Is the merA gene sufficient as a molecular marker of mercury bacterial resistance?

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INTRODUCTION

Mercury contamination is a serious environmental problem since Hg²⁺ can be found in water, soil, and air. Nowadays, environmental pollution by heavy metals, such as mercury, is mostly caused by human actions (Rytuba, 2003). The largest sources of anthropogenic mercury emission are: stationary combustion of coal, nonferrous metal production and cement production, artisanal and small-scale gold mining (The United Nations Environment Programme (UNEP), 2018). Mercury is toxic to a variety of organisms (Guzzi et al., 2021). However, some microorganisms are capable of survival in the presence of mercury, which can be used in the bioremediation of mercury-contaminated environments (McCarthy et al., 2017).

One of the genetic mechanisms providing mercury resistance is the bacterial mer operon, composed of the merR, merT, merP and merA genes (Fig. 1). The main reaction of mercury detoxification is the reduction of Hg²⁺ to Hg⁰. The reaction is catalyzed by mercury ion reductase (MerA) encoded by the merA gene (Barkay et al., 2003). MerA functions as a homodimer, and each subunit binds one flavin adenine dinucleotide (FAD) (Barkay et al., 2003; Lin et al., 2011). Due to the importance of mercury ion reductase, the merA gene or its fragments are commonly used as a molecular marker of bacterial resistance to mercury (Allen et al., 2013; de Luca Rebelo et al., 2013; Sotero-Martins et al., 2008; Wijaya et al., 2021; Zeyaullah et al., 2010). Bacteria that possess the mer operon with merA gene are classified as narrow spectrum ones able to detoxify only nonorganic mercury ions (Mathema et al., 2011). The merR gene encodes the regulatory protein (MerR) which controls the expression of the whole operon acting as a repressor in the absence of Hg²⁺ and as an inducer in the presence of Hg²⁺ interacting with the operator/promoter region as homodimer (Barkay et al., 2003; Lin et al., 2011). The genes of structure are merT and merP, which encode transport proteins. MerP acts in the periplasm space, and MerT is a membrane-spanning protein (Lin et al., 2011).

In this study, we tested the mercury resistance of selected bacteria isolated from Tussilago farfara L. that grow...
in a mercury-contaminated and noncontaminated area together with determining the presence of merA in their genomes. The parameters of bacterial growth kinetics were compared for isolates from contaminated and uncontaminated areas in medium supplemented and not supplemented with mercury. The question of whether merA is a sufficient molecular marker to determine bacterial mercury resistance is discussed.

MATERIALS AND METHODS

Tested bacteria

Eight gram-negative bacterial representatives of the microbiota of Tussilago farfara L. were analysed (data not published). Four of them were isolated from plants growing in postindustrial mercury-contaminated area, and four from mercury-noncontaminated area.

Determination of mercury

Total mercury concentrations were determined according to procedure: 0.5 g of each sample was digested with 6 ml of aqua regia and 2 ml of water. After mineralization, the solution was transferred to a volumetric flask, then 1 ml of 10% stannous chloride (SnCl₂) was added to each flask and diluted to 25 ml. Analyte was aspirated by a gas stream to analyzer. The method of cold-vapor atomic absorption spectrometry (CVAAS) was used after a wet acid digestion (with aqua regia) in a closed-vessels microwave oven. The absorbance was recorded when the mixture was stable. The total mercury content was measured using the Nippon Instruments Corporation RA-3 mercury analyzer (Sari et al., 2016).

Bacterial cultures and DNA isolation

Genomic DNA was extracted from 5 ml of 24-h bacterial cultures. Bacteria isolated from T. farfara L. grown in mercury-contaminated areas were cultured in 20 ml of standard Luria-Bertani (LB) medium with mercury concentration of 0.01% (Hg source HgCl₂ - 135 ppm HgCl₂, added to the medium as a 0.1 M HgCl₂ solution). Isolates of T. farfara L. grown in mercury-free areas were cultured in 20 ml of standard LB medium. The bacteria were cultured in 50 ml closed sterile falcon tubes placed horizontally on a laboratory shaker (140 rpm, 20°C). Genomic DNA extraction was conducted with the Genomic Mini AX Bacteria kit (A&A Biotechnology). The extraction of plasmid DNA from 10 ml of 24-h bacterial cultures (with and without addition of mercury) was performed with the Plasmid Mini AX kit (A&A Biotechnology).

