Molecular Analysis of Mercury-Resistant Bacillus Isolates from Sediment of Minamata Bay, Japan

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Bacillus isolates from Minamata Bay sediment were selected for the ability to volatilize mercury from a range of organomercurials (including methylmercury). Chromosomal DNA from 74 of 78 such strains hybridized with the mer mercury resistance operon DNA from marine Bacillus sp. strain RC607 (Y. Wang, M. Moore, H. S. Levinson, S. Silver, C. Walsh, and J. Mahler, J. Bacteriol. 171:83–92, 1989). The most frequent classes with regard to restriction nuclease site maps of the mer operon for the new isolates were identical to or closely related to the mer determinant of strain RC607. PCR amplification analysis with primers designed from the strain RC607 mer operon gave products of precisely the predicted size with the 74 Minamata Bay isolates.

Minamata Bay, Japan, was the site of one of the largest epidemics of methylmercury poisoning recorded (1, 17, 18). As part of the comprehensive analysis of methylmercury poisoning by the National Institute for Minamata Disease, microbiological sampling of Minamata Bay water and sediment began in 1982 (12, 13). Bacilli were most abundant in sediment and among mercury-resistant bacteria isolated from sediment (11–15). Multiple-organomercurial-volatilizing bacteria that could volatilize mercury from all mercurials tested (mercury mercaptide chloride, ethylmercury chloride, phenylmercury acetate, thimerosal, fluorescein mercapturic acid, and p-chloromercuribenzoate) were found only in sediment from Minamata Bay (14), and the frequency of multiple-organomercurial resistance among Minamata Bay isolates was at least 20 times higher than that found in isolates from a nearby unpolluted control site (14). This suggests that selection pressure exerted by organomercurials in the polluted sediment might still be environmentally important. To determine how similar mercurial-resistance determinants from a single ecologically important mercury-polluted site are, dot blot and Southern blot DNA-DNA hybridization and PCR analyses were carried out with 78 broad-spectrum organomercurial-resistant and organomercurial-volatilizing Bacillus strains (see references 11, 14 and 15). The results show that 74 of the Bacillus mer operon regions were closely homologous to one previously sequenced from a North American marine isolate (20).

The 78 multiple-organomercurial-volatilizing Bacillus strains included the 19 so-called class 8 organomercurial-volatilizing strains from the collection reported on in reference 14 plus an additional 59 strains (those from the 132 Bacillus isolates reported on in references 11 and 14 that were shown by atomic absorption measurements [as in references 11, 14 and 15] to volatilize mercury from the organomercurial substrates listed in Table 1 in 48 h at 30°C [data not shown]). The Bacillus isolates were identified according to the taxonomic scheme of Bergey’s Manual (4). Twenty strains were identified as B. subtilis, 17 isolated as Bacillus firmus, 24 were identified as Bacillus lentus, and 17 were identified as Bacillus subtilis (Table 1). All 78 strains could grow at 40°C but not at 10°C. All grew in broth with 7% (7 g/100 ml) NaCl added. All formed heat-resistant spores (data not shown). Plasmids were not found in any isolate by use of cesium chloride-ethidium bromide density gradients (according to references 5, 6 and 16 [data not shown]). On the basis of dot blot analysis (16), DNA from 74 of the 78 Bacillus isolates contained sequences related to the sequenced Bacillus sp. strain RC607 mer determinant (data not shown).

The mer operons of the 78 Minamata Bay Bacillus strains were grouped into seven classes by Southern blot mapping with eight restriction endonucleases. A representative Southern blot analysis of DNA from one member of each of classes I through VI is shown in Fig. 1, with digestion with either EcoRI plus BglII (Fig. 1A) or EcoRI plus SphI (Fig. 1B). The four strains of class VII did not show hybridization with Southern blot analysis at either 65°C (Fig. 1; high stringency) or 30°C (reference 2; low stringency; additional data not shown). The probe used in Fig. 1 was a mixture of strain RC607 EcoRI-SphI merA and merB fragments (Fig. 1A) or the merA probe alone (Fig. 1B). Each of classes I through VI gave a different pattern, as seen in Fig. 1.

