterial expression vectors have been developed with mercury resistance as the only selectable marker (3).

*Mycobacterium marinum*, which causes a tuberculosis-like disease in fish and skin infections in humans, is closely related to *Mycobacterium tuberculosis* and is a good model for mycobacterial infection studies (6). The genome of a clinical isolate of *M. marinum* (strain ATCC BAA-535) (14) carrying a plasmid encoding potential mercury resistance genes was recently published (22). Here, we report the DNA sequence and analysis of the first *Mycobacterium* mercury resistance operon. We characterized a previously unknown membrane protein (MerH) from *M. marinum*, which we found transports mercuric ions across the inner membrane of *Escherichia coli* via a pair of cysteine residues located in the first transmembrane (TM) region but only when coexpressed with MR. In *Mycobacterium smegmatis*, MerH and MR confer resistance to mercuric chloride, which may be a useful, naturally occurring, nonantibiotic selection marker in mycobacteria.

A 3.6-kb mer operon confers mercury resistance to *M. marinum* strain ATCC BAA-535. *M. marinum* strain ATCC BAA-535 (GenBank accession number NC_010612) contains a 23-kb plasmid (pMM23; GenBank accession number NC_010604) which harbors a 3.6-kb mer operon (Fig. 1) (22) containing five genes likely to be involved in mercury resistance (Table 1). The operon is predicted to contain genes encoding functions associated with regulation, transport, and reduction of mercuric ions and organomercurial breakdown, but the gene arrangement is unusual, with two of the genes being divergently transcribed from the remaining three (Fig. 1). These *mer* genes are surrounded by three other genes that may not participate directly in mercury resistance—a predicted glutathione reductase and two recombinases that may promote mobility of the genetic element.

*M. marinum* strain ATCC BAA-535 was found to be three to four times more resistant to mercuric chloride than was *M. smegmatis* mc²155 in two different growth media (see Table 5), and similar levels of mercury resistance were observed for *M. marinum* and *E. coli* expressing *mer* genes from *Tn501* on LB agar, which suggests that mercury resistance genes located on the pMM23 plasmid from *M. marinum* are functional.

One gene from the *mer* operon in pMM23.11 has no significant similarity to any protein in the nonredundant database but was predicted to be a membrane protein with four putative transmembrane helices. We hypothesized that this gene, designated *merH*, was involved in mercuric ion transport (Fig. 2B).

Characterization of the *merH* gene product in *E. coli*. When expressed in *E. coli* in place of MerT within a minimal mercury resistance operon, MerH could be recovered in the membrane protein fraction, indicating that it is inserted in the membrane (data not shown). Five fragments of *merH* were PCR amplified using primers listed in Table 2 and cloned using Ncol/BstBI1 in pYZ-TBL (4), making β-lactamase fusions at five different locations in MerH (Fig. 2B). β-Lactamase activity of the resulting hybrid proteins was measured in *E. coli* C43 cells (cul-
pBRmerH, the mercuric chloride MIC for E. coli mercuric ion transporter in E. coli (MIC of 15 μM) for control cells expressing no mercuric ion transporters (H9262) (Table 3). When merT, merR operon containing the 5′ mer region was replaced in plasmid pBRmerH, the mercuric chloride MIC for E. coli cells decreased from 111 to 81 μM but remained much higher than that for control cells expressing no mercuric ion transporters (MIC of 15 μM). This result suggests that MerH acts as a mercuric ion transporter in E. coli.

MerH was expressed in E. coli by replacing merT with merH within a cloned minimal Tn501 mer operon containing the merR, merT, merP, and merA genes in plasmid pBRmerT (Table 3). When merT was replaced by merH in plasmid pBRmerH, the mercuric chloride MIC for E. coli cells decreased from 111 to 81 μM but remained much higher than that for control cells expressing no mercuric ion transporters (MIC of 15 μM). This result suggests that MerH acts as a mercuric ion transporter in E. coli.