Bacteria identification

The 16S rRNA gene was amplified in a polymerase chain reaction (PCR) with a total volume of 25 μl which contained: 5 μl of genomic DNA (diluted to ~10 ng/μl); 12.5 μl of PCR Mix Plus (A&A Biotechnology); 5.5 μl of ddH₂O; 1 μl of primer 8F (5'-AGAGTTTGATCCTGGCTCAG-3') (10 μM) and 1 μl of primer 1492R (5'-GGTTACCTTGTAGTTACGACTT-3') (10 μM) (Heuer et al., 1997). Amplification was performed using the S1000 Thermal Cycler (Bio Rad) with the following parameters: initial denaturation 95°C for 3 min.; 35 cycles of denaturation 95°C for 30 sec.; annealing 59°C for 30 sec.; elongation 72°C for 1 min.; final elongation 72°C for 5 min. PCR products were separated and analyzed based on gel electrophoresis (agarose 2%, 70 V, 40 min.). DNA molecular marker DNA Marker 1 – range 100–1000 bp (A&A Biotechnology) was used.

merA gene identification

The presence of the merA gene was confirmed by amplifying the 200 bp fragment of the merA gene by a 25 μl PCR reaction consisting of 12.5 μl of DreamTaq Green PCR Master Mix (Thermo Scientific); 9.5 μl of ddH₂O; 1 μl of primer Ps_merA_For3: 5'-CGTTCGACGGCATTTCGGAACGATG-3' (10 μM); 1 μl of primer Ps_merA_Rev3: 5'-TGGCCGGGTCTTTCGTGAAAG-3' (10 μM) (primers designed based on the GenBank: X98999.3 using Primer3 and BLAST (Ye et al., 2012)) and 1 μl of genomic or plasmid DNA (isolated as described above). The amplification was performed using the S1000 Thermal Cycler (Bio Rad) with the following parameters: initial denaturation 95°C for 5 min.; 35 cycles of denaturation 95°C for 30 sec.; annealing 59°C for 30 sec.; elongation 72°C for 1 min.; final elongation 72°C for 5 min. PCR products were separated and analyzed using the Chromas Lite program. All consensus sequences were submitted to the GenBank database (rRNA_typestrains/16S_ribosomal_RNA).

Bacterial growth kinetics assessment

For the measurement of growth kinetics, 5 ml of 24-h bacterial cultures were centrifuged in LB and LB+0.01% Hg media (3 min, 5500 rpm, 20°C), the cell pellets were resuspended in 5 ml of fresh LB media and again collected by centrifugation and resuspension in 5 ml of LB. The newly resuspended cells were divided into two 2.5 ml cultures each, collected by centrifugation, and cell pellets were resuspended respectively in 3 ml of LB and 3 ml of LB+0.01% Hg medium.

200 μl of optimized to OD₅₆₀=0.1 (LB or LB+0.01% Hg medium, respectively) samples were placed in quadruplicates on a 96-well transparent culture plate and incubated in Microplate Reader for 48 hours with a custom-written shake program (140 rpm, 22°C), OD₅₆₀ measurement in five spots per well, every 27 minutes. For proper calculation of the kinetic parameters for bacterial growth, OD₅₆₀min measurement in five spots per well, every 27 minutes. For proper calculation of the kinetic parameters for bacterial growth, OD₅₆₀max values were calculated as ln(OD₅₆₀min)/(dtmax). To determine the maximum growth rate (μmax), the linear model was fitted. The duration of the lag phase was read from the plots. Doubling time (DT) was calculated according to Eqn. 1. The calculations of the standard deviation of the mean were conducted with Statistica software.

\[
DT = \frac{ln2}{\mu_{max}} \quad \text{(Eqn. 1)}
\]

μmax – maximum growth rate

RESULTS AND DISCUSSION

Eight tested bacteria isolated from the microbiota of T. farfara L. growing in mercury-contaminated (198.5±10.5 mg Hg/kg dry mass of soil) and noncontaminated (0.058±0.003 mg Hg/kg dry mass of soil) were identified by 16S rRNA sequence analysis. Four endophytic bacteria of T. farfara L. growing in mercury-uncontaminated soil, marked as ‘N’, disclosed the highest similarity to the three species of Pseudomonas, and one to Raoultella terrigena. Four other isolates, from T. farfara


Table 1. Bacterial isolates identification based on sequenced 16S rRNA gene in GenBank database

