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Regular paper

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 noncontaminated 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

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Abbreviations: CVAAS, cold vapour atomic absorption spectrometry; DT, doubling time of bacteria growth; FAD, flavin adenine dinucleotide G, isolates from microbiota of *Tussilago farfara* L. growing in mercury-contaminated soil; LB, Luria-Bertani media; LB+0.01% Hg, Luria-Bertani media with mercury concentration 0.01% (w/v) (Hg source $HgCl_2$, 135 ppm $HgCl_2$); N, isolates from microbiota of *Tussilago farfara* L. growing in mercury non-contaminated soil; NCBI, National Center for Biotechnology Information; μmax, maximum growth rate; OD_{600nm}, optical density at λ= 600 nm; PCR, Polymerase Chain Reaction; 16S rRNA, component of the prokaryotic ribosome 30S subunit

INTRODUCTION

Mercury contamination is a serious environmental problem since Hg^{2+} 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 Hg^{2+} to Hg^0. 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 Rebello *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 mer*R gene encodes the regulatory protein (MerR) which controls the expression of the whole operon acting as a repressor in the absence of Hg^{2+} and as an inducer in the presence of Hg^{2+} 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 ($SnCl_2$) 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 $HgCl_2$ – 135 ppm $HgCl_2$, added to the medium as a 0.1 M $HgCl_2$ 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 ddH_2O; 1 µl of primer 8F (5'-AGAGTTTGATCCTGGCTCAG-3') (10 µM) and 1 µl of primer 1492R (5'-GGTTACCTTGTTACGACTT-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 15 min.; 29 cycles of denaturation 95°C for 1 min.; annealing 54°C for 1 min.; elongation 72°C for 2 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 ddH_2O; 1 µl of primer Ps_merA_For3: 5'-CGTTCCACCGATTCCAGGACTG-3' (10 µM); 1 µl of primer Ps_merA_Rev3: 5'-TGGCCGGGTCTTCGTGGAAG-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 OD_{600n}=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 customwritten shake program (140 rpm, 22°C), OD_{600nm} measurement in five spots per well, every 27 minutes. For proper calculation of the kinetic parameters for bacterial growth, OD_{600nm} values were calculated as $\ln(OD_{600nm})$. 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.

$$DT = \frac{\ln 2}{\mu max} \quad \text{(Eqn. 1)}$$

µmax – maximum growth rate

RESULTS AND DISSCUSION

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-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

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 grimontii*	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 qingdaonensis*	NR_169411.1	99.14	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

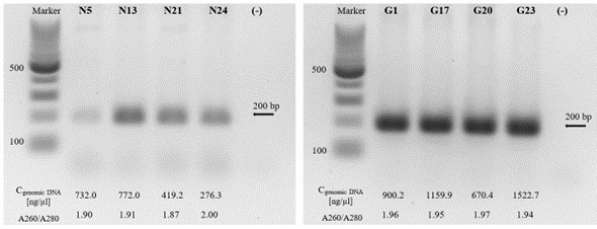

Figure 2. Results of gel electrophoresis of the PCR product, amplified 200 bp *merA* gene fragment.
Genomic DNA concentration and purity for each bacterial isolate (N and G – details in Table 1) are shown at the bottom of the figure

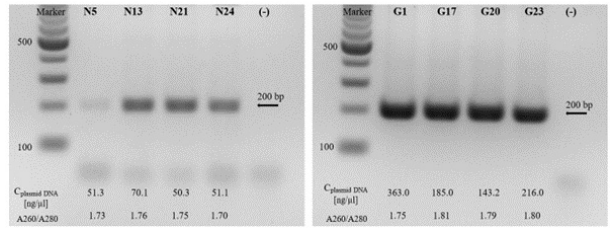

Figure 3. Results of gel electrophoresis of the PCR product, amplified 200 bp *merA* gene fragment.
Plasmid DNA concentration and purity for each bacterial isolate (N and G – details in Table 1) are shown at the bottom of the figure

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; 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 $2e^{-35}$) 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 hits 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

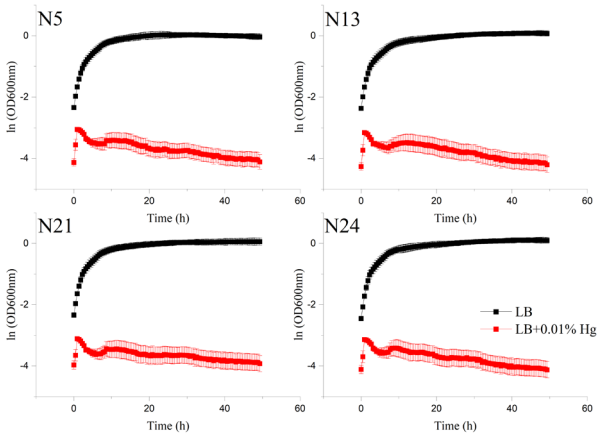

Figure 4. The growth curves of bacterial isolates from the microbiome of *Tussilago farfara* L. growing in mercury-free areas (N) cultured in LB or LB+0.01% Hg medium for 48 hours.
The error bars present the standard deviation of the mean

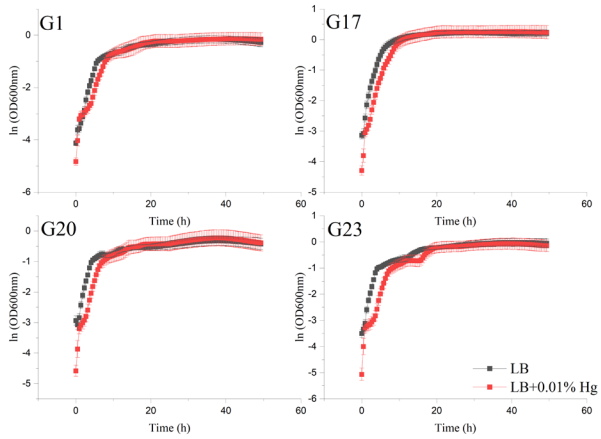

Figure 5. The growth curves of bacterial isolates from the microbiome of *Tussilago farfara* L. growing in mercury contaminated (G) areas cultured in LB or LB+0.01% Hg medium for 48 hours.
The error bars present the standard deviation of the mean

band intensity could be the result of different amounts of copies of the *merA* gene in the template DNA material (Figs. 2, 3).