In vivo 203Hg volatilization assays were performed as described elsewhere (17) in E. coli TG2 strains expressing either MerT, MerC, MerF, or MerH as the sole mercuric ion transporter within a minimal Tn501 mer operon (Fig. 3A). Mercury volatilization observed in the absence of a mercuric ion transporter was probably the result of cell permeabilization and was subtracted from all samples. MerT conferred the highest rate of mercury volatilization in E. coli, while MerH conferred intermediate rates, between the volatilization rates achieved with and without MerT, that were equivalent to the mercury volatilization rates achieved with MerF and MerC, mercuric ion transport proteins from Tn5053 and Tn21, respectively (8, 24). These data show that MerH is able to import mercuric ions into the E. coli cytoplasm for reduction by MR.

The following five combinations of cysteine-to-serine mutations were created in MerH using a two-step PCR method: C38S, C39S, C169S, C38S/C39S, and C38S/C39S/C169S (20) (Table 2). These were confirmed by sequencing both DNA strands. Mutants were cloned in place of merT in the pBRmerT plasmid and expressed from the Pmer7 promoter in E. coli TG2 upon induction with 0.4 μM HgCl2. Expression and membrane localization of the cysteine mutants were confirmed by 35S labeling of plasmid-encoded proteins (data not shown).

The effects of the cysteine mutations on mercuric ion resistance and transport were tested by HgCl2 MICs (Table 4) and 203Hg2+ volatilization assays (Fig. 3B) using E. coli TG2 cells expressing the different MerH cysteine mutants. The cysteine residues in MerH did not play an equal role in mercury resistance. Cysteine 169 did not participate in mercuric ion transport, since its mutation to serine did not decrease mercury resistance or mercuric ion transport activity compared to wild-type MerH. Mutation of cysteine 38 decreased mercury resistance by approximately 25%, whereas mutation of cysteine 39 completely abolished resistance. Both were predicted to be in the first TM region of MerH. Cells expressing the MerH double (C38S/C39S) or triple (C38S/C39S/C169S) mutants were as

![TABLE 1. Annotation of mercury resistance genes located on pMM23 from M. marinum*](http://jb.asm.org/)

| ORF no. | Gene name | BLAST resulta | Reference/observation |
|---------|-----------|---------------|-----------------------|
| pMM23.08 | Belongs to the superfamily of serine DNA recombinases; 99% identical to DNA resolvase of Mycobacterium abscessus | May be involved in mobility of the genetic element |
| pMM23.09 | Belongs to the family of FAD-dependent pyridine nucleotide-disulphide oxidoreductases; 43% identical to glutathione reductase of Gramella forsetii strain CHR28 | Unlikely to be involved in mercury resistance |
| pMM23.10 | merB | 64% identical to the organomercurial lyase from Streptomyces lividans | 18 |
| pMM23.11 | merH | No statistically significant match | Hypothetical membrane protein |
| pMM23.12 | merA | 67% identical to the mercuric ion reductase from Streptomyces sp. strain CHR28 | 15 |
| pMM23.13 | merR | 62% identical to the MerR protein from Streptomyces lividans pJOE796 | 18 |
| pMM23.14 | merT | 54% identical to the MerT protein encoded by the mer operon of Streptomyces lividans | 18 |
| pMM23.15 | Belongs to the superfamily of serine DNA recombinases; 99% identical to DNA inverase/resolvase of M. abscessus | May be involved in mobility of the genetic element |

*a The genes were compared against the non-redundant databases using FASTA and BLAST, and protein motifs were identified using Pfam, Prosite TMHMM and SignalP.

b FAD, flavin adenine dinucleotide.
sensitive to mercuric chloride as were cells expressing no mercuric ion transport proteins (Table 4).

Similarly, mutation of cysteine 38 significantly decreased Hg volatilization, and mutation of cysteine 39 completely abolished volatilization (Fig. 3B). As expected, the double and triple cysteine mutants of MerH were not able to transport mercuric ions.