<table>
<thead>
<tr>
<th>Origin</th>
<th>Isolate ID</th>
<th>Identification (closest relative)</th>
<th>Accession number</th>
<th>Sequence similarity rate [%]</th>
<th>Seq. length (nucleotide)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Tussilago farfara</em> (L) from mercury non-contaminated soil (N)</td>
<td>N5</td>
<td><em>Pseudomonas grimontii</em></td>
<td>NR_025102.1</td>
<td>99.22</td>
<td>1400</td>
</tr>
<tr>
<td></td>
<td>N13</td>
<td><em>Pseudomonas cedrina</em></td>
<td>NR_024912.1</td>
<td>99.71</td>
<td>1380</td>
</tr>
<tr>
<td></td>
<td>N21</td>
<td><em>Raoultella terrigena</em></td>
<td>NR_113703.1</td>
<td>99.64</td>
<td>1407</td>
</tr>
<tr>
<td></td>
<td>N24</td>
<td><em>Pseudomonas qingdaoensis</em></td>
<td>NR_169411.1</td>
<td>98.92</td>
<td>1390</td>
</tr>
<tr>
<td><em>Tussilago farfara</em> (L) from mercury-contaminated soil (G)</td>
<td>G1</td>
<td><em>Pseudomonas grimontii</em></td>
<td>NR_025102.1</td>
<td>99.14</td>
<td>1399</td>
</tr>
<tr>
<td></td>
<td>G17</td>
<td><em>Pseudomonas qingdaoensis</em></td>
<td>NR_169411.1</td>
<td>99.14</td>
<td>1389</td>
</tr>
<tr>
<td></td>
<td>G20</td>
<td><em>Pseudomonas reinekei</em></td>
<td>NR_042541.1</td>
<td>99.28</td>
<td>1389</td>
</tr>
<tr>
<td></td>
<td>G23</td>
<td><em>Raoultella terrigena</em></td>
<td>NR_113703.1</td>
<td>99.35</td>
<td>1393</td>
</tr>
</tbody>
</table>

Figure 2. Results of gel electrophoresis of the PCR product, amplified 200 bp *merA* gene fragment.

Genomic DNA concentration and purity for each bacterial isolate (N and G – details in Table 1) are shown at the bottom of the figure.

L. that grows in mercury-contaminated soil signed as ‘G’, were assigned to two species of *Pseudomonas* and one species of *Raoultella* (Table 1). High sequence similarity (>99%) to the corresponding 16S rRNA sequences from the database (Table 1) and the fact that all analyzed sequences were longer than 1300 bp, may confirm good quality of the analysis (Janda & Abbott, 2007).

The concentration of genomic and plasmid DNA obtained by extraction from identified bacteria (Table 1) was sufficient to perform the PCR reaction (Figs. 2, 3) and the purity of genomic DNA was higher (A260/A280 above 1.80) (Fig. 2) than that of plasmid DNA (Fig. 3) (Gallaher, 1998). The presence of PCR products was confirmed by electrophoretic mobility appropriate for the analyzed fragment of the *merA* gene (Figs. 2, 3).

Identification of the *merA* gene, which encodes mercury ion reductase, is commonly used to confirm the presence of the *mer* operon in environmentally derived samples (Wijaya et al., 2021; Zeyaullah et al. 2010). Usually, the annotated genome of bacteria obtained in such studies is available in databases. In this study there was no sequence of the *merA* gene available for identified bacteria; therefore, the most conservative region of the *merA* gene was preferable. To determine the conservative gene fragment BLASTn (NCBI) and BLASTx (NCBI) analyses were performed (Altschul et al., 1997; Pearson, 2013; Zhang et al., 2000). Search options were restricted only to the *Pseudomonas* genus, as six out of eight studied isolates were assigned to this genus.

One of the studies presents the conservative region of *merA* as a molecular marker of the bacterial *mer* operon (Sotero-Martins et al., 2008). The determined 431-bp fragment was considered as a conservative *merA* region based on bioinformatics analyses of all *merA* sequences available at the time of Sotero-Martins et al. (2008) research. The experimental studies on the 431 bp conservative *merA* region were then carried out on several bacterial species, but no representative of the *Pseudomonas* genus was tested. Thus in this study designing of primers to find the conservative region of *merA* in *Pseudomonas* sp. was indispensable and then resulted in determination of the 200 bp fragment considered conservative.

