Bioremediation of heavy metals by an unexplored bacterium, Pseudoxanthomonas mexicana strain GTZY, isolated from aerobic-biofilm wastewater system

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Abstract

We prompted first to characterize a wastewater bacterium, *Pseudoxanthomonas mexicana* GTZY, that efficiently transforms toxic mercury and arsenic and to explore its bioremediation capability and to reveal their relevant gene resistance operons. The Isolated strain was characterized for its phylogenetic, biochemical and phenotypic properties. The strain GTZY potentially removed 84.3% of mercury and their mercury volatilization (Hg(II) to Hg(0)) was confirmed using X-ray film method, and its respective *merA* gene was PCR amplified. In addition, strain GTZY was efficiently removed arsenate (68.5%) and arsenite (63.2%), and showed resistance up to >175 and >55 mM, respectively. Their genomic annotations were disclosed the linkage of *Tn2*-transposon and *int1* in both ends of *mer*operon (*merAPTR)*. The co-existence of arsP and arsH proteins in its intrinsic *ars* operon (*arsCPRH)* was extremely diverse from its ancestral species. We believe that mercury resistance-conferring *mer*operon of *P. mexicana* GTZY presumably derived horizontally from other species in the reactor, while arsenic resistance-conferring intrinsic *ars* operon was highly diversified and evolved from its ancestral species. By considering the potential of the strain GTZY to transform heavy metals, this can be used to recover contaminated sites.

Highlights

- First detailed heavy metals bioremediation study in the genus, *Pseudoxanthomonas*
- Isolate has great potential to resist, reduce and remove the toxic Hg(II) and As(V)
- An acquired mobile elements linked *mer* gene operon was observed in its chromosome.
- An intrinsic *ars* gene operon was extremely diverse from its ancestral species

1. Introduction

Heavy metals are inorganic ill-defined chemical compounds that ubiquitous in nature. In which, some heavy metals such as cadmium (Cd), arsenic (As), nickel (Ni), lead (Pb), chromium (Cr), mercury (Hg), copper (Cu) and zinc (Zn) are mainly persisting in the environments due to the core release of anthropical sources (Loganathan et al. 2015; Govarthanan et al. 2016b, a, 2018, 2019; Jobby et al. 2018; Briffa et al. 2020; Khalid et al. 2020). This persistence is considered as an emerging situation due to their potential toxicity in the entire ecosystem, including humans, animals and plants (Olmedo et al. 2013; Rebelo and Caldas 2016; Luo et al. 2020; Qin et al. 2021). Some heavy metals are considered highly toxic as they could cause human illness even at their relatively low levels. Among those, arsenic and mercury are the prime consideration for their chemical toxicity, persistant, frequency and potential to human exposure(Hou et al. 2020). According to the national priorities list released by the Agency for Toxic Substances and Disease Registry (ATSDR), among the 275 toxic substances, arsenic and mercury were ranked as first and third, respectively(Watters and Rayman 2014, 2018). These heavy metals are non-biodegradable and can not catabolized by microorganisms. Alternatively, these heavy metals can be transformed from toxic to non-toxic forms by the nuclear structural changes via microbial activities(Wuana and Okieimen 2011; Jebeli et al. 2017; Wang et al. 2020).

In general, microbial mercury bioremediation mainly occurs by the mechanism of reduction or volatilization, which is governed by *mer* operon in microorganisms(Boyd and Barkay 2012; Raj and Maiti 2019). In *mer* operon, merA protein is one of the main constituents along with Hg binding (*merP*), regulatory (*merR* and *D*), and mercury transporting proteins (*merT* and *E*), and are mainly located in the bacterial chromosome or mobile genetic elements (e.g. plasmids, integrons or transposons)(Oregaard and Sørensen 2007; Boyd and Barkay 2012; Møller et al. 2014; Naguib et al. 2018). Relying on the availability of toxic mercury (Hg^2), merR protein binds specifically into the promoter-operator region and it is followed by regulating the transcription of structural genes. As a result, it produces a key enzyme in the detoxifying system known as mercury reductase (encoded by *merA* gene), an NAD(P)H-dependent flavin oxidoreductase, reducing toxic mercury (Hg^2) into a volatile natured less toxic mercury (Hg^0)(Freedman et al. 2012; Dash et al. 2017). Likewise, microbial arsenic bioremediation mainly involves arsenic reduction (*arsC*), oxidation (*AoxAB, arsH*), methylation and intra-cellular bioaccumulation(Pepi et al. 2007; Rahman and Hassler 2014; Govarthanan et al. 2015, 2016a, 2019; Satyapal et al. 2018; Ben Fekih et al. 2018). The *ars* operon is an energy-gaining detoxifying respiratory system that facilitates the reduction of pentavalent arsenate (V) into trivalent-arsenite (III)(Suhadolnik et al. 2017). The interaction of transcriptional repressor protein (arsR) with promoter region of *ars* operon, followed by the binding of arsR protein with arsenate, dissociates the repressor protein from a specific DNA site permitting the *ars* operon's transcription(Páez-Espino et al. 2015; Li et al. 2016). This organoarsenic reduction process is governed by the *arsC* encoding arsenic reductase enzyme. The *ars* operons, such as *arsRB, arsRBC, arsRDABC* and *arsHRBC* are widely demonstrated in the gram-negative bacteria(Dey et al. 2016; Ben Fekih et al. 2018; Taran et al. 2019).

The existence and sustainability of these metal resistance operons among the various bacterial communities are usually maintained and transmitted with the help of mobile genetic elements via horizontal gene transfer(Møller et al. 2014; Suhadolnik et al. 2017). In some bacteria, this metal resistance sustainability is preserved intrinsically in the bacterial chromosome, the consequence of vertical gene transfer from its ancestral species. Many bacterial species reportedly have arsenic and mercury resistance operons in their chromosome or mobile genetics elements(Møller et al. 2011; Ghaly et al. 2017; Agarwal et al. 2019). However, so far, none among the species in the genus *Pseudoxanthomonas* have been demonstrated for any heavy metal resistance and their respective operons. The 25 members of this genus are gram-negative, rod-shaped, non-pathogenic that are long been known for their prevalence in diverse ecological sources, including soil, water, plants, animal and human tissues(Thierry et al. 2004; Rani et al. 2010; Kittiwongwattana and Thawai 2016; Selvaraj et al. 2018). Although the genus *Pseudoxanthomonas* is well characterized for its biodegradation capability and surfactant production(Choi et al. 2013; Talwar and Ninnekar 2015; Biswas et al. 2017; Astuti et al. 2019; Lu et al. 2019; Purwasena et al. 2020), it is unremarked in many environmental processes, particularly in terms of its bioremediation capability. This study thereby deals (i) isolation and characterization of Hg and As resistant strain GTZY from an bioreactor treating antibiotic-containing wastewater, (ii) evaluation of Hg and As removal efficiency of strain GTZY in batch-culture experiments,

(iii) disclosure of the *mer* and *ars* resistance operon from strain GTZY and (iv) phylogenetic distribution and relationship of identified *mer* and *ars* operon of strain GTZY with other bacterial communities.

2. Materials and Methods

2.1. Bacterial strain

The bacterial samples were collected from the aerobic-biofilm wastewater reactor treating aminoglycoside antibiotic, streptomycin (50 mg L^{-1}) (State key laboratory of RCEES, CAS Institute, Beijing, China). The ten-fold serial dilution method was used to isolate and enrich bacterial isolate from the collected sludge samples. Inoculated (100 µL aliquots) plates were incubated at 30 ºC for 12–18 h. Tryptone soy agar (TSA) and Reasoner's 2A agar (R2A) media were used for the purpose of bacterial isolation. Gram's staining was routinely performed to check the microbial purity. Initially, there were 23 bacterial isolates harvested from the wastewater reactor. To obtain the potential heavy metal transforming bacteria, the purified isolates were further screened on the growth media amended with As (V); 1 µM and $HgCl_2$; 10 µM. Among the 23 isolates, strain GTZY was choosen for further studies due to its fast growing ability and surviving capability in the presence of increased concentration of As (V); 100 µM and $HgCl_2$; 50 µM. These screened isolates were stored as 40% glycerol stock for further studies.

2.2. General characterization of bacterial isolate

The morphological, biochemical and physiological characterization of isolated bacterial strain was performed based on the standard microbiological methods. The optimum temperature and pH were identified by culturing the strain on the nutrient agar media in the various temperatures (25–40 ºC) and pH (5–8). After 12–18 h incubation, bacterial colony morphology was determined using a single colony which grown on the nutrient agar, TSA, LB and R2A media. The hanging drop method was performed to check the motility of isolates under the light microscopy. Gram's staining solutions were manually prepared and isolates were viewed under the light microscopy magnifications (100×). A salt tolerance test was carried out by preparing Brain-heart infusion agar plates with the addition of various NaCl concentrations 0–7% (w/v). Activitiy of various enzymes (catalase, oxidase, urease and indole), hydrogen sulfide production, utilization of various carbon sources, nitrate reductability and hydrolysis of biochemicals (gelatin, starch and casein) tests were also carried out by the standard microbial methods.