Figure 2 summarizes the results of the restriction nuclease mapping of the 74 Minamata Bay bacillus isolates that gave hybridization with the Bacillus RC607 mer DNA probes (20). The positions of the merA gene for mercuroxidase and the merB gene for organomercurial lyase for classes I through IV in Fig. 2 were deduced from the restriction map of class I and the location of these genes in the published DNA sequence (20). The maps for classes V and VI are the best current versions from combined Southern blot and PCR analysis. The restriction nuclease site map of the 6-kb mer region of class I strains in Fig. 2 was identical, as far as could be determined, to that of the Boston harbor Bacillus sp. strain RC607 (19, 20). This was the largest class, with 38% of the 78 bacillus strains analyzed. The restriction nuclease site map from Southern blotting for class II (in Fig. 1 and 2) differed from that in class I only by the loss of the BglII cutting site in merA. Class III (a single strain) has a restriction nuclease site map identical to that of class I, except that the overall length between the EcoRI sites is 5.8 kb, 0.2 kb smaller than with class I (Fig. 2). Class IV isolates showed a mer operon restriction site map similar to that of DNA from the class I sequence, except...
FIG. 1. Southern blot DNA-DNA hybridization of chromosomal DNA from seven representative Minamata Bay isolates (lanes 1 to 7, classes I to VII, respectively) with *mer* probe DNA. (A) EcoRI- and BglII-digested chromosomal DNA; (B) EcoRI- and SphI-digested chromosomal DNA. Probe and lane P, the EcoRI- and SphI-digested 3.8-kb fragment with *merA* (from plasmid P46, a pUC9 derivative containing the 3.8-kb EcoRI-SphI fragment of plasmid pYW40) and the 2.9-kb fragment with *merB* (from plasmid P47, a pUC9 derivative containing the 2.9-kb SphI-EcoRI fragment of plasmid pYW40) (A) and the 3.8-kb EcoRI-SphI fragment with *merA* (B). Both are from *Bacillus* sp. strain RC607 (19, 20), and the *Escherichia coli* strains with the probe plasmids were provided by I. Mahler (Brandeis University). *Bacillus* chromosomal DNA was prepared by the method of Marmur (8). 32P-labelled EcoRI-SphI DNA fragments from plasmids pYW46 and pYW47 were used as a mixed probe (A), or the DNA fragment from plasmid P46 alone was used (B). Probe DNA was labelled with [α-32P]dCTP with the Random Primer DNA Labelling Kit (Takara Shuzo Co., Osaka, Japan). Membranes for hybridization were prepared with Probe Tech 2 (Oncor Inc., Gaithersburg, Md.). Hybridization was carried out at 45 and 65°C for 16 to 20 h in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)-1× Denhardt's solution-0.1% sodium dodecyl sulfate-10 μg of poly(A) per ml (16).

![Southern blot DNA-DNA hybridization](image)

**FIG. 2.** Restriction nuclease maps of *mer* operons of 74 Minamata Bay sediment *Bacillus* isolates. Six classes (I through VI) are illustrated, with the numbers of isolates shown in parentheses. Restriction endonuclease sites are marked above the maps, and the relative positions and orientations of the *merA* and *merB* genes are shown below.

### TABLE 1. Distribution of restriction nuclease site patterns among species of the 78 *Bacillus* isolates

<table>
<thead>
<tr>
<th>Class (no. of strains)</th>
<th><em>B. subtilis</em></th>
<th><em>B. firmus</em></th>
<th><em>B. lentus</em></th>
<th><em>B. licheniformis</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>I (30)</td>
<td>20</td>
<td>9</td>
<td>1</td>
<td>12</td>
</tr>
<tr>
<td>II (16)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>III (1)</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IV (7)</td>
<td>2</td>
<td>1</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>V (19)</td>
<td>3</td>
<td>15</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>VI (1)</td>
<td></td>
<td></td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>VII (4)</td>
<td>2</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total no. of strains</td>
<td>20</td>
<td>17</td>
<td>24</td>
<td>17</td>
</tr>
</tbody>
</table>

* Distribution of *Bacillus* species among the 78 broad-spectrum organomercurial-volatilizing strains. The strains tested include the 19 so-called class 8 broad-organomercurial-volatilizing strains reported on in reference 14 plus 29 *Bacillus* isolates reported on in references 11 and 15 that were found by atomic absorption spectroscopy (data not shown) to also volatilize mercury from all tested organomercurial compounds (mercuric chloride, 5 to 20 μg/ml; methylmercuric chloride, 0.1 μg/ml; ethylmercuric chloride, 0.1 μg/ml; thimerosal, 0.5 μg/ml; phenylmercuric acetate, 0.5 μg/ml; fluorescein mercuric acetate, 1 μg/ml; and p-chloromercuribenzoate, 0.1 μg/ml).

for the missing BglII site (in *merA* as in class II), the missing NcoI site, and additional PstI sites in the *merB* region. The class IV restriction site map also differs from those of classes I, II, and III downstream from *merB* (Fig. 2).