MerH cannot transport Hg\(^{2+}\) across the cytoplasmic membrane of *E. coli* without MR. Cells expressing mercuric ion transporters in the absence of MR were expected to show a

![FIG. 2. Sequence and topology of MerH. (A) Predicted protein sequence of MerH aligned with MerT, MerC, and MerF based on TM domain locations. Note that MerH has no significant similarity to the others at the primary sequence level. TM regions are shown in boxes and conserved cysteine residues in boldface type. (B) Topology of MerH determined by fusion to β-lactamase. Filled and open circles show fusions where the β-lactamase was found active and inactive, respectively. Carbenicillin MICs of strains expressing MerH–β-lactamase hybrid proteins are indicated in boldface type in mg ml\(^{-1}\).](http://jb.asm.org/)

### TABLE 2. List of primers used in this study

| Primer name | Primer sequence (5'–3') | Purpose |
|-------------|------------------------|---------|
| MerH_F      | 5' CCA TTC GAA AGG AGA ACC CTG ATG 3' | merH amplification |
| MerH_R      | 5' ACT AGA GAT CTA AGG GCC GC 3' |
| MerH_C38S_F | 5' GCC ACT TGG AGC TCC CTC GTG GTG 3' |
| MerH_C38S_R | 5' CAC ACC GAG TCA GCT CAA AGT GGC 3' |
| MerH_C39S_F | 5' GCC ACT TGG AGC TCC CTC GTG GTG 3' |
| MerH_C39S_R | 5' CAC ACC GAG TCA GCT CAA AGT GGC 3' |
| MerH_C3839S_F | 5' GCC ACT TGG AGC TCC CTC GTG GTG 3' |
| MerH_C3839S_R | 5' CAC ACC GAG TCA GCT CAA AGT GGC 3' |
| MerH_NcoI_F | 5' AGG GAG AAC CCC CAT GGC TGT G 3' | MerH topology |
| HBL1        | 5' AGT CAG GAG GGT ACC GCC GAT GG 3' |
| HBL2        | 5' GAT CAC GAG GGT ACC GCC GAT CAG 3' |
| HBL3        | 5' GGA AAA GAG GGT ACC CAC CGC GC 3' |
| HBL4        | 5' GCG GCT GGG GTG ACC GAA CAG 3' |
| HBL5        | 5' GAG ACN CCA TAT GAC TGC GCT TCA TGA 3' |
| MerA_NdeI_F | 5' GGG TCG TCA TGC TCA AGC GC 3' |
| MerA_HindIII_R | 5' GGT GGA AAG TCT TCA GCT GCC GCA G 3' |
| MerH_NdeI_F | 5' GAG ACC CCA TAT GAC TGC GCT GCC CAC 3' |
| MerH_HindIII_R | 5' AGG GAT AGG CTT TCA AGC GCC GCA G 3' |

*Boldface type indicates particular sequence features (start codons, stop codons, and ribosome-binding sites). Italicized residues represent introduced restriction sites. Underlined sequences show differences from the original sequence for point mutations.*
hypersensitive phenotype to mercuric chloride, since they specifically import mercuric ions (21). Plasmids expressing different mercuric ion transporters (MerT, MerC, MerF, and MerH) without expression of MR were constructed and transformed into E. coli TG2 cells (Table 3) to test the HgCl₂ sensitivity of resulting strains (Fig. 3C). Expression of MerT or MerC in the absence of MR resulted in an HgCl₂-hypersensitive phenotype in TG2. Their sensitivity to mercuric chloride increased by approximately fourfold compared to that of cells expressing no mercury resistance transporters. In the case of MerF, a 1.5-fold increase in HgCl₂ sensitivity was observed. However, for MerH, this hypersensitive phenotype was not observed. Cells expressing MerH unexpectedly showed a slight increase in mercury resistance (1.2-fold increase) compared to cells expressing no mer genes. These data suggest that MerH transports mercuric ions across the inner membrane of E. coli cells only when MR is present in the cytoplasm.

Apart from the predicted length and number of TM regions, the major difference between MerH and other mercuric ion transporters is that MerH lacks a second pair of cysteine residues predicted to be located in the cytoplasm (Fig. 2A). In MerT, MerC, and MerF, this pair of cysteine residues has been shown to be involved in mercury transport, since their mutation resulted in a reduced rate of mercury volatilization (13, 24). These data suggest that MerH is not able to import mercuric ions to the E. coli cytoplasm because it lacks the cysteine pair in this compartment. In order to achieve the transport of Hg²⁺ into the cytoplasm, MerH may require MR cysteine residues to act as acceptors for mercuric ions, or MR may be required to cause a productive conformational change in MerH. It is also possible that these cytoplasmic cysteines are required for Hg²⁺ transport in E. coli and not in the natural host, M. marinum.