2.3. 16S rRNA gene amplification and phylogenetic tree analysis

The genomic DNA isolation and *16rRNA* gene PCR amplifications were carried out as described in the previous study(Selvaraj et al. 2018). In brief, the genomic DNA was harvested from the bacterial samples using the TIANamp bacteria DNA kit (Tiangen, China), and the isolated DNA was quantified using Nanodrop 1000 spectrophotometer (Nanodrop, USA). The 16SrRNA fragments were amplified using the universal primers (27F-5'-AGAGTTTGATCCTGGCTCAG-3' and 1492R-5'-AAGGAGGTGATCCAGCCGCA-3'), sanger sequenced (Biobase, China) and interpreted by the Ribosomal Database Project (RDP) and

GenBank-National Center for Biotechnology Information (NCBI) database. The MEGA6.06 and Clustal W softwares were used to constructing a phylogenetic tree for isolated strain against its closest 19 bacterial strains. The Neighbour-joining method and bootstrap analysis were executed for the bacterial phylogeny and statistical sequence analysis, respectively. In bootstrap analysis, 1000 replicates were applied for determining the confidence values of bacterial phylogenetic tree nodes. Finally, the partial *16S rRNA* gene sequence was submitted in the GenBank.

2.4. Determination of mercury MIC

After bacterial isolation, the bacterial efficiency on mercury resistance was maintained in LB media by the supplement of mercury chloride solution (10–30 μM; $HgCl_2$). The bacterial isolates were grown overnight on the LB media added with 10 μM of $HgCl_2$. Subsequently, those cultures were resuspended with 2–5 mL of Tris-buffered saline (TBS) solution, and absorbance ($OD_{600\ nm}$) was adjusted to 0.100 OD. Further, 125 μL of appropriate bacterial dilutions were inoculated on the 96-well plate containing 125 μL of LB broth (in triplicate) amended with various concentrations (0, 1, 5, 10, 20, 30, 40, 50, 60, 70 and 80 μM) of $HgCl_2$ and incubated at 30 ºC for 72 h in the dark. After the incubation, bacterial growth was observed by measuring the absorbance ($OD_{600\ nm}$) using a microplate reader. The inhibition effect of mercury on the microbial growth, values of MIC and MIC_{50} were calculated.

2.5. Mercury removal assay

Overnight grown bacterial cultures (in triplicate) added with 10 μM of $HgCl_2$ were subjected for the mercury removal assay. Every 12 h of incubation, 4 mL of culture was centrifuged for 10 min at 7500 rpm,thereby recovered bacterial pellets were resuspended in 2.8 mL of Tris-buffered saline solution. In the resuspended solution, 0.4 mL of 5-diphenyl-3-thiocarbazone (Dithizone) reagent solution (0.50 mg of dithizone mixed with 20 mL of acetone) was added, followed by the addition of 0.4 mL of sulphuric acid (2 N) to maintain the acid pH (pH 2–3) and finally added by 0.4 mL of Dioxane (Naguib et al. 2019). The acidic pH of the solution enhanced the formation of orange-red colour by coupling of dithizone and mercury (II). These final solutions were diluted to 5 mL using distilled water and checked mercury removal values at 488 nm in a UV/Visible spectrophotometer. Meanwhile, a similar experimental set up was carried out using dead bacterial cells (autoclaved cells) for treating as control. The standard curve was also prepared for $HgCl_2$ solution without bacterial suspension. The detection limit for this method was estimated as < 1 ppb (Nordlander). The actual Hg concentrations during the incubation period was measured and thereby the mercury removal percentage was calculated as follows,

$$\%\ \text{removal of mercury} = \frac{\text{Initial Hg concentration} - \text{residual Hg concentration}}{\text{Initial Hg concentration}} \times 100$$

2.6. Mercury volatilization assay

A simplified X-ray film method was used to confirm the mercury volatilization from Hg (II) state to Hg (0) state by the strain GTZY. Overnight grown culture added with 10 µM; $HgCl_2$ was centrifuged at 6000 rpm for 5 min. Harvested bacterial pellets were resuspended into a 200 µL assay solution containing 0.07 M phosphate buffer (pH 7), 0.2 mM magnesium acetate, 5 mM sodium thioglycolate and 50 µM $HgCl_2$. The microtiter plate containing the above-mentioned solution were fully covered using an X-ray film (Kodak scientific imaging, Hyderabad, India)(Møller et al. 2011, 2014) and incubated at 35 ºC in the dark. A similar solution mixture that without bacterial cells and bacterial suspension without $HgCl_2$ were utilized as control samples.

2.7. Determination of arsenic MIC

The bacterial arsenic resistance was evaluated by adding the different concentrations of sodium arsenate (10–400 mM) and sodium arsenite (10–200 mM) into the overnight grown bacterial culture, while the control samples have no metals. The cultures were resuspended with 2–5 mL of TBS solution with an absorbance ($OD_{600\ nm}$) of 0.100. Further, 125 µL of appropriate bacterial dilutions and 125 µL of LB broth (in triplicate) were added into the 96-well plate containing suspended concentrations of arsenic and incubated at 30 ºC for 48 h in the dark. After the incubation, tested bacterial samples were estimated for their optical density at 600 nm using a microplate reader. The inhibition effect of arsenic on the microbial growth, values of MIC and MIC_{50} were calculated. .

2.8. Arsenic removal assay

After the bacterial isolation, the efficiency of arsenic resistance was maintained by adding sodium arsenate (100 mM) and sodium arsenite (100 mM) into the bacteria growth media (NA). Arsenic utilization by bacterial isolate was determined by the Silver Diethyldithiocarbamate (SDDC) method(Tahir et al. 2012). The detection limit for this method was evaluated as 1–5 ppb. Initially, the isolate (1%) was inoculated (in triplicate) in LB broth added with 5 µM sodium arsenate and sodium arsenite solution (as sources for As (V) and As (III), respectively) and incubated at 30 ºC for various time intervals (viz. 8, 12, 18, 24, 48 and 72 h). After the definite time points, the actual arsenic concentrations during the incubation period was estimated using the SDDC method. Briefly, the production of arsine carrying hydrogen passed through a reduction column impregnated with lead acetate and then into the absorbing solution SDDC dissolved pyridine. The end red-coloured product (arsine with SDDC solution) was measured spectrophotometrically at 535 nm.

2.9. Arsenic reduction and oxidation assay

The ability of the bacterial isolate to oxidize Arsenic (As III) and reduce Arsenic (As V) was checked by the silver nitrate solution(Dey et al. 2016). After 24 and 48 h of incubation, 100 µL of silver nitrate solution (0.1 M) was added into the grown bacterial culture (100 µL) amended with 1 µM sodium arsenate and sodium arsenite. If the culture broth turns brown, it represents the presence of silver arsenate, while if the culture broth turns yellow, represents the presence of silver arsenite.

2.10. merA and arsC genes sequencing

The extracted and purified genomic DNA was used as a template DNA to screen mercury (*merA*) and arsenical (*arsC*) resistance genes. The designed *merA* (*merA*-FP- 5'-CTACCCCGCGCAACAGGACA-3' and *merA*-RP- 5'-TGATGGCGCCTTGCGCATTG-3') and *arsC* (*arsC*-FP-5'-TCAGGACGAGGCGCCGATCT-3' and *arsC*-RP-3'- ATGGACCGCCCCTACAACCT-5') primers were used to amplify the partial regions *merA* and *arsC*. The purified genomic DNA (50 ng) was used as a template for preparing the PCR reaction. The triplicate PCR mixture (50 µL) comprised of 1× PCR buffer (with $MgCl^{2+}$), 2.5 mM of the dNTPs mix, 0.5 µM of each primer, and 1 U of Taq DNA polymerase (Takara, Bio Inc., Shiga, Japan). The PCR conditions were performed as follows: initial denaturation stage at 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 56°C for 30 s, extension at 72°C for 2 min and a final extension at 72°C for 10 min. The amplified specific PCR products were resolved using 1.2% agarose (Biowest, Hong Kong) gel and sequenced (Sanger sequencing). The gene sequences were analyzed using BLASTN.

2.11. Annotations of mer and ars gene operons

The extracted total genome of isolated strain GTZY was sequenced using the third-generation single-molecule real-time (SMRT) technology (Pacific Biosciences Inc., USA), and the sequenced genome was submitted to the GenBank. The whole-genome sequence of *Pseudoxanthomonas mexicana* strain GTZY (Accession No.: CP060028) was used for the resistance gene cluster's interpretation. The *mer* and *ars* resistance gene clusters related data were initially annotated using the online ORF prediction server, Rapid Annotations using Subsystems Technology (RAST), and their manual annotations were performed using BLAST. A Python application software, Easyfig (ver. 2.1) was used for the linear comparison and visualization of *mer* and *ars* resistance gene clusters. In addition, the BLAST comparison between the gene annotated files was manually uploaded, output files (blastn) generated, interactively coloured and enabled a rapid transition using the software, Easyfig. The SnapGene Viewer (ver. 4.1.3) was used for the gene annotation and visualization.