The *mer* operon restriction nuclease map of the 19 class V *Bacillus* isolates (the second largest class among Minamata Bay isolates) bears little relationship to the map for class I isolates. However, DNA from both classes hybridized effectively with strain RC607 *merA* and *merB* probe DNA (Fig. 2 and additional data not shown). The EcoRI fragment length for class I isolates was 6.7 kb, but *mer* DNA probing two EcoRI fragments from class V isolates, totalling about 1 kb longer (Fig. 2). This
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may be similar to the situation encountered in comparison of restriction nuclease site maps of mer transposons Tn501 and Tn21 from gram-negative bacteria, in which sequence analysis showed that the DNA sequences were 80% identical but that only 25% of the restriction nuclease sites remained in common (3, 9, 10).

The single class VI isolate gave rise to a restriction nuclease map that of the class V isolates, except for the additional BglII site shown. The mer DNA of class V and VI strains appeared to have a lower degree of homology to the probe DNA (than those of classes I through IV), since 10 times more chromosomal DNA was needed to obtain approximately similar Southern blot results (data not shown). Finally, four so-called class VII mercury-volatilizing Bacillus isolates are not shown in Fig. 2, since restriction site maps could not be constructed in the absence of Southern blot DNA-DNA hybridization. Strain RC607 mer DNA had been previously reported to lack detectable homology to the DNA of some other mercury-resistant Bacillus spp. (19). Additional cloning experiments will be needed to determine the structure of the mer operons in the class VII strains.

The distribution of restriction nuclease site patterns of the mercury resistance regions of different Bacillus species in Minamata Bay sediment correlated with species designations (Table 1). The most frequently found class (class I) was found most often in B. subtilis isolates, whereas the next two most abundant classes (II and V) occurred most often in B. radius and B. lentus isolates, respectively. This might reflect differing environmental niches and/or different histories of transfer from strain to strain of the mercury resistance determinants.

Seven synthetic oligonucleotide primers (identical to sequences of strain RC607) were used in PCR experiments to measure the sizes of the Bacillus merA and merB genes and the regions between. These are listed in the legend to Fig. 3. For merA, the two forward primers corresponded to the first six amino acids of mercuric reductase, Met-Lys-Lys-Tyr-Arg-Val, and a highly conserved internal region, amino acids 174 through 179 (Ile-Ile-Gly-Ser-Gly-Gly), with the reverse primer corresponding to the C-terminal six amino acids, Leu-Ser-Cys-Cys-His-Ile. PCR products of the expected length were obtained for merA (with both forward primers, 1,893 and 1,374 nucleotides, respectively [Fig. 3A]) and merB (654-nucleotide expected length [Fig. 3B]) with total cellular DNA from all 74 Bacillus