**merA and merH from M. marinum confer mercury resistance on M. smegmatis.** The merH and merA genes were separately and jointly cloned in pVV16 (an E. coli-Mycobacterium shuttle vector) and constitutively expressed in M. smegmatis mc²155 (Table 3). Transformants were selected on 25 µg ml⁻¹ kanamycin and 50 µg ml⁻¹ hygromycin. After 4 days of incubation at 37°C, single colonies of mc²155 expressing different mer genes were observed under the microscope. Expression of merA slightly affected the mc²155 colony morphology but did not affect the growth rate. Expression of merA with merH...
resulted in a significant change in the morphology of the colonies (which were much smoother) but did not affect the growth rate. However, expression of \textit{merH} from the constitutive \textit{hsp60} promoter of \textit{pVV16} was toxic to the cells. Ring-shaped colonies appeared on L agar plates, and cells could not be grown in L broth. The effects of \textit{merA} and \textit{merAH} expression upon mercury resistance in \textit{M. smegmatis} were determined by \textit{MIC} assays. Levels of mercury resistance of \text{mc}2155 strains expressing \textit{merA} or \textit{merA} and \textit{merH} from the \textit{hsp60} promoter in the \textit{pVV16} vector were determined on L agar with antibiotic selection or 7H11 agar without antibiotic selection pressure.

\begin{table}[h]
\centering
\begin{tabular}{|l|c|}
\hline
\textbf{MerH protein or mutant} & \textbf{\textit{HgCl}_2 \text{MIC (\textmu}M\text{)}}^b \\
\hline
Wild type & 81 ± 4 \\
C38S mutant & 71 ± 4 \\
C39S mutant & 22 ± 4 \\
C38S/C39S mutant & 22 ± 4 \\
C169S mutant & 81 ± 4 \\
C38S/C39S/C169S mutant & 22 ± 4 \\
\ldots & 18 ± 4 \\
Empty vector \textit{pBR322} & 18 ± 4 \\
\hline
\end{tabular}
\caption{Effects of cysteine-to-serine mutations in MerH upon mercury resistance conferred on \textit{E. coli} \text{TG2} expressing a minimal \textit{mer} operon (\textit{merRHPA})}
\end{table}

\footnotesize{\begin{enumerate}
\item[^a] —, no transporter expressed.
\item[^b] Values are the mean MIC ± the standard deviation.
\end{enumerate}}
TABLE 5. Comparison of mercury resistance levels of E. coli (expressing mer genes from Tn501), M. marinum, and M. smegmatis mc²155 (parental strain or strain expressing mer genes from the M. marinum mer operon)

| Strain               | L agar containing 25 μg/ml kanamycin | L agar | 7H11 agar |
|----------------------|--------------------------------------|--------|-----------|
| E. coli              | 15 ± 4                                |        |           |
| E. coli (Tn501)      | 118 ± 8                               |        |           |
| M. marinum           | 110 ± 8                               | 177 ± 8|           |
| M. smegmatis mc²155 | 30 ± 4                                | 59 ± 4 |           |
| M. smegmatis mc²155 (pVV16) | 4 ± 2                      | 59 ± 4 |           |
| M. smegmatis mc²155 (pVV16-merA) | 15 ± 4                   | 103 ± 8|           |
| M. smegmatis mc²155 (pVV16-merAH) | 37 ± 4                     | 110 ± 8|           |

* Values are mean MIC ± the standard deviation.

after 3 to 4 days of incubation at 37°C (Table 5). Expression of merA in M. smegmatis resulted in a fourfold increase in mercury resistance, while coexpression of merA and merH in resulted in a 10-fold increase. These data show that the merA gene product is sufficient to confer some mercury resistance on M. smegmatis but that expression of the putative Hg²⁺ transport gene (merH) is required for maximal mercury resistance. On 7H11 agar plates, M. smegmatis also showed maximal mercury resistance upon coexpression of merA and merH but with much greater MICs, suggesting that the kanamycin selection of pVV16 strongly decreased mercury resistance. However, expression of merAH in mc²155 conferred sufficient mercury resistance (>30 μM) to allow selection of recombinant M. smegmatis cells on HgCl₂, indicating that these two genes can be used as a non-antibiotic-selectable marker in mycobacteria.

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