3. Results

3.1. Characterization of bacterial isolate

The isolated bacterial strain was aerobic, gram-negative, rod-shaped and highly motile. The colonies were pale yellow, circular, convex and mucoid-natured on the solid nutrient media (Fig. S1). The isolate can grow in the media up to 40 ºC and pH 8, while its optimum temperature and pH were 34–37 ºC and 7–7.5, respectively. Isolate can tolerate the presence of sodium chloride up to 8% (w/v); however, its optimum concentration was 3.5–5.5%. Their biochemical and phenotypic characterizations were given in Table S1. Biochemically, the isolate turned positive for catalase while remained negative for oxidase and urease, which in turn ferments glucose and mannitol but not sucrose and lactose. The hydrolysis test turned positive for the gelatin and casein, but remained negative for starch. Moreover, it showed negative results for indole production, nitrate reductase, Voges-Proskauer (VP) test, and positive for the Tween-20 and Methyl Red (MR) test. The length of the 16S rDNA gene sequence was 1424 bp, and it shared the closest

relationship (99.39%) with *P. mexicana* CP4 (Accession No.: MT549102) in the phylogenetic tree by the neighbour-joining method (Fig. 1). Hence, the isolated bacterium was named as *Pseudoxanthomonas mexicana* strain GTZY and submitted to the GenBank (Accession No.: MZ242608).

3.2. MIC, volatilization and bioremediation of mercury

The effect of mercury on microbial growth was studied. Further, MIC and MIC_{50} were determined by supplementing different mercury concentrations into LB broth inoculated with strain GTZY. After 48 h of incubation, mean values of OD_{600nm} were plotted against 0–80 µM of $HgCl_2$. The MIC value of mercury against strain GTZY was 33.4 µM of $HgCl_2$, and its MIC_{50} value was at 40.8 µM of $HgCl_2$. Microbial growth was notably inhibited by 35.5 µM of $HgCl_2$, and it was completely inhibited (0.21 OD_{600nm}) at 49.7 µM of $HgCl_2$ (Fig. 2A). After 60 h of incubation, the residual concentration of Hg was 1.7 ± 0.2 µM and it was sustained for the remaining time periods (Fig. S3A). The mercury reducing ability of the strain GTZY was confirmed by a mercury volatilization assay conducted at 48 h of incubation. The strain formed a foggy area (dark circle) due to the reaction between Hg (0) and Ag emulsion on the X-ray film (Fig. S2). However, the isolate started to produce foggy formation from 4 h onwards. Hence, the strain GTZY reduced inorganic Hg (II) and formed volatile Hg (0), which reacted with the film. There was no interference of foggy circles observed in the control samples.

Mercury removal by the strain GTZY was determined using the spectrophotometric dithizone method. Initial and residual Hg concentrations were evaluated against the standard calibration curve of various concentrations of Hg. The strain GTZY removed 16.3% of Hg from 4 h onwards, and their removal process was stopped at 48 h of incubation (Fig. 2B). The strain GTZY removed a maximum of 84.3% mercury at 48 h and their removal rate was maintained until 96 h of incubation. At the same time, no mercury removal was found in the control samples.

MIC, reduction and bioremediation of arsenic metals

The isolate GTZY was resistance to the high concentrations of arsenate and arsenite solutions. Also, it removed the arsenic compound at various time periods. Isolate GTZY was efficiently removed arsenate (68.5%) and arsenite (63.2%) at 72 h of incubation (Fig. 3). At 12 h of incubation, the removal rate (%) of arsenate and arsenite were accounted for 34.2% and 28.3%. Meanwhile, no notable removal of arsenical compounds was observed in the control samples. The MIC values of isolate towards arsenate and arsenite were > 175 and > 55 mM, and their corresponding MIC_{50} were 240 and 60.5 mM, respectively. The bacterial growth was completely arrested with 330 mM; arsenate and 69.4 mM; arsenite (Fig. S4). After 60 h of incubation, the residual concentration of As (III) and As (V) were 22 ± 2 µM and 27.5 ± 2.5 µM (Fig. S3B), thereafter it was sustained for the remaining time periods. After 24 and 48 h of incubation, 100 µL of silver nitrate solution was added to both bacterial cultures (As III and V), and the suspensions were turned into the light yellow colour, which indicated the presence of As (III). Meanwhile, there was no brownish colour observed which indicating the absence of silver arsenate. The result confirmed that isolate GTZY could reduce As (V) to As (III), while the isolate could not oxidize As (III) to the As (V).

3.3. Resistance genes sequencing

From the specific gene PCR, *arsC* (495 bp) and partial *merA* (550 bp) genes were amplified successfully and this was confirmed by the agarose gel electrophoresis (Fig. S5). The amplified DNA fragments were sequenced and annotated in GenBank. The partial sequence of *merA* from isolate GTZY was closely similar (coverage: 96%, similarity: 83.37%) to the *merA* sequence of *Lysobacter oculi* strain 83 − 4 (Accession No.: CP029556), which encodes for mercury (II) reductase enzyme. *arsC* gene of isolate GTZY was closely similar (coverage: 100%, similarity: 99.88%) with the *arsC* encoding arsenate reductase enzyme of *Stenotrophomonas acidaminiphila* strain ZAC14D2_NAIMI4 (Accession No.: CP012900).

3.4. Mercury gene cluster and its interpretation

From the genome analysis of strain GTZY, an entire set of mercury resistance gene cluster, named *merAPTR* (2829 bp) was identified (Fig. 4A). The gene cluster was possessed 1695 bp of *merA* (mercury (II) reductase), 279 bp of *merP* (mercury (II) binding protein), 351 bp of *metT* (mercuric transport protein) and 408 bp of *merR* (mercuric (II) resistance transcription regulator) genes. This gene cluster was flanked by a linkage of *Tn2*-family transposase, *ISII* (579 bp) and a site-specific integrase, *int1* (1080 bp) on both sides. IS-II family transposase was located adjacent to the gene encoding lead, cadmium, zinc and mercury-transporting ATPase (*zntA*). The entire *mer* gene cluster consists of 34 restriction enzyme sites in its respective gene sites.

Seven different bacterial mercury gene clusters (*Thermomonas* sp., *Stenotrophomonas acidaminiphila, Pseudomonas aeruginosa, Cupriavidus metallidurans, Achromobacter xylosoxidans, Delftia lacustris* and *Burkholderia cenocepacia*) along with the strain GTZY were chosen for the gene cluster interpretation, and their comparative visualization was given in Fig. 5. The strain GTZY *mer* gene cluster's downstream and upstream were highly conserved against three of seven bacterial species. Besides, it had the closest relationship (coverage: 100%, similarity: 99.89%) with the mercury gene cluster of *Thermomonas* sp. XSG (Accession No.: CP061497) and were followed by the relationship (coverage: 100%, similarity: 99.82%) with *Stenotrophomonas acidaminiphila* strain ZAC14D2 (Accession No.: AM743169) and *Pseudomonas aeruginosa* H47921 (Accession No.: CP008861) (coverage: 99%, similarity: 82.17%). From these interpretations, it was clearly evidenced that *merA* encoding mercury (II) reductase and *merP* encoding mercury (II) binding protein were invariably conserved in mercury gene clusters of all the bacterial species except *Cupriavidus metallidurans* CH34 (Accession No.: CP000352). Meanwhile, *merR* encoding mercury (II) transcription regulator was invariably conserved in all the bacterial species except *Achromobacter xylosoxidans* FDAARGO 984 (Accession No.: CP066291).