FIG. 3. PCR amplification of the merA gene (A) and of the merB gene and the region between merA and merB (B) from Minamata Bay sediment strains. Lanes M contain as size markers a HindIII digest of lambda phage: from top to bottom, 23.1, 9.4, 6.6, 4.4, 2.3, and 2.0 kb and 564 and 125 bp, respectively, for eight fragments. (A) Lanes 1 through 7, chromosomal DNA templates from representative class I to VII strains and merA primers P17 and P18; lanes 8 through 14, merA primers P31 and P18. (B) Lanes 1 through 7, merB primers P19 and P20; lanes 8 through 14, gap region primers P41 and P42. PCR reactions were run in a PC-700 Program Control System (ASTEC, Japan) thermal cycler, with 1 μg of template chromosomal DNA or 50 ng of template plasmid DNA and 50 pmol each of the forward and reverse primer DNAs. Twenty cycles (95°C, 0.5 min; 55°C, 0.5 min; 72°C, 1 min) were run in a buffer containing 0.1 mM each dNTP, 50 mM HEPES buffer (pH 7.9), 1.5 mM MgCl₂, and 50 mM KCl, with Taq DNA polymerase (Perkin-Elmer Cetus, Norwalk, Conn.). For the merA gene, the two forward primers and one reverse primer were primer P17 (forward; 5’CCGAATTCTGAAAAAATATCGAGT3’), corresponding to the first 6 amino acids of the MerA protein with an EcoRI restriction site added at the 5' end; primer P31 (forward; 5’TTGAATTCTAGGTTCTGAGC3’), corresponding to amino acids 174 through 179 and also with an EcoRI restriction site added at the 5' end; and primer P18 (reverse; 5’CCGAATTCTATCAGCAGCAAG3’), corresponding to the carboxy-terminal 6 amino acids of MerA and with an EcoRI restriction site added at the 5' end. For the merB gene, forward primer P19 (5’CCGAATTCTAGAAAATCGAATCAG3’, with an EcoRI restriction site added at the 5' end), corresponding to the first 5 amino acids of the MerB protein, and reverse primer P20 (5’TTGAATTCTAATACTGACAGCAT3’, corresponding to the carboxy-terminal 5 amino acids and with an EcoRI restriction site added at the 5' end) were used. For the region between the merA and merB genes, forward primer P41 (5’CCGAATTCTAGGAATATACGAGTC3’, with an EcoRI restriction site added at the 5' end), corresponding to the 6 amino acids (623 through 628) of the MerA protein, and reverse primer P42 (5’TTGAATTCTCGATCTGCCAT3’, corresponding to the first 5 amino acids of MerB and with an EcoRI restriction site added at the 5' end) were used.
strains of groups I through VI (Fig. 3 and additional data not shown), indicating close homologies to the sequences of the primers used and similar spacing between primers. Weak PCR signals of appropriate size were observed with template DNA from class VII strains (Figs. 3 and data not shown). With primers for the region between merA and merB that were identical to nucleotides encoding the C terminus of MerA and the N terminus of MerB of strain WP4 (20), strongly produced PCR products were found and all had the same size, about 2 kb, slightly larger than the 1,893-nucleotide internal merA product (Fig. 3B).

To understand the impact of microbes on mercury transformations in Minamata Bay and the effects of mercury pollution on the microbial population, one would like to have collections of microbial strains dating back to the initial identification of the cause of Minamata disease (the late 1960s). Unfortunately, the current study is of bacteria isolated after the dredging of sediment with more than 25 μg of Hg per g was almost complete (1, 17). These bacteria showed a lower frequency of mercury resistance (14) and lower sediment mercury content (14) than at earlier times. The recent Bacillus strain collection has now been analyzed in detail for the range of organomercurials that could be detoxified (Table 1) and for the structure of the mer operon genes (Fig. 2). Remarkably, the restriction nuclease site map based on Southern blot hybridization analysis for the most abundant class of Minamata Bay isolates was identical to that from the Massachusetts Bacillus strain (20). The PCR analysis shown in Fig. 3 (and additional data not shown) confirms and extends this conclusion. The sizes of the four sets of PCR products measured from the six classes of restriction nuclease maps were identical, within the limits of analysis by agarose gel electrophoresis, and furthermore were the same as that expected from the DNA sequence of Bacillus sp. strain RC607 (19, 20). It is apparent that the determinants for mercury resistance in sediment bacteria are not locally specialized but are representative of the global distribution of such determinants, as found also for determinants of resistance to antibiotics carried by plasmids of bacteria that inhabit humans. In future analysis of other mercury-polluted sites, it will be of interest to determine if subtle physiological differences occur in the mercurial detoxification process with different map classes and different Bacillus species. There may be subtle adaptation to different environments within the Minamata Bay sediment. Relevant microenvironmental information is not available, however, for the current collection of 78 isolates of bacilli.

We have studied bacteria remaining at the end of a period of gradually reduced mercury levels, starting in 1971 (with the cessation of industrial release of mercury) (1, 17, 18). The gradual decrease in sediment mercury from 1959 (when organomercury was first suggested to be the cause of Minamata disease) and before dredging removed most remaining mercury in the late 1980s suggests that natural processes (largely microbial) had removed between 75 and 90% of the mercury in Minamata Bay sediment (1, 17, 18). Nevertheless, environmental selection for organomercurial resistance remained (14), and it is important to study in detail the microbial genes responsible for natural bioremediation in important mercury pollution sites. In the future, such studies should be started as soon as possible and should be extended to mercury-methylating bacteria as well as to those that remove methylmercury.

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REFERENCES