All isolates were able to grow in LB medium (Figs. 4, 5). 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 $Max_{OD_{600nm}}$ the values 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 sulfhydryl, phosphoryl, carboxyl, 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 inhibited the growth of bacteria probably by disrupting proper protein functions (Broussard *et al.*, 2002). That was probably the mechanism behind the inhibition of the growth of N bacteria that was observed in our experiments.

The growth curves for G bacteria were similar under both conditions with and without mercury addition, but differed between isolates (Fig. 5). The shortest lag phase for both conditions presented G17 – *Pseudomonas qingdaonensis,* the longest lag phase for media with mercury addition presented G1 – *Pseudomonas qingdaonensis* and G23-*Raoultella terrigena,* other parameters for these two were comparable. The visible drop in the value of OD_{600nm} for G23 was observed in the 20th hour of culture (Fig. 5, Table 2).

The maximal growth rate for G isolates was lower in the medium with mercury addition, and also the DT was longer (Table 2). In both culture conditions, the maximal growth rate was the highest for G1 – *Pseudomonas qingdaonensis*, the lowest values of μmax for both conditions present G20 – *Pseudomonas reinekei* (Table 2). The doubling time of bacteria cells depends on the strain of the bacteria and the culture conditions and can vary from a few minutes to a few days (Štumpf *et al.*, 2020). The difference in DT for G isolates in LB media was significant between G1 and G20, but disappeared in media with mercury addition (Table 2) despite the fact that G1 and G20 were identified as different species (Table 1, Table 2).

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 (Vermeersch *et al.*, 2019). The observed duration of the lag phases was max. 4 h in media with LB+0.01% Hg (135 ppm $HgCl_2$) (Table 2). In the study presented by Irawati *et al.* (2012) the addition of 50 ppm $HgCl_2$ to the bacterial culture resulted in the extension of the lag phase in 8 hours and the addition of 100 ppm $HgCl_2$ to the bacterial culture resulted in the extension of the lag phase in 16 hours. The maximal OD_{600nm} 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 $HgCl_2$ 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 $HgCl_2$ in media) (Irawati *et al.*, 2012) or for bacteria isolated from the Kor River (20 ppm $HgCl_2$ in media) (Kafilzadeh & Mirzaei, 2008). The bacteria isolates from Kor River reach the maximal OD_{600nm}=1.2 (Kafilzadeh & Mirzaei, 2008), while the isolate G17 (*Pseudomonas qingdaonensis)* isolate at almost seven times higher concentration of mercury in media reaches $Max_{OD_{600nm}}$ 1.30±0.07. Moreover, the other isolates of the G group had $Max_{OD_{600nm}}$ ~ 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 the mercury resistance mechanism but also for possible application in bioremediation techniques.

Table 2. Growth kinetic parameters of N and G bacteria for both conditions studied.
For N in LB, a linear fit was performed for the first 2 hours (•), because there was no observed growth, the data analysis for N isolates cultivated in LB+0.01% Hg medium was not carried out. For G isolates, linear fit was performed from the end of the lag phase up to the fourth hour of cultivation in LB media (*) and up to the eighth hour of cultivation in LB+0.01% Hg media (**)

Isolate ID	μmax [h^{-1}]	DT [h]	lag phase [h]	$Max_{OD_{600nm}}$
LB media				
N5•	0.635±0.057 R_2=0.986	1.09±0.10	0	1.03±0.04
N13•	0.648±0.057 R_2=0.984	1.07±0.10	0	1.08±0.04
N21•	0.645±0.060 R_2=0.984	1.08±0.11	0	1.06±0.07
N24•	0.711±0.067 R_2=0.989	0.98±0.10	0	1.11±0.04
G1*	0.616±0.050 R_2=0.992	1.13±0.10	1	0.85±0.09
G17*	0.518±0.039 R_2=0.976	1.34±0.11	0.5	1.25±0.05
G20*	0.497±0.038 R_2=0.994	1.40±0.12	1	0.72±0.09
G23*	0.570±0.042 R_2=0.991	1.22±0.10	1	0.98±0.11
Isolate ID	μmax [h^{-1}]	DT [h]	lag phase [h]	$Max_{OD_{600nm}}$
LB+0.01% Hg media				
N5	–	–	–	0.05±0.01
N13	–	–	–	0.04±0.01
N21	–	–	–	0.04±0.01
N24	–	–	–	0.04±0.01
G1**	0.396±0.052 R_2=0.988	1.75±0.26	4.0	0.88±0.10
G17**	0.328±0.030 R_2=0.978	2.11±0.21	2.7	1.30±0.07
G20**	0.311±0.036 R_2=0.939	2.23±0.29	3.2	0.80±0.11
G23**	0.349±0.057 R_2=0.956	1.99±0.39	4.0	0.94±0.07

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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