3.5. Arsenic gene cluster and its interpretation

From the genome analysis of the strain GTZY, an entire set of arsenic resistance gene clusters, *arsCPRH* (2791 bp), was identified (Fig. 4B). This gene cluster possessed 495 bp of *arsC* (arsenate reductase), 1026 bp of *arsP* (arsenate permease protein), 336 bp of *arsR* (arsenate repressor protein) and 819 bp

arsH (arsenate resistance protein). The identified *ars* gene cluster comprised of 41 restriction enzyme sites in its respective gene sites. Three different bacterial mercury gene cluster (*Dokdonella koreensis, Luteimonas* sp. and *Lysobacter oculi*) and the strain GTZY were chosen for the gene cluster interpretation and visualization (Fig. 6). However, strain GTZY *arsCPRH* gene cluster's downstream and upstream were more conserved against only to *Dokdonella koreensis* DS-123 (Accession No.: CP015249) (coverage: 94%, similarity: 79.14%) and least conserved with *Lysobacter oculi* 83 − 4 (Accession No.: CP029556) (coverage: 64%, similarity: 77.51%). The arsenic resistance genes *arsP, arsR* and *arsH* were conserved among all four bacterial genera. In the meantime, the *arsC* gene was observed only in the isolate GTZY and *Dokdonella koreensi* DS-123. The arsenic gene cluster's arrangement in *Luteimonas* sp. (*arsPRH)* and *Lysobacter oculi (arsRPH*) were extremely different from the strains, GTZY and *Dokdonella koreensis (arsCPRH).*

4. Discussion

Only a few gene clusters encoding toxic chemical degradationhave been reported so far in *Pseudoxanthomonas* species(Choi et al. 2013; Wang et al. 2013). Thereby, in this study, we looked into the bioremediation capability of two different heavy metals (Hg and As) and their related resistance gene operons in the environmental non-pathogenic bacterium, *P. mexicana* strain GTZY, for the first time. Initially, PCR amplification was confirmed the presence of metal resistance genes (*merA* and *arsC*). Interestingly, further genome annotation studies revealed the occurrence of corresponding resistance operons set (*merAPTR* and *arsCPRH)* in the genome of strain GTZY. In particular, 2829 bp size mercury operon was located initially with mercury ion reducing reductase enzyme (*merA*), which has been proven to volatilize or reduce the toxic Hg (II) to non-toxic Hg (0), then it might extrude passively from the microbial cellular environment (Fig. 8)(Sotero-Martins et al. 2008; Agarwal et al. 2019). This cytoplasmic enzyme helps to minimize the environmental re-pollution of mercury (II) caused by the mercury efflux-containing bacteria, which usually pumped out mercury rather than mercury reduction (Boyd and Barkay, 2012). In *merAPTR*, periplasmic Hg (II) binding protein-encoding *merP* and Hg (II) transporting inner membrane protein-encoding *merT* were exactly located at 10 and 12 bp upstream of *merA* gene, respectively. It is followed by the cytoplasmic mercury regulatory protein-encoding *merR*, located before the transporter gene and 729 bp downstream of the *merA* gene. The gene cluster orientation and specifications of the strain GTZY's *mer* operon were found to be in a close relationship (99.72 %) only with *mer* operons of other gram-negative bacteria, *Thermomonas* sp. (Accession No.: CP059266) and *Stenotrophomonas* sp. (Accession No.: AM743169). Despite the orientation (*IS2-merA-merP-mer-T-merR-int1*) of *mer* operon of *Pseudomonas* sp. and *Delftia* sp. was similar to the *mer* operon of strain GTZY; they have only 82.17 % and 79.19 % of gene similarities, respectively. More than 80 % of bacterial communities were demonstrated for the presence of their *mer* operon embedded in their chromosomal DNA(Boyd and Barkay 2012) and the strain GTZY does the same, and no plasmids were detected in the strain GTZY. The ancestor species of *Pseudoxanthomonas* was not detected for any *mer* operon, excluding the process of strain GTZY does mercury bioremediation. Besides, another strain *Pseudoxanthomonas mexicana* GTZY2 (Accession No.: CP060731) isolated from a wastewater reactor

(control) not exposed to any antibiotics was not detected for any heavy metals related operons in its genome. However, *mer* operon of the strain GTZY has distinctly flanked both ends by incorporating *Tn2* family transposase (*IS-II*) and a site-specific integrase (*int1*). Previously, members of *Bacillus, Stenotrophomonas, Pseudomonas, Paenibacillus, Enterobacter* and *Exiguobacterium* were reported for the *mer* operon linked with elements of transposons (*TnMERI1, Tn5083, Tn6294* and *Tn5085*)(Ramírez-Díaz et al. 2011; Wang et al. 2013; Matsui et al. 2016; Agarwal et al. 2019) and integron gene (*int1*)(Ghaly et al. 2017). These two mobile genetic fragments were proven for the transmission of functionally specific genes among the various bacterial communities(Wang et al. 2013; Ghaly et al. 2017; Ben Fekih et al. 2018). Hence, we speculated that *mer* operon of strain GTZY is not to be inherited through vertical gene transfer from its ancestral species of *Pseudoxanthomonas;* instead, it might be received from other related bacterial communities by the horizontal gene transfer mechanisms such as conjugation, transposition or phage attack with *mer* operon. Besides, it seems that the strain GTZY can be a perfect fit for the heavy metal selected genome plasticity and interaction with other bacterial communities, which often have consequences with evolved bacterial species in the toxic heavy metals contaminated sites. Since the strain GTZY was isolated from the high-dose streptomycin treating reactor, it could have a high fitness cost that leads to the out-competence by wild type sensitive strains existing in the heavy metal contaminated sites.

On the other side, the strain GTZY possessed an arsenic gene operon (*arsCPRH*) in its chromosome with the size of 2791 bp. The identified *arsCPRH* of strain GTZY was diverse from commonly known prokaryotic *ars* operons, *arsRBC* and *arsRDABC* identified in the *Escherichia coli, Staphylococcus xylosus, Pseudomonas aeruginosa, Pseudomonas fluorescens, Acidiphilium multivorum* and *Staphylococcus aureus*(Páez-Espino et al. 2015; Chen et al. 2016; Ben Fekih et al. 2018) rientation of strain GTZY and their gene components had only shown matching with a soil bacterium, *Dokdonella koreensis* DS-123 (Accession No.: CP015249). The multiple sequence alignment between these two *ars* operons revealed their maximum gene cluster coverage and basepairs similarities accounted for 94 % and 79.14 %. It is thereby likely that identified *ars* operon was distinct from other bacterial genera containing *ars* operons. Also, the identified *ars* operon from the strain GTZY has not flanked with any mobile genetic elements in both ends and was located intrinsically in a chromosome, which might be received from its ancestral strains by the process of vertical gene transfer. From the genome interpretation study, we confirm the existence of *ars* operon in its ancestral bacterial species, *Pseudoxanthomonas suwonensis* 11-1 (Accession No.: CP002446), isolated from cotton waste compost(Weon et al. 2006) and *Pseudoxanthomonas spadix* BD-a59 (Accession No.: CP003093), known for BETX biodegradation(Choi et al. 2013) (Fig. 7). This further reveals that the arsenic gene cluster orientation *(arsC-arsP-arsR-arsH)* of strain GTZY as extremely divergent (< 38% similarity) from their inherited BD-a59 *(arsH-arsB-arsC-arsR)* and 11-1 *(arsR-arsH-arsB-arsC)* strains. In particular, *arsR* and *arsH* of the strain GTZY were similar with the nucleotides of BD-a59 and 11-1 strains, while *arsC* was diverse in their ancestral strains, respectively. The genome of strain GTZY is comprised of the membrane permease transporter encoding *arsP* genewhile,BD-a59 and 11-1 strains are located with the most frequent *acr3* family efflux transporterencoding *arsB* gene, which extrudes arsenate (III). Apart from

arsCPRH, strain GTZY has also been distributed with additional *arsB* (963 bp), *arsR* (219 bp) and *arsC* (420 bp) genes in its chromosome.

The presence of *arsP* and its co-existence with the *arsH* gene in the *ars* operon of strain GTZY makes them versatile among other known bacterial *ars* operons. The *arsP* encoding efflux related permease system is known for the detoxification or extrudes the toxic organic arsenicals (III) such as roxarsone and monomethylarsonous acid (MMA)(Shen et al. 2014; Chen et al. 2015b). The *arsP* gene encoding ArsP permease was initially identified in the *ars* operon of *Campylobacter jejuni* (Wang et al. 2009), and later it was believed to be a prokaryotic cell membrane pump that could extrude arsenical roxarsone (III)(Shen et al. 2014). The recent genomic interpretation study suggests that the ancestral origin of arsP and their homologs, existence in the bacteria, archaea and a few other eukaryotes (Yang et al. 2015). The existence of a gene encoding flavoprotein, arsH, utilizing NADP+ to resist and oxidize the toxic methylarsenite (MAs (III)) into MAs (V) was reported in *Campylobacter jejuni* strain(Li et al. 2016). These arsH enzymes are widely distributed in gram-negative, mostly *Gammaproteobacteri*a including *Pseudomonas putida, Sinorhizobium meliloti, Acidithiobacillus ferrooxidans, Rhodopseudomonas palustris, Ochrobactrum tritici, Thiobacillus ferrooxidans, Herminiimonas arsenicoxydans* and *Pseudomonas aeruginosa*, but not in gram-positive bacteria (Yang et al. 2005; Chen et al. 2015a; Yang and Rosen 2016; Falgenhauer et al. 2017). The ArsP protein from both *P. putida* and *S. meliloti* was evaluated as an organoarsenical oxidase enzyme conferring resistance to trivalent methyl As derivatives. We assumed that *arsCPRH* of strain GTZY has highly evolved from its ancestral species, in response to the environmental mutations, genetic reshuffling and recombination with other bacterial communities.