The alignment of the proposed 431 bp fragment (Sotero-Martins et al., 2008) with 200 bp in TBLASTx (NCBI) presented here was performed (Altschul et al., 1997). To improve search sensitivity, DNA sequences were translated to protein sequences (Pearson, 2013). The 200 bp *merA* gene fragment studied showed 80% identity (E-value 2e-39) with a 431 bp fragment proposed by Sotero-Martins et al. (2008) as a molecular marker of mercury resistance encoded by the *mer* operon.

The BLASTx analysis of the 431 bp fragment presented here with >97% of identity with mercury reductase in different species. Presented here a 200 bp sequence in the same BLASTx analysis presented more than 20 hits with 100% identity with mercury reductase in *Pseudomonas* sp., but also some hits with another species.

The most specific primers for the longer fragment or whole *merA* gene could only be designed if the genome of isolated bacteria is sequenced and annotated, then the most accurate comparison would be possible in BLAST programmes.

The presence of *merA* gene was confirmed in all isolates from microbiota of *T. farfara* L. growing in mercury-contaminated soil (G) and mercury-noncontaminated soil (N) (Figs. 2, 3). For G isolates, all bands were equal intensive despite differences in DNA concentration and purity, but in N bacteria, the intensity of the bands differed between isolates (Figs. 2, 3). *mer operon* can be found on transposons, plasmids, and the bacterial chromosome (Osborn et al., 1997). Subsequent PCR confirmed the presence of 200-bp *merA* gene fragment also in the plasmid DNA of bacteria studied (Fig. 3). The template for *merA* amplification from tested bacteria was present in genomic and plasmid DNA. The variability in...
The growth curves of N isolates in LB medium were similar with no lag phase and the saturation growth phase from the 10th hour (Fig. 4), and the µmax values were also comparable (Table 2). Contrary to N isolates, G isolates have a noticeable lag phase in LB media, the Max of OD600nm for the G isolates were lower than the values for the N isolates (Fig. 5, Table 2). Despite the presence of the merA gene, N isolates cannot grow in medium with 0.01% (w/v) addition of mercury (Fig. 4). After 10 hours in all N cultures, inhibition of the growth of the bacterial culture was noticed. Mercury ions bind to sulphydryl, phosphoryl, carbosy, amide, and amine groups in proteins; in the result, proteins precipitate, and enzyme activity is inhibited. Most proteins after mercury binding remain inactive (Broussard et al., 2002). The studied high concentration (0.01%) of mercury was toxic for N isolates. Under our experimental conditions the growth of N bacteria that was observed in our experiments differed between isolates (Fig. 5). The shortest lag phase from the 10th hour (Fig. 5) was observed in the Kor River isolates despite the 7 times higher concentration of mercury in media) (Kafilzadeh & Mirzaei, 2008). The bacteria isolates from Kor River reach the maximal OD600nm=1.2 (Kafilzadeh & Mirzaei, 2008), while the isolate G17 (Pseudomonas qingdaonensis) isolate at almost seven times higher concentration of mercury in media reaches Max of OD600nm=1.30±0.07. Moreover, the other isolates of the G group had Max of OD600nm ~ 0.8, slightly lower than Kor River isolates despite the 7 times higher concentration of mercury. This result indicates a higher tolerance for the presence of mercury in the environment in the studied G bacteria, and therefore, a potentially more promising application in bioremediation. The values of the growth kinetic parameters calculated growth kinetics parameters (µmax, DT) for G isolates in media with mercury addition presented the same application potential of isolated bacteria. However, G17 (Pseudomonas qingdaonensis) had the shortest lag phase, which could be useful for further application in bioremediation.

The studied bacterial isolates, thanks to their ability to grow at a high concentration of mercury, i.e., 0.01% (w/v), are good models not only for further investigation of the mercury resistance mechanism but also for possible application in bioremediation techniques.
Our experiment suggests that confirmation of the presence of merA gene is not sufficient enough to confirm the genetic background of bacterial mercury resistance at the concentration studied. The need for further investigation of the molecular mechanism and determination of other molecular markers of the resistance to mercury of bacteria based on the mer operon is evident. Subsequent research will attempt to make this.

REFERENCES


McCarthy D, Edwards GC, Gustin MS, Care A, Miller MB, Sunna A (2017) An innovative approach to bioremediation of mercury con...


