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

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Gene encoding mercuric ion reductase, merA is a crucial component of the mer operon for reduction of nonorganic mercury ions into less toxic form. The merA gene or its fragments are commonly used as a molecular marker of bacterial resistance to mercury. In this study, it was tested whether the merA gene can be considered as a molecular marker of mercury bacterial resistance. For this purpose, the presence of the mer operon in bacteria isolated from the microbiota of Tussilago farfara L. growing in post-industrial mercury-contaminated and non-contaminated areas was verified by merA gene identification. Mercury resistance was determined by analyzing the bacterial growth parameters in standard Luria-Bertani (LB) medium with mercury concentration of 0.01% (w/v) and in medium without mercury addition. The results obtained showed that the merA gene was present in all T. farfara L. bacterial isolates growing in both mercury-contaminated and non-contaminated soils, however, only the isolates from mercury-contaminated areas were able to grow under mercury conditions. Although merA is commonly regarded as a molecular marker of bacterial mercury resistance, results of our research indicate the need for a verification of that statement/thesis and further investigation of bacterial mercury resistance to indicate other its key markers, structures, or mechanisms.

Keywords: mer operon, mercuric ion reductase, microbiota, Tussilago farfara L., mercury contamination, bioremediation

Received: 10 May, 2022; revised: 18 June, 2022; accepted: 18 June, 2022; available on-line: 17 August, 2022

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

Figure 1. The generic mer operon structure, genes present in all operons (Lin et al., 2011)
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'-GGTTACCTTGTTACGACTT-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 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 10 min. The PCR product was purified using Syngen GEL/PCR Mini Kit. Sequencing was done by Genomed Company using the Sanger method. The obtained FASTA files were processed in the Chromas Lite program. All consensus sequences were submitted to the GenBank database (rRNA_typestrains/16S_ribosomal_RNA).

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'-CGTTCCACCGGATTCGACTG-3' (10 µM); 1 µl of primer Ps_merA_Rev3: 5'-TGGCCGGCTTCTCGTGGAAG-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 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.

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 OD₀₅₀₅ = 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), OD₅₅₀₅ measurement in five spots per well, every 27 minutes. For proper calculation of the kinetic parameters for bacterial growth, OD₅₅₀₅ values were calculated as ln(OD₅₅₀₅) = ln(OD₅₅₀₅₀). 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.

\[
DT = \frac{\ln 2}{\mu_{\text{max}}}
\] (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 analyzes. Four endophytic bacteria of T. farfara L. growing in mercury-contaminated 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 L., were compared for isolates from contaminated and uncontaminated areas in medium supplemented and not supplemented with mercury.
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Table 1. Bacterial isolates identification based on sequenced 16S rRNA gene in GenBank database

| Origin                        | Isolate ID | Identification (closest relative) | Accession number | Sequence similarity rate [%] | Seq. length (nucleotide) |
|-------------------------------|------------|-----------------------------------|------------------|-----------------------------|-------------------------|
| Tussilago farfara (L) from mercury non-contaminated soil (N) | N5         | Pseudomonas grimmortii            | NR_025102.1      | 99.22                       | 1400                    |
|                               | N13        | Pseudomonas cedrina               | NR_024912.1      | 99.71                       | 1389                    |
|                               | N21        | Raoultella terrigena              | NR_113703.1      | 99.64                       | 1407                    |
|                               | N24        | Pseudomonas qingdaonensis         | NR_169411.1      | 98.92                       | 1390                    |
| Tussilago farfara (L) from mercury-contaminated soil (G) | G1         | Pseudomonas grimmortii            | NR_025102.1      | 98.14                       | 1389                    |
|                               | G17        | Pseudomonas qingdaonensis         | NR_169411.1      | 99.14                       | 1389                    |
|                               | G20        | Pseudomonas reinekei              | NR_042541.1      | 99.28                       | 1389                    |
|                               | G23        | Raoultella terrigena              | NR_113703.1      | 99.35                       | 1393                    |

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) (Gallagher, 1998). The presence of PCR products was confirmed by electrophoretic mobility appropriate for the analysed 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). The 200 bp merA gene fragment studied showed 98% identity (E-value 2e-31) 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 equally 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 bacterial isolates from the microbiome of Tussilago farfara L. growing in mercury-free areas (N) cultured in LB or LB+0.01% Hg medium for 48 hours. The error bars present the standard deviation of the mean.

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.

The extension of the lag phase in LB+0.01% Hg media was noticeable (Fig. 5, Table 2). This delay indicated adaptation to the new environment, in our case, the presence of mercury (Vermersch et al., 2019). The observed duration of the lag phases was max. 4 h in media with LB+0.01% Hg (135 ppm HgCl₂) (Table 2). In the study presented by Irawati et al. (2012) the addition of 50 ppm HgCl₂ to the bacterial culture resulted in the extension of the lag phase in 8 hours and the addition of 100 ppm HgCl₂ to the bacterial culture resulted in the extension of the lag phase in 16 hours. The maximal OD₆00nm for G isolates under conditions with and without mercury were not significantly different (Table 2).

All four studied G isolates were able to grow in LB+0.01% Hg (135 ppm HgCl₂ in media) concentration (Fig. 5) which was higher than previously reported data for bacteria isolated from a gold mine in Indonesia (50 ppm, 100 ppm HgCl₂ in media) (Irawati et al., 2012) or for bacteria isolated from the Kor River (20 ppm HgCl₂ 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 gingdaonensis) isolate at almost seven times higher concentration of mercury in media reaches Max,OD₆00nm 1.30±0.07. Moreover, the other isolates of the G group had Max,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 gingdaonensis) 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.

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Table 2. Growth kinetic parameters of N and G bacteria for both conditions studied.

| Isolate ID | µmax [h⁻¹] | DT [h] | lag phase [h] | Max[µg/dm³] |
|------------|------------|-------|--------------|-------------|
| N5         | 0.328±0.042| 0.98±0.10| 0             | 0.98±0.04   |
| N13        | 0.648±0.057| 1.02±0.10| 0             | 0.05±0.01   |
| N21        | 0.71±0.067 | 0.98±0.10| 0             | 0.05±0.01   |
| N24        | 0.616±0.050| 1.13±0.10| 0             | 0.85±0.09   |
| G1         | 0.518±0.039| 1.34±0.11| 0.5           | 1.25±0.05   |
| G20        | 0.497±0.038| 1.40±0.12| 1             | 0.72±0.09   |
| G23        | 0.570±0.042| 1.22±0.10| 1             | 0.98±0.11   |

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.

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