Initially, *P. mexicana* GTZY isolate was characterized for its phylogeny, morphology and biochemical properties, and was found specifically relevant to the properties of various reported members of *Pseudoxanthomonas.* Like other heavy metal transforming bacteria, strain GTZY did not utilize the mercury and arsenic as main carbon sources in the basal media (data not shown). Since these heavy metals are not catabolized by the heavy metal transforming bacteria, their bioremediations were usually evaluated by the removal, reduction, oxidation and volatilization assays(Wuana and Okieimen 2011; Jebeli et al. 2017; Wang et al. 2020). TZY was successfully fulfilled the above chemical strategies against the mercury and arsenic metals in the growth media.

The strain GTZY can reduce inorganic Hg (II) and into foggy forming volatile Hg (0) in 4-h incubation. Previously, Mahbub et al. (2017) have proved the ability of *Sphingopyxis* sp. SE2 to volatilize or remove 44 % of mercury (II) in 6-h incubation. This study found 49.6 % of mercury removal achieved by the strain GTZY in 6 h. However, *Sphingobium* sp. SA2 was recorded for the maximum Hg (II) volatilization and reduction in 6 h account for 79 % (Mahbub et al. 2016, 2017). The strain GTZY's mercury volatilization and reduction ability were further confirmed by the occurrence of the *merA* gene encoding for mercury (II) reductase. Also, the isolate was proven to resist (> 33.4 µM) and remove (84.3 %) the toxic form of mercury (II) in 48-h incubation. The Minimum inhibitory concentrations (MIC and MIC_{50}) of strain GTZY against Hg (II) was observed to be 33.4 µM and 40.8 µM;, respectively, whereas in other bacterial classes, *Alphaproteobacteria*, *Betaproteobacteria, Flavobacteria, Firmcutes* and *Sphingobacteria,* MICs were

observed in the range of 5-30 μM; Hg (II)(Møller et al. 2011, 2014). After 60 h incubation, the concentration of Hg (II) was remained stable at 1.9 ± 0.2 μM along with strain GTZY. In the meantime, thermophilic species *Aquificae* was proven to be sustainable at 3.0 μM; Hg (II) in the basel media(Freedman et al. 2012). These results represent that the strain GTZY could be useful for preventing the formation of a more toxic form of methyl mercury (MeHg). In contaminated environments, high concentration and precipitation of Hg (II) always end up with more accumulation of toxic MeHg(Eckley et al. 2020). Hence, this is arguably a better solution to reduce the formation of toxic MeHg, in which a high concentration of Hg (II) should be cleaned up first so that the strain GTZY would be more suitable for the detoxification.

On the other side, strain GTZY can remove (68.5 %) and resist (> 175 mM) As (V) in the culture media at 72-h incubation. The Minimum inhibitory concentrations of strain GTZY towards arsenate and arsenite were > 175 and > 55 mM. Previous studies revealed the arsenate and arsenite MICs of *Gammaproteobacteria, Firmcutes* and *Kocuria,* in which the ranges of 75 – 125 mM and 10 – 50 mM, respectively, whereas in the *Bacillus fusiformis* ORAs1*, B. thuringiensis* ORAs2*,, Pseudomonas* sp. ORAs5, *Aeromonas molluscorum* ORAs6, MICs of arsenate and arsenite were > 16.68 mM and > 133.47 mM, respectively(Pepi et al. 2007). In addition, strain GTZY was reduced organic As (V) into As (III) form in the liquid solution, and it was further confirmed by the amplification of the *arsC* gene (495 bp) encoding arsenate reductase. Meanwhile, the strain GTZY was also able to remove (63.2 %) and resist (> 55 mM) As (III); however, it could not oxidize organic As (III) into As (V) form in the liquid solution. Similarly, Dey et al. (2016) isolated *Bacillus* sp. and *Aneurinibacillus aneurinilyticus* and found that both strains could remove As (V) (51.45–51.99 %) and As (III) (50.37–53.29 %). Besides, the strains also resisted the arsenate (4500 ppm) and arsenite (550 ppm). However, both strains have oxidized the arsenite, but none of them reduced arsenate into arsenite. Many previous reports suggest different detoxification mechanisms of arsenic resistance bacteria, including arsenic reduction and oxidation, heavy metal efflux pumping, metal adsorption to the cell surface, bacterial cell membrane binding and complex formation with exopolysaccharides (Zhang et al. 2016; Govarthanan et al. 2016a). Despite the absence of arsenite oxidase enzyme, we presume that the strain GTZY might be removed the arsenite (III) by following any of the above-proposed detoxification mechanisms.

Conclusion

From this study, it is therefore concluded that *Pesudoxanthomoas mexicana* strain GTZY has the potential for the bioremediation of highly toxic heavy metals such as mercury and arsenic. In short, this is the first investigation that opens up the resistance mechanism of *mer* (2.8 kb) and *ars* (2.7 kb) operons in the chromosome of *P. mexicana*. The existence of *mer* operon might be derived via horizontal gene transfer from other bacterial communities, because of the linkage of mobile genetic elements (*IS2* and *int1*) in its both terminuses. The occurrence of *ars* operon could be rendered vertically from its ancestral *Pseudoxanthomonas* species. This intrinsic *ars* operon was highly diversified and evolved from its ancestral species, which might be due to their mutations, genetic reshuffling and recombination with other bacterial communities. Initially, strain GTZY was isolated from an aerobic wastewater reactor and

characterized for its biochemical, physiological and phylogeny, besides the isolated strain showed its potential resistance towards heavy metals (Hg and As) and their increased removal and reduction in short periods. The evaluations from this study was further confirmed by the existence of resistance genes (*merA* and *arsC*). It is thereby claimable that the strain GTZY could be an exceedingly valuable addition to the mercury and arsenic-contaminated sites for time-

Declarations

Author Contributions: Conceptualization, Methodology, Software using, Data analysis, Writing—original draft preparation - Abdul Raheem Nelofer; Project administration, Conceptualization - Selvaraj Ganesh-Kumar (corresponding author) and S. Balachandran. Software using - Soorangkattan Saravanan, Software using, Data analysis- Karuppanan Kalimuthu; Software using, Data analysis - Ganesan Govindarajan; Data analysis - Rajah Dilip Kumar; Shivani Ramamurthy Baluraj - Data analysis; Software using, Data analysis - Imran Hasan; All authors have read and agreed to the published version of the manuscript.

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Declarations

Ethics approval: Not applicable.

Consent to participate: All persons who meet authorship criteria are listed as authors.

Consent for publication: All authors certify that they have participated sufciently in the work to take public responsibility for the content. Furthermore, each author certifes that this material or similar material has not been and will not be submitted to or published in any other publication.

Competing interests: The authors declare no competing interests.

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Figures

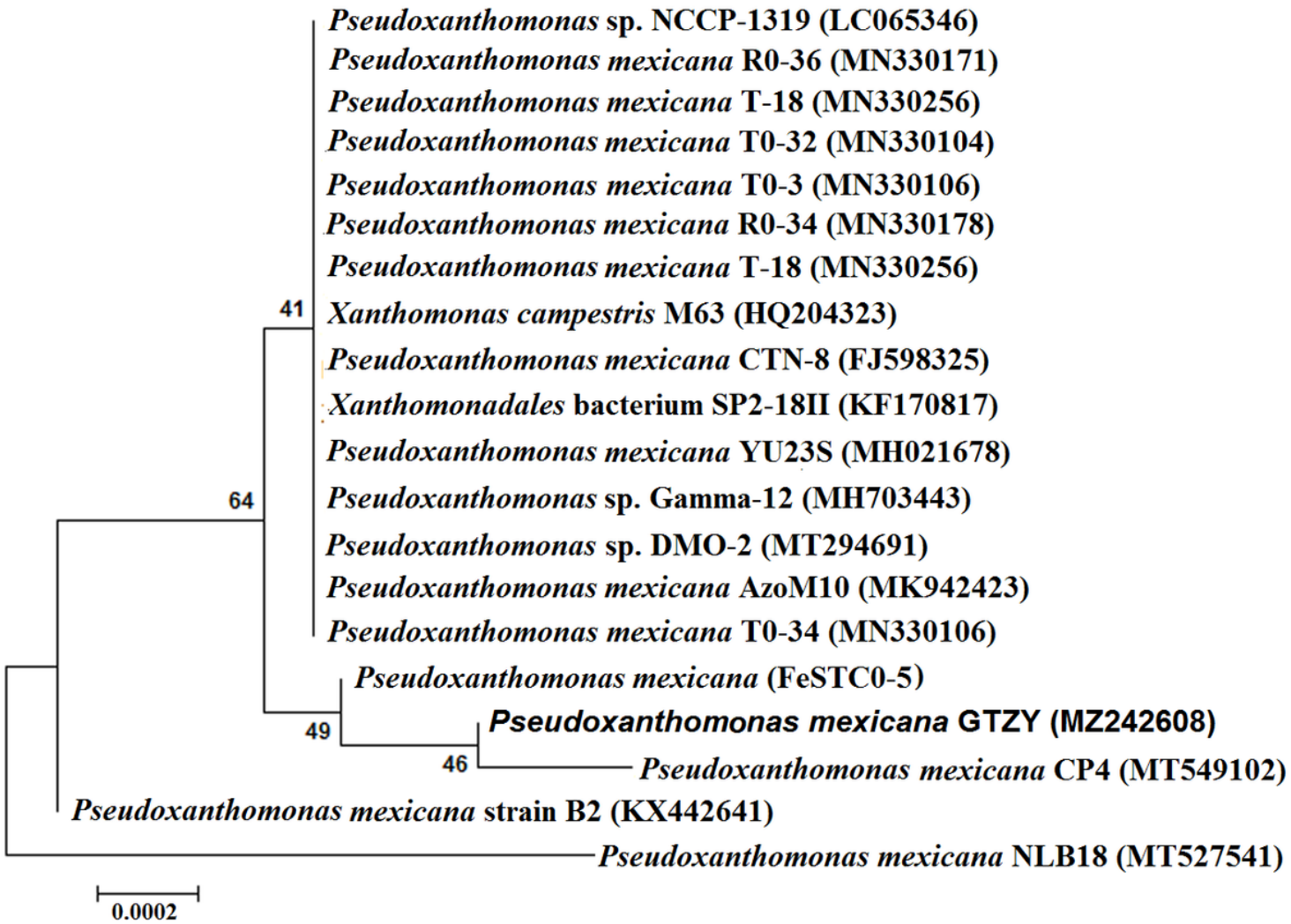

Figure 1

A phylogenetic tree showing the clustering of twenty 16S rRNA gene sequences along with an isolated bacterial strain GTZY. The neighbour-joining constructed tree was evaluated by the bootstrap method. The scale bar 0.0002 represents the nucleotide substitution level, and numbers indicate bootstrap values.

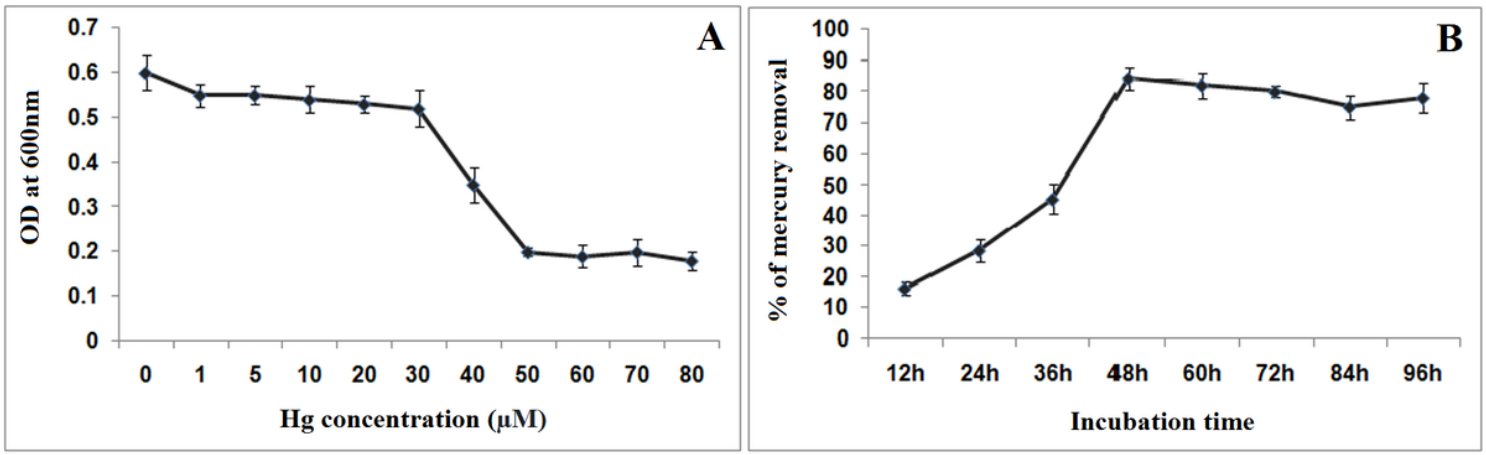

Figure 2

The effect of mercury on the growth of strain GTZY with different concentrations of mercury amended LB media. The microbial growth was estimated by the absorbance at 600 nm (A). Percent removal of mercury by the strain GTZY was determined at various periodic intervals (B).

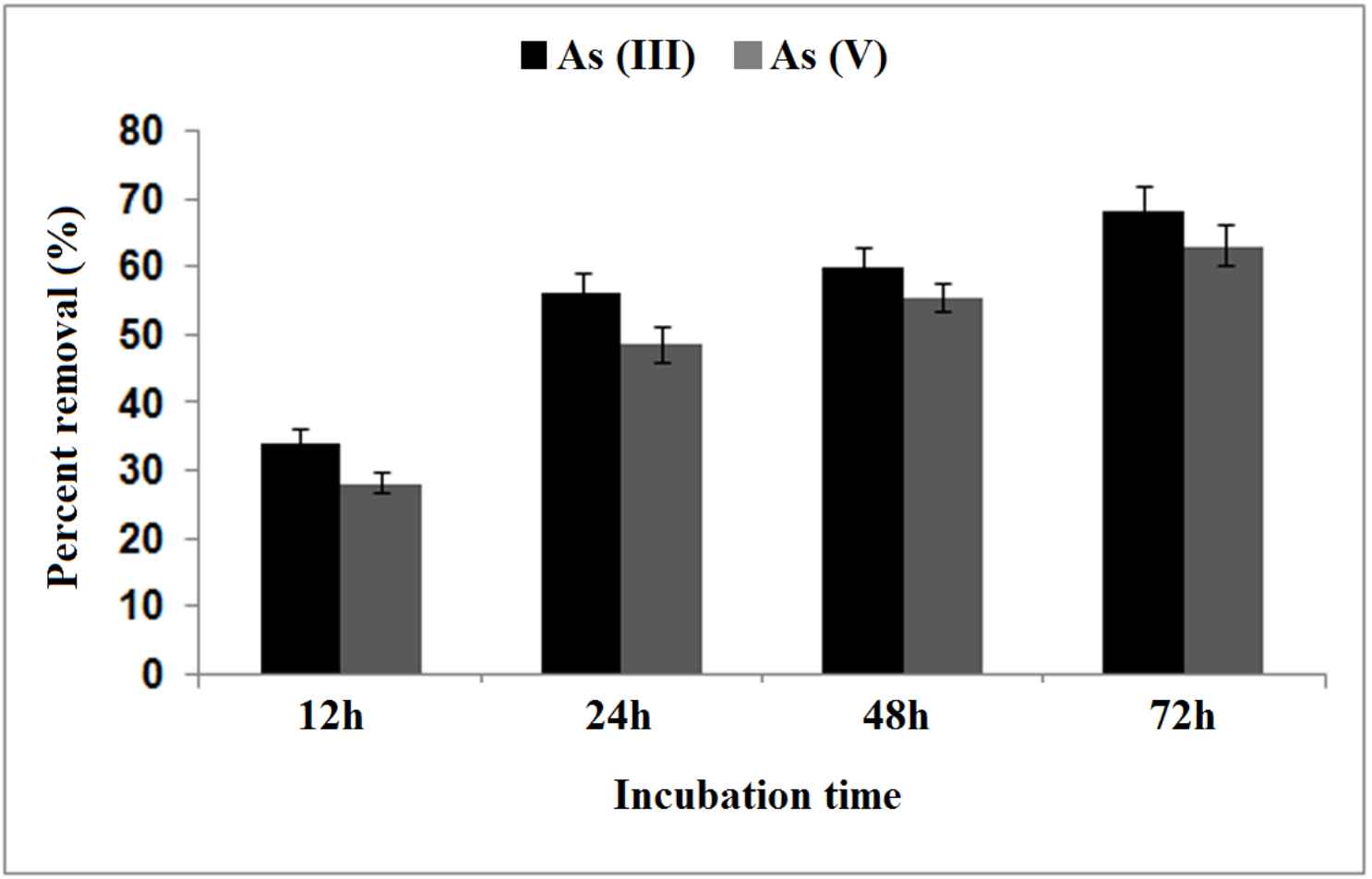

Figure 3

Percent removal of arsenite (III) and arsenate (V) by the isolate GTZY was estimated using Silver Diethyldithiocarbamate (SDDC) method. The experimental strain (1 %) was inoculated in LB broth amended with 5 µM sodium arsenate and sodium arsenite solution (as sources for As (V) and As (III), respectively) and incubated at 30 ºC.

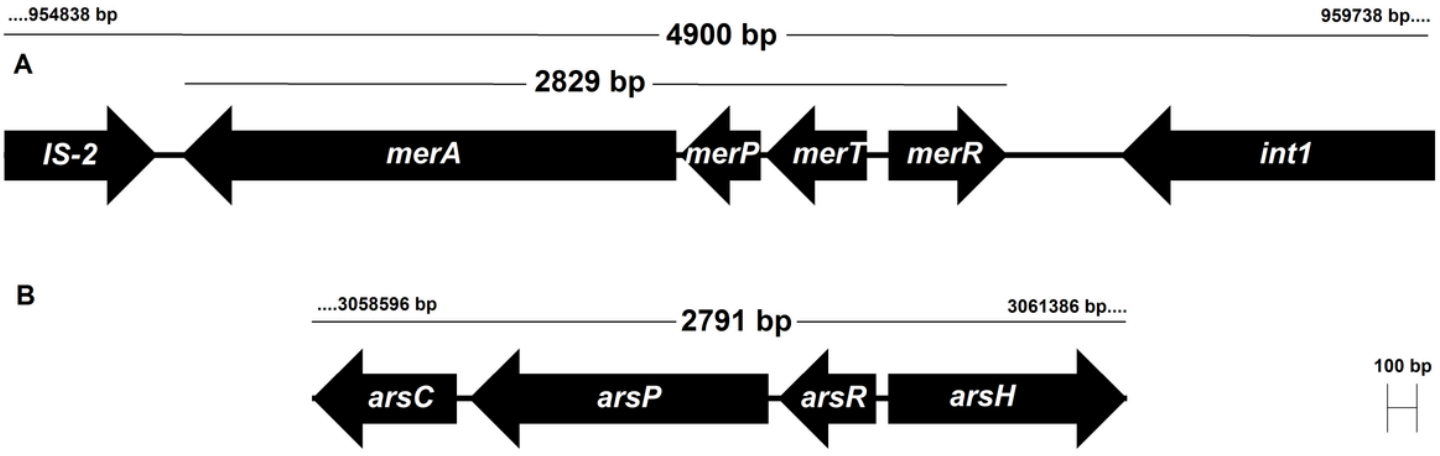

Figure 4

The genetic organization of heavy metal resistance operons from *Psudoxanthomonas mexicana* GTZY. Arrows indicate the orientation of transcription and its open reading frame. Arrow length indicates the basepairs length of particular genes. The particular gene descriptions are given in detail in the text. (A) Mercury resistance operon and (B) Arsenic resistance operon.

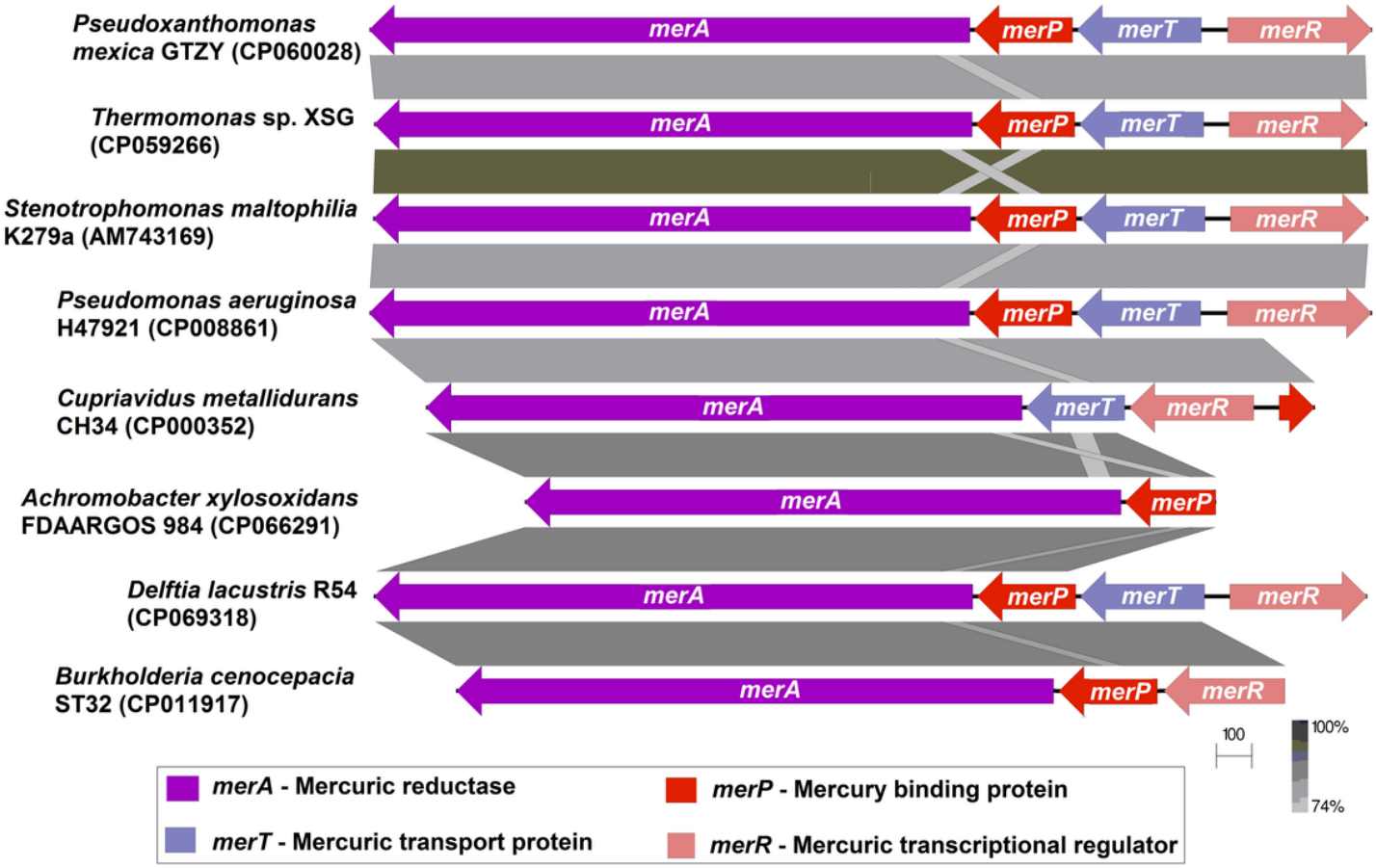

Figure 5

A phylogenetic distribution and genetic organization of mercury resistance operon of *Psudoxanthomonas mexicana* GTZY. *mer* gene clusters of different bacterial strains were annotated by BLASTn and compared with the EasyFig tool. The *mer* operon of strain GTZY has the closest relationship (coverage: 100 %, similarity: 99.89 %) with the *mer* operon of *Thermomonas* sp. XSG (Accession No.: CP061497). The degree of sequence homology (%) between the operons was shown by the grey shades, and shade intensity indicates the relationship level between the *mer* operons. Arrows indicate the orientation of transcription and its open reading frames. The bar indicates 100 bp lengths of nucleotides.

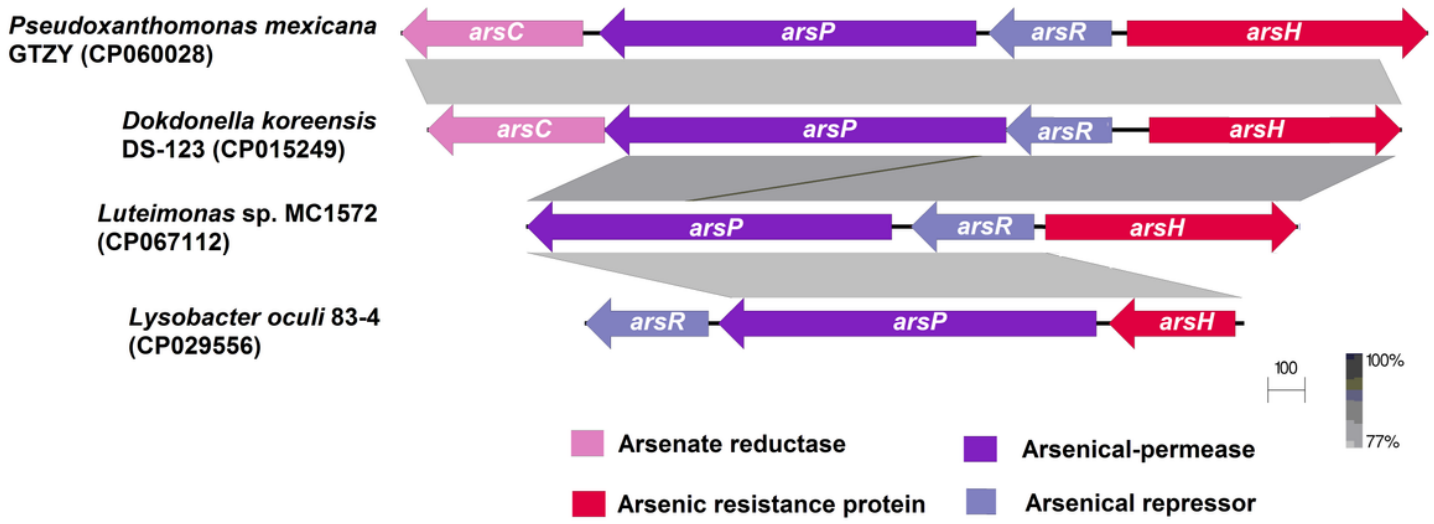

Figure 6

A graphical representation of BLASTn comparison of arsenic resistance operon of *Psudoxanthomonas mexicana* GTZY along with various bacterial species. *ars* operon of *Dokdonella koreensis* DS-123 (Accession No.: CP015249) is the only bacterial strain shown the maximum relationship (coverage: 94 %, similarity: 79.14 %) with *ars*operon of isolate GTZY and least relationship (coverage: 64 %, similarity: 77.51 %) with *Lysobacter oculi* 83-4 (Accession No.: CP029556).

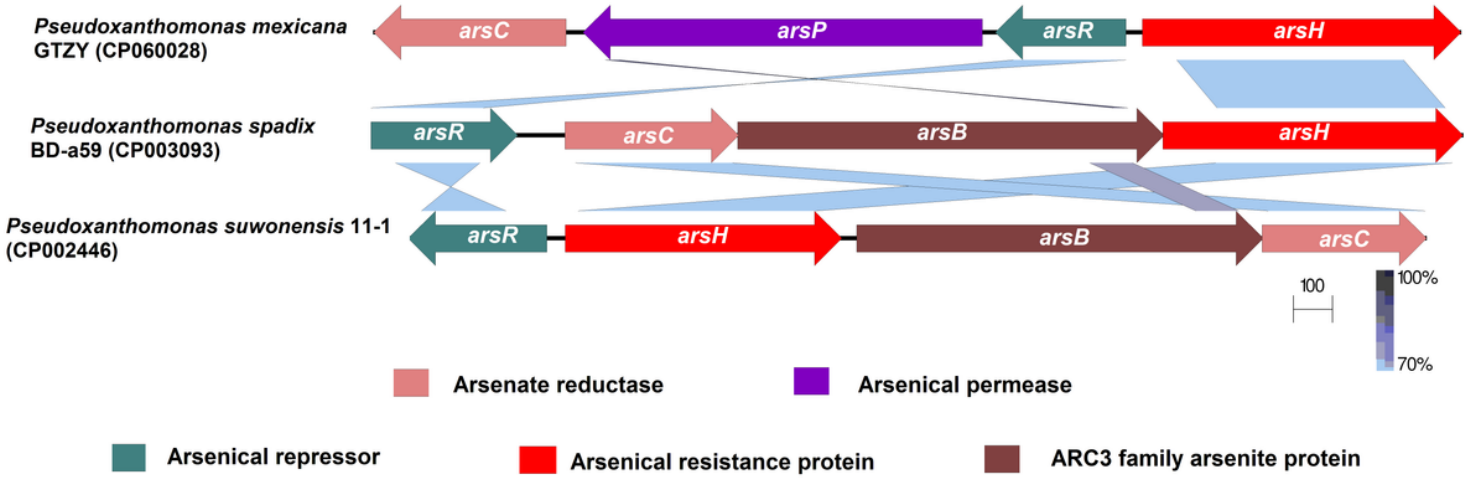

Figure 7

The genomic comparison of intrinsic ars operon of *Psudoxanthomonas mexicana* GTZY along with its bacterial ancestral species (EasyFig visualization). An extremely diverse relationship (< 38 %) was detected against ancestral strains, *Pseudoxanthomonas spadix* BD-a59 (Accession No.: CP003093), reported for BETX biodegradation (Choi et al. 2013a) and *Pseudoxanthomonas suwonensis* 11-1 (Accession No.: CP002446) isolated from cotton waste compost.

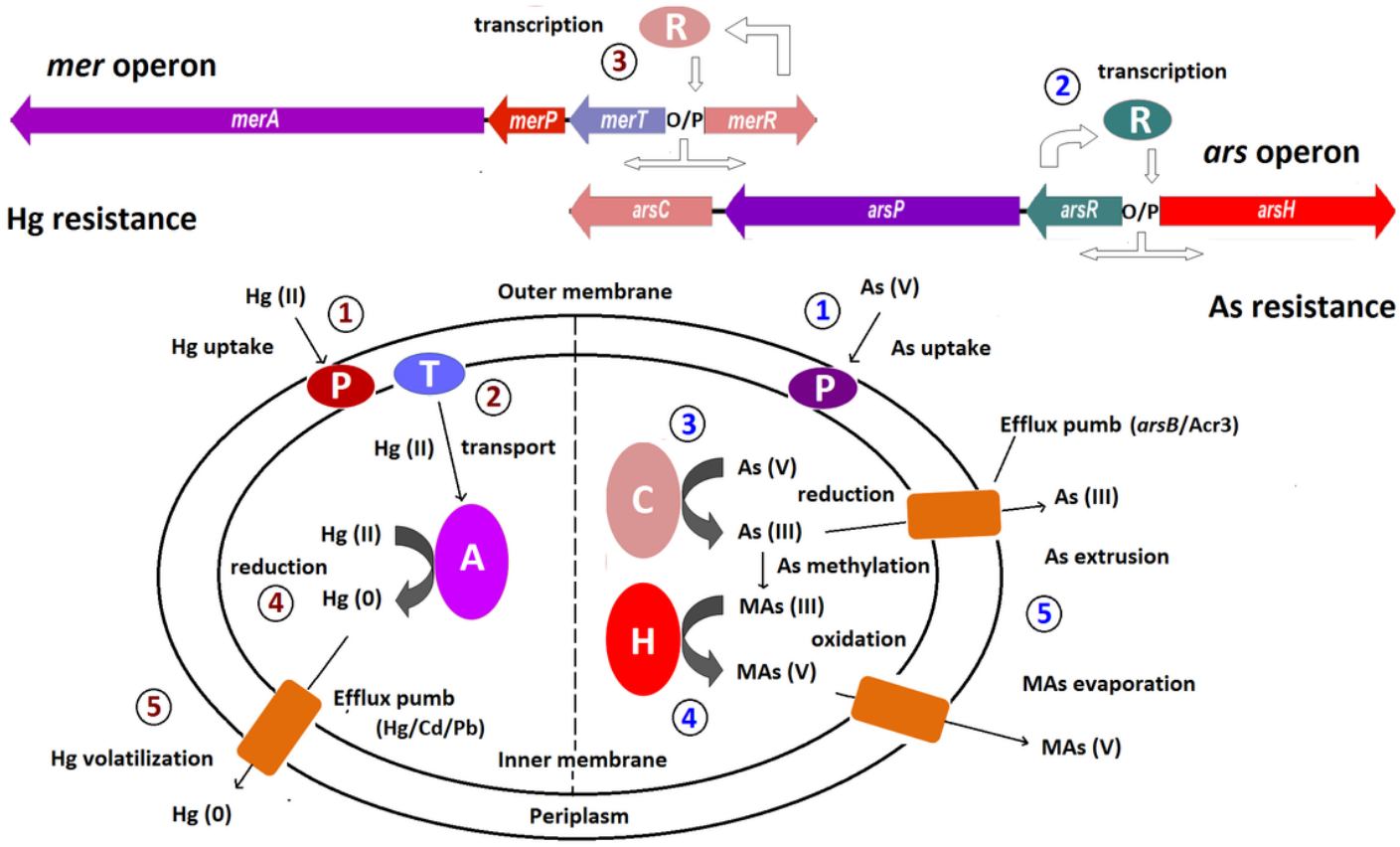

Figure 8

The role and cellular mechanisms of *mer* and *ars* systems occurred in the *Pseudoxanthomonas mexicana* strain GTZY. P – Periplasmic permease facilitate the cellular uptake of heavy metals (Hg and As), R – Regulator initiates the transcription of corresponding genes, T – Inner membrane protein transport the Hg (II) to reducing enzyme, A – *merA* encoding mercury reductase reducing toxic Hg (II) to non-toxic Hg (0), C – *arsC* encoding arsenic reductase reducing As (V) to As (III), H – *arsH* encoding arsenical (III) oxidase enzyme transferring toxic MAs (III) to less toxic MAs (V). Reduced or oxidized metals extrude via cell membrane efflux system. The numbers represent the steps of heavy metal detoxification. The reduced As (III) and Hg (0) are further extruded via cell membrane efflux system, *arsB* encoding Ars3-family transporter and Hg/Cd/Pb transporting protein, respectively.

Supplementary Files

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- SupportingInformations.docx
- GraphicalAbstract.png